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Plenary Session

1

DNA degradation during apoptotic cell death

S Nagata

Department of Genetics, Osaka University Medical School, Osaka, Japan

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Apoptosis is a principal mechanism in metazoans by which superfluous or potentially harmful cells are eliminated. Deregulation of this process leads to a variety of diseases such as cancer and autoimmune diseases. Stimuli that can induce apoptosis are relatively diverse, and include the death factors (Fas ligand, tumor necrosis factor and TRAIL), DNA damage, and oxidative stress. Regardless of the origin of the apoptotic stimulus, commitment to apoptosis leads to activation of caspases, a family of cysteine proteases. Cleavage of a select group of cellular substrates by caspases is responsible for the morphological and biochemical changes that characterize apoptotic cell death. The degradation of nuclear DNA into nucleosomal units is one of the features of apoptotic cell death, and is mediated by a caspase-activated DNase (CAD). Cells deficient in CAD undergo cell death without the DNA fragmentation, but CAD-null mice did not show any adverse phenotypes. A close examination of the apoptotic cells in these mice indicated that apoptotic cells are always in macrophages. It seems that at an early stage of apoptosis, the dying cells present an 'eat me signal' on their surface. This signal is recognized by macrophages for engulfment, and DNase II in the lysosomes of macrophages degrades DNA of apoptotic cells. Mice deficient in both CAD and DNase II genes were established, and the development of various organs was found to be severely impaired in these mutant mice. The mice accumulated a large amount of undigested DNA in macrophages in various tissues during development. This accumulation of DNA in macrophages activated the innate immunity to induce the expression of the interferon β gene. The interferon thus produced seems to be responsible for the impaired tissue development. These results indicate that the degradation of DNA during apoptotic cell death is an essential step of apoptosis to maintain mammalian homeostasis.

2

Paracrine pathways of cartilage destruction in osteoarthritis

S Abramson, M Attur, M Dave, M Leung, J Patel, P Gomez, A Amin

Division of Rheumatology, NYU School of Medicine – Hospital for Joint Diseases, New York, USA

Arthritis Res Ther 2003, **5(Suppl 3)**:2 (DOI 10.1186/ar801)

Osteoarthritis (OA) has been considered a biomechanically driven, degenerative disease of cartilage. However, the OA disease process affects not only the cartilage, but also the entire joint structure; and within the bone, cartilage and synovium of affected joints, profound metabolic changes transpire, which include the production of growth factors, nitric oxide (NO), prostaglandins (PGs), leukotrienes (LTs), IL-1 β , tumor necro-

sis factor alpha, IL-6, and IL-8. The autocrine production of IL-1 β by OA cartilage has been of particular interest, since both *ex vivo* human and *in vivo* animal studies indicate that IL-1 antagonists effectively attenuate cartilage degradation. Microarray technology has demonstrated differential expression in OA cartilage of a variety of IL-1-induced, NF κ B-dependent genes. Among IL- β -induced products of OA cartilage are various eicosanoids, which include E₂, PGD₂, LTB₄, PGF_{1 α} , PGF_{2 α} , and thromboxane. Treatment of OA cartilage with cyclooxygenase (COX) inhibitors increases LTB₄ production threefold to fivefold, indicating shunting of arachidonate from the COX to the 5-LO pathway. Functional analyses of individual eicosanoids reveals that PGD₂, in contrast to its derivative PGJ₂, stimulates catabolic processes, including NO and PG production. Lipoxin and 15-epi-lipoxin are also spontaneously released by OA cartilage, where they act to inhibit the spontaneous production of NO, PGE₂, IL-8 and IL-6. Consistent with the notion that OA is not simply a degenerative disease of cartilage, gene expression analysis of circulating peripheral blood mononuclear cells (PBMCs) shows upregulation of mRNA for IL-1 β , COX-2, IL-6, and IL-8 in OA (but not normal) PBMCs. OA PBMCs produce threefold to fivefold more PGE₂ in response to stimulation with IL-1 β than do normal cells. Thus, PBMCs, like chondrocytes and synovial cells, are activated in OA, and merit evaluation as sensors of inflammatory processes in the OA joint.

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Fifty years' experience in research for pathogenesis of rheumatoid arthritis

NJ Zvaifler

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Abstract not submitted for publication

4

Interferons and IRF/Stat transcription factors in the regulation of immunity, oncogenesis and bone remodeling

T Taniguchi, A Takaoka, H Takayanagi, K Honda

Department of Immunology, Graduate School of Medicine, University of Tokyo, Japan

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Analysis of the interferon (IFN)- α/β system over the past two decades revealed the critical roles of the IRF and Stat families of transcription factors in the regulation of this and other cytokine systems. We proposed operation of the positive feedback mechanism of the IFN gene induction, which is mediated by IRF-3 and IRF-7. We demonstrate that this mechanism is critical not only for innate immune response against viruses, but also for adaptive immune responses through induction of the maturation of dendritic cells. We also present our recent findings on a new link between the IFN signaling and tumor suppressor p53. In fact, the IFN-mediated induction of p53 gene is critical for boosting the p53-dependent apoptotic response in tumor suppression and antiviral immunity. Bone remodeling is central to maintaining the integrity of the skeletal system, wherein the

developed bone is constantly renewed by the balanced action of osteoblastic bone formation and osteoclastic bone resorption. We found that IFNs and IRF/Stat factors are uniquely involved in the regulation of bone remodeling, and summarize our data on how these cytokines and transcription factors participate in maintaining the bone homeostasis.

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5

Delineating biologic pathways involved in skeletal growth and homeostasis through the study of rare Mendelian diseases that affect bones and joints

M Warman

Department of Genetics and Center for Human Genetics, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio, USA

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We have found that the secreted glycoprotein, lubricin, is mutated in patients with the autosomal recessive disorder camptodactyly–arthropathy–coxa vara–pericarditis syndrome. To better define lubricin’s role in articulating joints we have been studying lubricin knockout mice. Knockout mice recapitulate features observed in patients with camptodactyly–arthropathy–coxa vara–pericarditis, and therefore allow us to learn more about the protein’s *in vivo* function. We find that synovio-cytes from knockout mice have different growth characteristics than do wild-type synovio-cytes. We also find that the articular cartilage surface in knockout mice loses superficial zone chondrocytes and becomes covered by proteinaceous material. These results indicate that lubricin protects cartilage surfaces and helps regulate synovial cell growth.

We had found that Wnt-inducible-secreted protein 3 (WISP3) is mutated in patients with the autosomal recessive disorder progressive pseudorheumatoid dysplasia. Patients with progressive pseudorheumatoid dysplasia require joint replacement surgery for what resembles end-stage osteoarthritis by their second decade of life. WISP3 is in very low abundance since we have only been able to detect mRNA by reverse transcription-polymerase chain reaction, and not by northern blot or *in situ* hybridization. Furthermore, we have not been able to detect endogenous WISP3 protein using a sensitive polyclonal antibody. Therefore, to determine the role of WISP3 in maintaining joints we created Wisp3 knockout mice. Surprisingly these mice did not develop joint failure, but they did seem to have altered timing of their secondary centers of ossification. We are currently exploring whether this altered timing is relevant to the pathogenesis of cartilage failure in human patients.

We have been searching for the gene responsible for autosomal recessive acromesomelic dysplasia. This is an interesting disorder because it primarily affects postnatal, rather than prenatal, growth. Also interesting is the fact that many carrier parents of affected individuals appear to have isolated short stature. Consequently, the discovery of the gene responsible for this disease may provide insights into pathways that effect growth.

Message from WHO

Assessing the burden of musculoskeletal conditions: a joint World Health

Organization–Bone and Joint Decade project

N Khaltav¹, B Pflieger¹, AD Woolf², C Mathers¹, K Akesson³, JM Hazes⁴, D Symmons⁵

¹World Health Organization, Geneva, Switzerland; ²Royal Cornwall Hospital, Truro, UK; ³Malmö University Hospital, Sweden; ⁴University Hospital of Rotterdam, The Netherlands; ⁵University of Manchester, UK *Arthritis Res Ther* 2003, **5(Suppl 3)**:174 (DOI 10.1186/ar805)

Introduction The Monitor Project of the Bone and Joint Decade (BJD) was developed to quantify the global burden of musculoskeletal condi-

tions and to develop strategies for their prevention. Experts within the Monitor Project have been working with officers at the World Health Organization (WHO) to estimate morbidity and mortality associated with rheumatic conditions.

Objectives To determine the burden of major musculoskeletal conditions and limb trauma in terms of mortality and disability.

Methods A Scientific Group meeting of experts in the areas of musculoskeletal conditions and limb trauma was held in January 2000 in Geneva in order to produce a WHO Technical Report on the impact of rheumatoid arthritis (RA), osteoarthritis (OA), osteoporosis, major limb trauma, and spinal disorders (low back pain [LBP]). Data on incidence, prevalence and severity for the conditions were collected by world region, gender, and age groups. Estimates of the economic burden of each condition were also made, as were descriptions of relevant health domains and states. Epidemiological models were formed for RA, OA and LBP. Computer software was used to combine the data on incidence and/or prevalence with estimates of case fatality, case remission, and average duration to establish the number of years of life lost for each condition in agreement with mortality data. Estimates for the distribution of disabilities associated with treated and untreated forms of each condition were made to help determine the number of years living in disability for each condition.

Results The work of the WHO/BJD collaboration has resulted in a WHO Technical Report (in press) as well as burden estimates for the WHO Global Burden of Disease 2000 study. Results comparing years of living with disability for 1990 data with those for 2000 data are presented in Table 1 for RA, OA, LBP, and a residual musculoskeletal condition category. The burden appears to have increased during the time period. Other results show that the impact of musculoskeletal conditions varies worldwide and is influenced by social structure, expectation, and economics.

Table 1

Years living with disability for musculoskeletal conditions^a

Condition	Global Burden of Disease 1990			Global Burden of Disease 2000		
	Male	Female	Total	Male	Female	Total
RA	859	2309	3168	1205	3092	4297
Osteoarthritis	5341	7934	13,275	5549	8667	14,216
Low back pain	–	–	–	1180	1045	2225
Other	398	1099	1437	596	1598	2194

^aYears of life lost data not presented due to the low mortality associated with these conditions. LBP was not considered in the Global Burden of Disease 1990 study. RA, rheumatoid arthritis. *Sources:* [1,2] and unpublished World Health Organization data.

Conclusions The WHO/BJD collaboration has been successful in accumulating and publishing vital data describing the burden of musculoskeletal conditions and limb trauma.

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Message from APLAR

Fortieth Anniversary of APLAR

P Pispati

President – APLAR

Arthritis Res Ther 2003, **5(Suppl 3)**:175 (DOI 10.1186/ar806)

What an honour it is to come to this exotic Miyazaki Island in this wonderful country of Japan. I bring to you warm greetings and felicitations

from 22 APLAR countries ranging from Iraq/Syria in the West to Japan/New Zealand in the East. APLAR is most proud that Japan is a leading and outstanding member of the APLAR community of nations. This is a significant year for APLAR. We are celebrating the 40th Anniversary of APLAR. We find no better occasion or location to make this announcement from the outstanding platform of the GARN Conference.

Ladies and Gentlemen, 3.7 billion people reside in APLAR countries; some highly developed, some economically prosperous and some developing nations. Rheumatology as a science and practice is firmly established in most APLAR member countries, including developing nations. There are highly qualified well-trained rheumatologists available who enjoy adequate practical laboratory support and are given to good therapeutics. There are centres of excellence where good education and training in rheumatology is imparted.

Yet, there are some countries that are not yet members of APLAR and in which organised rheumatology centres hardly exist. It is the aim of APLAR today to undertake and help initiate rheumatology services in these countries. This is an ambitious and onerous task that we have undertaken.

Exactly a year from now APLAR will hold its outstanding International Congress in Rheumatology in the picturesque Jeju Island in South Korea. We promise you an innovative, creative, stimulating scientific programme replete with warm hospitality. Do come to the APLAR Congress in South Korea in September 2004 – you will never ever regret it. The GARN Conference is certain to generate new data, new ideas with the plethora of brilliant scientists assembled under one roof here. We have come to learn a lot and to develop positive ideas into an action programme in the APLAR region. This is precisely why I am here to take away a message of advances in science and to give this message of co-operation and goodwill to the brilliant success of this GARN Conference. MAY PEACE AND HARMONY PREVAIL. MAY GOD BLESS US ALL.

Topics Symposium (1) Rheumatology (1): RA

6

Premise: traditional disease-modifying antirheumatic drugs are less effective and safe than targeted therapy with biologic response modifiers in rheumatoid arthritis

R Fleischmann

Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA

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Introduction Multiple traditional disease-modifying antirheumatic drugs (DMARDs), including hydroxychloroquine, gold, penicillamine, sulfasalazine, leflunomide, methotrexate, azathioprine, and cyclosporine, have been approved for use in patients with rheumatoid arthritis. Most patients discontinue these medications by 2 years due to loss of efficacy and/or toxicity. There are currently four biologic response modifiers (BRMs) commercially available: etanercept, infliximab, adalimumab, and anakinra. The question is whether these BRMs have a superior efficacy:tolerability ratio, allowing patients to remain on therapy significantly longer.

Objectives To compare clinical trial data of traditional DMARDs and BRMs, alone or in combination, with respect to efficacy and safety.

Results It is impossible to compare efficacy or safety results of clinical trials of different agents as trial designs, primary endpoints and statistical analyses have changed considerably over the years. The best measure of the effectiveness of a particular medication, therefore, is how long patients remain on therapy that would assume a significant clinical benefit without serious adverse events (AE). Less than 50% of patients treated with traditional DMARDs remain on therapy for 2 years [1]. Combination therapy of DMARDs in patients with established disease does not fare better. Significant AEs that occur with these agents include hematological, hepatic, pulmonary, renal, infectious and mucocutaneous reactions. Each of the BRMs that target tumor necrosis factor alpha have far superior results with respect to continuing therapy: 60% of infliximab patients >2 years, 52% of patients with etanercept >4 years and 56% of patients with adalimumab >5 years [2]. Rare AE that do occur include reactivation of latent tuberculosis and other opportunistic infections, demyelinating disease, congestive heart failure and possibly lymphoma. With proper screening and

caution, however, patients should be identified if they are at risk and not treated, thus sparing these AEs. Patients treated with BRMs do not develop the multiorgan adverse events noted with traditional DMARDs. **Conclusions** Anti-tumor necrosis factor BRMs appear to have superior efficacy and safety than do traditional DMARDs alone or in combination. For this reason, the use of a BRM should be strongly considered in a patient with rheumatoid arthritis who has not reached maximal efficacy or who develops a significant AE with the use of traditional DMARDs.

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7

The use of animal models to find and understand arthritis genes

R Holmdahl

Medical Inflammation Research, Lund University, Lund, Sweden

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Inbred animals are useful for studies of the identification of genes associated with rheumatoid arthritis (RA) since they are more efficient tools for identification of genes controlling complex diseases. There are several arthritis models, which each may reflect various variants of the heterogeneity of RA in humans. Examples are collagen-induced arthritis (CIA) and pristane-induced arthritis, which both fulfill the clinical diagnostic criteria for RA.

Type II collagen (CII) is immunogenic and contains peptides that can be bound to major histocompatibility complex (MHC) class II and can be presented to T cells, whereas pristane is not immunogenic by itself. Both diseases are genetically complex and the susceptibility is, as RA, dependent on many polymorphic genes operating in concert. So far two of these genes have been identified; the MHC class II Ab gene in the mouse [1] and the Ncf1 gene in the rat [2]. The Ncf1 protein is a part of the NADPH oxidase complex involved in generation of the inducible oxidative burst. The discovery of the Ncf1 polymorphism led to a new proposed pathway in which oxygen radicals modify antigen presentation and the resulting activation of autoreactive T cells. This hypothesis has now been further documented by the identification of an Ncf1 mutation in the mouse that reproduces the effects earlier observed in the rat. Mice with the deficient Ncf1 allele, and expressing the MHC class II allele Aq, binding CII peptides, could be shown to be dramatically more susceptible to CIA, and also developed a chronic form of arthritis. Interestingly, the immune response to CII was enhanced by the Ncf1 deficiency linking the Ncf1 pathway to the adaptive immune response.

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8

Synovial mast cells require C5a receptor (CD88) for arthritis induction

D Lee¹, M Brenner¹, C Gerard², C Benoist³, D Mathis³, G Watts¹

¹Division of Rheumatology, Immunology and Allergy, Brigham & Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; ²Department of Pediatrics, Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA; ³Section on Immunology and Immunogenetics, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts, USA

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A critical role for mast cells in arthritis pathogenesis has been demonstrated in the K/BxN serum transfer mouse model; however, the mecha-

nisms by which synovial mast cells are activated to induce arthritis remains unknown. Previous studies have demonstrated a requirement for both IgG Fc receptors and complement components (C3 and C5) as well as the receptor for the anaphylotoxin C5a (C5aR, CD88) in K/BxN serum transfer arthritis. Knowing that human synovial mast cells express CD88 while mast cells at other anatomic sites lack CD88 expression, we hypothesized that synovial mast cells may require activation via CD88 for arthritis induction in the K/BxN model. To test this hypothesis, we engrafted wild-type (C57BL/6 [B6]) and CD88-deficient (CD88^{-/-}) bone marrow-derived mast cells (BMMC) into mast cell-deficient W/Wv recipient mice. After allowing 10 weeks for engraftment, we challenged B6 and CD88^{-/-} BMMC-engrafted W/Wv mice as well as control, non-engrafted W/Wv and wild-type mice with arthritogenic K/BxN serum. We find that CD88^{-/-} BMMC-engrafted W/Wv mice clinically display resistance to arthritis induction equivalent to that seen in non-engrafted W/Wv mice, while B6 BMMC engraftment restores arthritis sensitivity to W/Wv mice. Histologic analyses confirm engraftment of mast cells in synovial and other tissues in CD88^{-/-} BMMC-recipient W/Wv mice. These results demonstrate an *in vivo* requirement for CD88 expression by synovial mast cells for induction of inflammatory arthritis in the K/BxN arthritis model.

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Albumin-based drug delivery as novel therapeutic approach for rheumatoid arthritis

A Wunder¹, U Muller-Ladner², E Stelzer³, E Neumann², H Sinn¹, S Gay⁴, C Fiehn⁵

¹Department of Radiochemistry and Radiopharmacology, German Cancer Research Center, Heidelberg, Germany; ²Department of Internal Medicine I, University of Regensburg, Regensburg, Germany; ³Cell Biophysics/Cell Biology Program, European Molecular Biology Laboratory Heidelberg, Heidelberg, Germany; ⁴WHO Collaborating Center for Molecular Biology and Novel Therapeutic Strategies for Rheumatic Disorders, University of Zurich, Zurich, Switzerland; ⁵Clinic of Internal Medicine V, University of Heidelberg, Heidelberg, Germany
Arthritis Res Ther 2003, **5(Suppl 3)**:9 (DOI 10.1186/ar810)

We reported recently that albumin is a suitable drug carrier for targeted delivery of methotrexate (MTX) to tumors. Due to pathophysiological conditions in neoplastic tissue, high amounts of albumin accumulate in tumors and are metabolized by malignant cells. MTX, covalently coupled to human serum albumin (MTX-HSA) for cancer treatment, is currently being evaluated in phase II clinical trials. Because the synovium of patients with rheumatoid arthritis (RA) shares various features observed also in tumors, albumin-based drug targeting of inflamed joints might be an attractive therapeutic approach. Therefore, the pharmacokinetics of albumin and MTX in a mouse model of arthritis was examined. Additionally, uptake of albumin by synovial fibroblasts of RA patients and the efficacy of MTX and MTX-HSA in arthritic mice were studied. The results show that, when compared with MTX, significantly higher amounts of albumin accumulate in inflamed paws, and significantly lower amounts of albumin are found in the liver and the kidneys. The protein is metabolized by human synovial fibroblasts *in vitro* and *in vivo*. MTX-HSA was significantly more effective in suppression of the onset of arthritis in mice than was MTX. In conclusion, albumin appears to be a suitable drug carrier in RA, most probably due to effects on synovial fibroblasts, which might increase the therapeutic efficacy of and reduce side effects of MTX.

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SKG mice, a new genetic model of rheumatoid arthritis

S Sakaguchi, T Takahashi, H Hata, T Nomura, N Sakaguchi

Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan
Arthritis Res Ther 2003, **5(Suppl 3)**:10 (DOI 10.1186/ar811)

We have established a mouse strain, designated SKG mice, which spontaneously develop chronic autoimmune arthritis. The arthritis resembles rheumatoid arthritis in the proliferative synovial inflammation accompanying infiltration of CD4⁺ T cells, formation of pannus eroding cartilage and bone, development of autoantibodies including rheuma-

toid factor, and various extra-articular manifestations. It can be adoptively transferred to histocompatible athymic mice by peripheral CD4⁺ T cells or thymocytes, or to histocompatible SCID mice by bone marrow cells. Thus, the abnormality in this model seems to be expressed in the bone marrow-derived cellular components, leading to thymic generation and activation of CD4⁺ T cells recognizing/attacking normal self-antigens in the joints. In genetic analysis, the offspring of crosses between SKG mice (which has a BALB/c genetic background) and normal BALB/c mice, whether the mother was SKG or BALB/c, developed no arthritis. In contrast, arthritis occurred in approximately 50% of the N2 generation obtained by crossing the nonarthritic F1 hybrids with SKG mice; the arthritides in the N2 generation showed a similar clinical course and severity as in SKG mice. Thus, the genetic abnormality is presumably of a single gene locus, designated as the *skg* gene, and inherited in an autosomal recessive fashion with nearly 100% penetrance of the trait in homozygotes raised in our conventional environment. Linkage analysis between the development of macroscopically evident arthritis and the homozygosity of chromosome-specific microsatellite markers by utilizing the N2 generation of backcrossing the F1 generation of SKG and *Mus musculus castaneus* to SKG mapped the *skg* locus to the centromeric portion of chromosome 1, with the lod score of the locus as infinite. Positional cloning of the *skg* gene revealed that the gene encodes a signal transduction molecule in T cells. Altered signal transduction from the T-cell antigen receptor through the mutated molecule changes the thresholds of T cells to thymic selection, leading to positive selection of otherwise negatively selected autoimmune T cells. This genetically determined 'selection shift' of the T-cell repertoire towards high self-reactivity and resulting thymic production of pathogenic autoimmune T cells may be a primary cause of and also a predisposing factor for RA in humans.

11

The role of IL-17 in the development of arthritis in mouse models

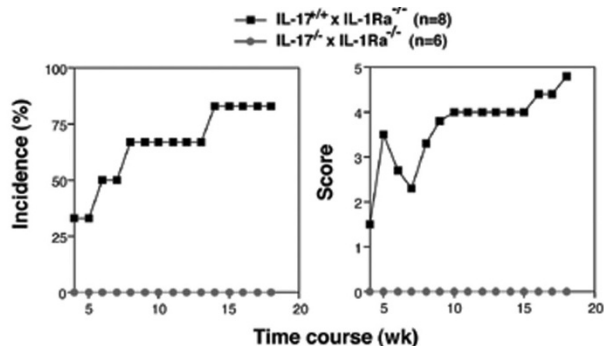
Y Iwakura, S Nakae, R Horai, S Saijo

Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan
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Introduction IL-17 is a T-cell-derived proinflammatory cytokine, which is suspected to be involved in the development of rheumatoid arthritis (RA) because this cytokine is found in sera and synovial tissues of RA patients. The pathogenic roles of IL-17 in the development of RA, however, still remain to be elucidated.

Objective To elucidate the roles of IL-17 in the development of arthritis, we produced IL-17-deficient (IL-17^{-/-}) mice, and examined the effect of deficiency on the development of arthritis in two etiologically different, spontaneous RA models, the HTLV-I transgenic mouse model and the IL-1 receptor antagonist (IL-1Ra)-deficient mouse model, as well as the type II collagen-induced arthritis model [1].

Figure 1



Development of arthritis in IL-1Ra^{-/-} is completely suppressed by the deficiency of IL-17.

Methods IL-17^{-/-} mice were produced by replacing exon 1 and exon 2 of the *il-17* gene with a neomycin resistance gene [2]. HTLV-I transgenic mice carrying the HTLV-I *env-pX* region and IL-1Ra^{-/-} mice were produced as described elsewhere [3,4].

Results Both HTLV-I transgenic mice and IL-1Ra^{-/-} mice develop arthritis spontaneously due to autoimmunity caused by excess T-cell activation. The development of arthritis in HTLV-I transgenic mice was markedly suppressed in IL-17^{-/-} mice, and that in IL-1Ra^{-/-} mice was abolished completely (Fig. 1).

Moreover, type II collagen-induced arthritis was markedly suppressed in IL-17^{-/-} mice. We found that crosslinking of OX40 led to promote IL-17 production on CD4⁺ T cells, and OX40 expression was augmented in IL-1Ra^{-/-} mice due to excess IL-1 signaling, resulting in the overproduction of IL-17. IL-17 was responsible for the priming of collagen-specific T cells and collagen-specific IgG_{2a} production.

Conclusion These observations suggest that IL-17 acts downstream to IL-1, and plays a crucial role in the development of arthritis by activating autoantigen-specific cellular and humoral immune responses.

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Topics Symposium (2) Immunology

12

Cytokine production by dendritic cells genetically engineered to express IL-4: induction of Th2 responses and differential regulation of IL-12 and IL-23 synthesis

D Fox, Y Morita, R Gupta, K Seidl, K McDonagh

Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Arthritis Res Ther 2003, **5(Suppl 3)**:12 (DOI 10.1186/ar813)

We recently showed a therapeutic effect of bone marrow-derived dendritic cells (DCs) retrovirally transduced with IL-4 in murine collagen-induced arthritis, a Th1-mediated autoimmune disease. We have now further investigated the functional characteristics of these engineered cells. We hypothesized that the ability of DCs to regulate the type of immune response may depend in part on their capacity to produce IL-12 and IL-23. IL-4-transduced DCs produced increased levels of IL-12p70 following ligation of CD40. Quantitative mRNA analysis revealed that IL-4-transduced DCs expressed higher levels of IL-12p35 mRNA, but lower levels of mRNA for IL-23p19 and the common subunit p40 found in both IL-12 and IL-23, compared with control DCs. Thus, expression of the IL-12 and IL-23 subunits is differentially regulated in IL-4-transduced DCs. Similar results were obtained using *in vitro* differentiated myeloid DCs cultured in 10–50 ng/ml IL-4. IL-4 led to diminished secretion of IL-23 protein after activation of these cells by CD40 ligand. *In vivo* studies demonstrated that IL-4-transduced, antigen-pulsed DCs led to lower antigen-specific T-cell production of IFN- γ . Delayed type hypersensitivity induced by IL-4-transduced antigen-pulsed DCs was diminished compared with control DCs. Our results indicate that therapeutic suppression of Th1-mediated autoimmunity and induction of Th2 responses *in vivo* by IL-4-transduced DCs occurs despite their potential to produce increased levels of IL-12, but reflects, in part, decreased production of IL-23.

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GRAIL: a gene related to anergy in lymphocytes

G Fathman, L Soares, N Anandasabapathy, C Seroogy

Division of Immunology and Rheumatology, Department of Medicine, Stanford University Medical School, Stanford, California, USA

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T-cell anergy may serve to limit autoreactive T-cell responses *in vivo*. Anergy induction *in vitro* is blocked by calcineurin inhibitors and by

inhibition of protein synthesis. In order to look for a potential anergy specific gene, we examined early changes in gene expression in murine CD4⁺ T-cell clones after antigen-T-cell receptor signaling in the presence (activation) or absence (anergy) of B7 co-stimulation. *GRAIL* (Gene Related to Anergy in Lymphocytes) was a novel transcript whose expression was markedly induced in anergic T cells *in vitro* compared with activated or resting T cells. *GRAIL* is a novel murine type I transmembrane protein that localizes to the endocytic pathway and bears homology to several RING Zinc-finger proteins. *GRAIL* functions as an E3 ubiquitin ligase. Expression of *GRAIL* in retrovirally transduced T-cell hybridomas dramatically limits activation-induced IL-2 production *in vitro*. Substitution of histidine for asparagine at two positions in the ring finger (H2N2 *GRAIL*) blocks enzymatic function of *GRAIL*. Retroviral transduction of hematopoietic stem cells to express *GRAIL* reiterates the anergy phenotype in resultant CD4⁺ T cells, including inability to secrete IL-2 or proliferate following antigen stimulation. Expression of the enzymatically inactive (dominant-negative) form of H2N2 *GRAIL* blocks anergy induction in T cells *in vivo*. These data demonstrate that *GRAIL* is necessary and sufficient to induce anergy in CD4⁺ T cells.

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Characterization of signaling complexes at the T-cell antigen receptor

L Samelson

Laboratory of Cellular and Molecular Biology, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland, USA
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Engagement of the T-cell antigen receptor (TCR) induces the assembly of signaling complexes comprised of adapter molecules and enzymes. We use multiple approaches in our characterization of these complexes and the process of signal transduction mediated by the TCR. Our genetic approach is the study of mutations in a critical adapter molecule, LAT. Some of these mutations lead to an interesting lymphoproliferative disorder. Signaling complexes can be studied *in vitro* using biochemical and biophysical methods to determine the rules of signal complex formation. Finally, we visualize the formation and fate of multi-protein complexes at the site of TCR activation. To do so, we express various fluorescently-tagged signaling proteins in T cells and observe clusters of molecules containing the TCR and important adapters and enzymes. Direct imaging of signaling molecules has revealed the highly dynamic nature of molecular interactions at the TCR.

15

Toll-like receptor family: receptors essential for microbial recognition and immune responses

S Akira, M Yamamoto, K Takeda

Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Japan

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Toll-like receptors (TLRs) play a critical role in the detection of invading pathogens within the body and subsequent immune response against them. We have generated knockout mice for individual TLRs and showed that individual TLRs recognize distinct microbial components. TLR4 is essential for the response to LPS. TLR2 is involved in the recognition of lipoproteins and peptidoglycan, together with TLR1 or TLR6. TLR9, TLR3, TLR5 and TLR7 recognize deoxyribonucleotides, ribonucleotides, flagellin, and imidazoquinolines, respectively. The TLR is a type 1 transmembrane receptor that is composed of an extracellular leucine-rich repeat domain and a cytoplasmic domain homologous to that of the IL-1R family. Upon stimulation, TLR recruits IL-1 receptor-associated kinase via adaptor MyD88, and finally induces activation of NF- κ B. Cytokine production in response to each TLR ligand is completely abolished in MyD88-deficient cells, indicating that MyD88 is an essential signaling molecule shared among the IL-1R/Toll family. However, several novel adaptor molecules have recently been identified. Evidence is now accumulating that differential utilization of these adaptors may activate overlapping as well as distinct signaling pathways, and finally may give rise to distinct biological effects exerted by individual TLR families.

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The actions of BAFF in B-lymphocyte maturation and its effects on the development of autoimmune disease

F Melchers

Department of Cell Biology, Biozentrum of the University of Basel, Basel, Switzerland

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BAFF, a member of the family of tumor necrosis factor ligands, is essential for the development of peripheral, mature, long-lived B lymphocytes. It binds to three different receptors (BCMA, TACI and BAFF-R), which are all members of the family of tumor necrosis factor receptors. Defects in the genes encoding either BAFF or BAFF-R abolish the generation of mature B cells. BAFF is made by myeloid cells while BAFF-R is expressed preferentially on B cells. BAFF induces polyclonal maturation of resting, short-lived immature cells to resting, long-lived mature B cells without proliferation. Lupus erythematosus-prone mice have elevated levels of BAFF in their blood, and treatment of these mice with BAFF decoy receptor (BCMA-Ig) prevents the onset of this autoimmune disease. Human lupus patients also show elevated levels of BAFF in their blood. Treatments with BAFF-neutralizing agents should prevent, delay or, at least, slow down the disease.

17

Proteomic surveillance of autoimmunity in osteoarthritis

T Kato¹, Y Xiang¹, T Sekine¹, H Nakamura¹, S Imajoh-Ohmi², H Fukuda², K Nishioka¹

¹Department of Bioregulation, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan;

²Laboratory Center for Proteomics Research, Institute of Medical Science, University of Tokyo, St Marianna University School of Medicine, Tokyo, Japan

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Objectives To understand immunological aspects of osteoarthritis (OA), which has been considered a degenerative disease, we compared profiles of autoimmunity comprehensively between OA and rheumatoid arthritis (RA) using analysis of the chondrocytes' proteome.

Methods Proteins extracted from normal articular chondrocytes were separated by two-dimensional electrophoresis. Western blotting (WB) was then used to detect antigenic protein spots, using 20 serum samples from either OA or RA patients. Mass fingerprinting was used for identification of the detected autoantigens. The identified proteins were prepared as recombinant fusion proteins with maltose binding protein (TPI-MBP) to confirm their antigenicity and to investigate the frequency of the autoantibodies, epitope localization, and clinical significance by ELISA and WB, using serum samples obtained from 93 patients with OA, 54 patients with RA, and 43 patients with SLE.

Results Sixty-two autoantigens were detected in responses to OA and RA serum samples, in which 19 protein spots were detected only in the OA group. One of these apparently OA-specific protein spots, detected in four out of 20 OA patients but not in RA patients, was identified as human triose phosphate isomerase (TPI). All four positive sera against this spot reacted to a fusion protein of TPI-MBP but not MBP alone. Also, TPI-MBP affinity-purified antibodies from these sera only reacted to the spot that was identified as TPI in the two-dimensional electrophoresis membrane. The frequencies of the anti-TPI IgG in the sera from OA, RA, and SLE patients were 24.5%, 5.6%, and 4.7%. Further frequencies of the autoantibody in synovial fluid from 29 OA patients and 19 RA patients were found to be 24.1% and 0%, respectively. All the positive samples were further confirmed by WB. In the epitope mapping using eight truncated recombinant TPI proteins, multiple epitopes were identified, one of which was recognized in more than 90% of the positive serum samples. Clinically, the X-ray grading was lower in the anti-TPI-positive OA group than that in the anti-TPI-negative group.

Conclusion The overall profile of autoimmunity in OA differs from that in RA, which may reflect OA-specific pathological roles of autoimmunity. The autoantibodies to TPI, detected in OA predominantly and produced by the antigen-driven mechanism, would have potential as a diagnostic marker for OA.

Topics Symposium (3) Locomotor Science

18

Regulation of synovial cell function by adenovirus vector-mediated gene transduction

S Tanaka, H Seto, H Oda, K Nakamura

Department of Orthopaedic Surgery, The University of Tokyo, Tokyo, Japan
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It has been recently demonstrated that synovial fibroblasts (SFs) contain a multipotent mesenchymal cell population. To examine the chondrogenic potentialities of SFs *in vitro* and *in vivo*, SFs were isolated from knee joints of rabbits or rheumatoid arthritis patients, and infected with adenovirus vectors carrying LacZ (control), constitutively active forms of activin receptor like kinase (ALK)-3 or ALK-5 genes. Efficient gene transduction was confirmed by β -galactosidase staining of the LacZ virus-infected SFs. Northern blotting of type II collagen and aggrecan genes showed clear induction of these genes in SFs infected with ALK-3 virus, while no chondrogenic phenotypes were observed in LacZ or ALK-5-infected cells. ALK-3 virus-infected SFs were also positively stained by Alcian blue staining and type II collagen immunostaining. When transplanted into cartilage defects of rabbit knee joints, ALK-3 virus-infected rabbit SFs produced repair cartilage of hyaline morphology containing a type II collagen-positive matrix that restored the articular surface. These results suggest that adenovirus vector-mediated ALK-3 gene expression can induce chondrogenic differentiation of synovial fibroblasts, and that they are promising candidates for cell-based therapies for articular cartilage defects.

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Role of BH3-only protein Bim in autoimmune and degenerative diseases

P Bouillet, S Cory, J Adams, A Strasser

Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute, Melbourne, Australia

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Background Apoptosis is the physiological process used by an organism to eliminate cells that are no longer needed, have been damaged or are dangerous. Defects in the control of apoptosis have been implicated as a cause or a contributing factor in a variety of diseases. Proteins of the Bcl-2 family are major regulators of this process.

We have previously shown that Bim is required for certain apoptotic responses, for hematopoietic cell homeostasis and as a barrier against autoimmune disease.

Objectives We study Bim-deficient mice in order to understand the role of Bim in homeostasis and to evaluate its role in the ontogeny of certain autoimmune and degenerative diseases.

Methods We intercrossed Bim-deficient mice with mouse strains used as models for such diseases, namely *bcl-2-KO*, *PKD1-KO*, *IL7R-KO* and *Lurcher* mice, and analysed double mutants.

Results We have shown that Bim is essential for the development of tolerance to self-antigens [1]. Using six different model systems, we have demonstrated that, during development in the thymus, Bim plays a major role in the elimination of the T lymphocytes that bear a T-cell receptor (TCR)/CD3 complex that engages self-antigens (negative selection). Thus, Bim appears essential for apoptosis of autoreactive T lymphocytes and B lymphocytes in central as well as peripheral tolerance.

By intercrossing mice with mutations in *bcl-2* and *bim*, we have generated mice lacking both genes and shown that all the deficiencies caused by the absence of Bcl-2 (runting, polycystic kidneys, lymphopenia, hair greying) could be efficiently rescued by the concomitant absence of Bim [2]. This result demonstrates that BH3-only proteins can be involved in the induction of certain degenerative diseases.

By contrast, removal of Bim does not prevent the degeneration of cerebellar Purkinje cells and granular neurons in *Lurcher* mice.

We are currently investigating the consequences of the loss of Bim in *PKD1*-deficient and *IL7R*-deficient mice.

Conclusions Our results indicate that BH3-only proteins may be at the origin of certain autoimmune and degenerative diseases, and may be targets of interest for the research of new drugs against such diseases.

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Regulation of RANKL signaling in arthritic bone destruction

H Takayanagi

Department of Immunology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan and PRESTO, Japan Science and Technology Corporation (JST), Japan

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Formation of osteoclasts is induced by a tumor necrosis factor family cytokine, RANKL (receptor activator of NF- κ B ligand). To maintain the normal bone homeostasis and to prevent the pathological bone resorption, RANKL signaling must be strictly kept in control.

During the course of our study on the bone loss in rheumatoid arthritis (RA), we found that RANKL expressed on synovial fibroblasts is responsible for osteoclastogenesis from synoviocytes. However, it has been also reported that RANKL-expressing T cells are involved in osteoclastogenesis in RA. We then focused on the regulation of osteoclast differentiation by T cells. Using mice lacking a receptor component for IFN- γ , we revealed that T-cell production of IFN- γ strongly suppresses osteoclastogenesis by interfering with the RANKL signaling pathway [1]. We have shed light on a new biological function of IFN- γ , which is to protect against calcified tissue destruction upon T-cell activation, demonstrating that activated T cells not only positively regulate, but also negatively affect osteoclastogenesis.

To explore the molecular targets for suppressing bone destruction in RA, we performed a genome-wide screening of RANKL-inducible genes. We found that RANKL induces IFN- β , a critical cytokine for antiviral defense. Mice deficient in IFN- β signaling exhibited severe osteopenia accompanied by enhanced osteoclastogenesis, suggesting that IFN- β is essential for normal bone remodeling by suppressing excessive osteoclast differentiation [2]. In addition, we revealed beneficial effects of IFN- β in animal models of pathological bone resorption. We have recently identified that the transcription factor NFATc1 is specifically induced by RANKL [3]. We demonstrate that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL stimulation, and the ectopic expression of NFATc1 causes the precursor cells to undergo efficient differentiation without RANKL signaling. Thus, NFATc1 may be a master switch regulator for the terminal differentiation of osteoclasts, functioning downstream of RANKL signaling. The activation of NFATc1 by RANKL is regulated by calcium-dependent phosphatase, calcineurin, and calcineurin inhibitors such as FK506 and cyclosporin A strongly suppress osteoclastogenesis. The possibility of NFATc1 as a therapeutic target of bone destruction in RA will be discussed.

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Transcription mechanism of chondrogenesis

H Asahara

The Scripps Research Institute, La Jolla, California, USA

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The precise patterning of a developing skeletal framework relies on appropriate control of chondrogenesis and on subsequent cartilage

development. This multistep process, where mesenchymal cells differentiate into chondrocytes and then chondrocytes progress through each developmental zone of the cartilage, is tightly regulated by a number of key signaling molecules, which include PTH-related protein, fibroblast growth factor, bone morphogenetic protein and transforming growth factor. Importantly, these factors have been shown to promote phosphorylation of transcription factor cAMP response element (CREB) at its Ser133, which activates specific gene expression with recruitment of its transcriptional co-activator CREB binding protein (CBP). Recently, CBP has been shown to have intrinsic histone acetyltransferase activity, which suggests a potential link between gene expression and chromatin acetylation. In this regard, the important role of CBP in CREB-dependent gene expression has been demonstrated using a novel *in vitro* chromatinized template transcription assay. With these findings, the importance of the CREB/CBP transcription complex for chondrogenesis was examined by expressing a potent dominant-negative CREB inhibitor (A-CREB). Consistent with the robust Ser133 phosphorylation of CREB during chondrogenesis, A-CREB blocked chondrogenesis from mesenchymal stem cells. During chondrogenesis, specific chromatin factors are activated by the CREB/CBP pathway and the chromatin factors promote chondrocyte-specific gene expression via association with multiple transcription factors that are known to be involved in chondrocyte differentiation. These findings suggest that, in addition to DNA-binding type transcription factors, chromatin factors, which alter the chromatin code, may play a critical role for cell differentiation and tissue-specific gene expression in cartilage development.

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Cellular therapies in musculoskeletal diseases: synovial-derived mesenchymal stem cells

F Luyten

Department of Rheumatology, University Hospitals KU Leuven, Leuven, Belgium

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We have identified and characterized a population of adult human mesenchymal stem cells (MSCs) from the synovial membrane (SM) capable of differentiation to cartilage, bone, skeletal muscle, and adipose tissue *in vitro*. SM-MSCs can be obtained without irreversible damage, are easily expandable with limited senescence, and are phenotypically stable throughout expansion and after storage in liquid nitrogen. The use of MSCs is restricted by the as yet insufficient knowledge of the long-term stability of the repair tissue and by their tendency to differentiate towards other cell lineages. Their multilineage potential may present a risk of heterotopic tissue formation, and *in vivo* preclinical studies in experimental models relevant to specific clinical applications should therefore be performed.

Several applications will be discussed, including autologous cell transplantation for the repair of joint surface defects, a procedure that should be carried out using cells that are stably committed to the articular cartilage phenotype, naturally resistant to vascular invasion, mineralization, and ossification. Therefore, we investigated whether human SM-MSCs can acquire *in vitro* the expression of the markers reported to be associated with the stable-chondrocyte phenotype, and whether this is associated with the capacity to form stable cartilage *in vivo*.

Further exploration of the plasticity of the SM-MSCs led to the unexpected finding that they participate in the repair of skeletal muscle in two animal models. We characterized the myogenic differentiation of this cell population in a nude mouse model of skeletal muscle regeneration, and demonstrated that they contributed to myofibers and to long-term persisting functional satellite cells. When administered into dystrophic muscles of immunosuppressed mdx mice, human SM-MSCs restored, at least in part, muscle function in this mouse model of Duchenne muscular dystrophy.

Many challenges remain in the area of cellular products, including product specification and quality control, and proper prospective, multi-center, randomized, clinical studies comparing this with standard treatments. There is no doubt that a clear global regulatory path is needed for the development of these novel approaches, as lack of transparency and conflicting legislation in regulation is one of the major threats preventing proper development of the field.

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Role of bone morphogenetic protein signals during skeletal development

N Tsumaki¹, M Horiki¹, T Kimura², H Yoshikawa¹

¹Department of Orthopaedic Surgery, Osaka University Medical School, Osaka, Japan; ²Department of Orthopaedic Surgery, Toyama Medical and Pharmaceutical University, Toyama, Japan
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Development of the skeletal components of limbs is initiated by mesenchymal cell condensation to form primordial cartilage, followed by endochondral ossification. Cartilage serves as the template for the formation of most bones. During development, proliferating chondrocytes differentiate into hypertrophic chondrocytes. In the final step in endochondral bone formation, the hypertrophic cartilage is invaded by blood vessels and osteoprogenitor cells, and the calcified cartilage is subsequently replaced by bone. Bone morphogenetic proteins (BMPs) were originally identified as secreted signaling molecules that could induce endochondral bone formation. Subsequent molecular cloning studies have revealed that the BMP family consists of various molecules, including members of the growth and differentiation factor (GDF) subfamily. BMPs have diverse biological activities during development of various organs and tissues, and the precise roles of BMP signals during mammalian skeletal development have yet to be determined. We previously isolated the promoter/enhancer sequence of the $\alpha 2(XI)$ collagen chain gene; this sequence is responsible for the chondrocyte-specific expression during mouse development. Using this sequence, we created transgenic mice that overexpress BMP4, GDF5 and Noggin (a BMP antagonist) in cartilage. Overproduction of BMP4 or GDF5 in cartilage increased cartilage production and enhanced chondrocyte differentiation. Noggin-expressing transgenic mice, in which BMP signals seem to be blocked in cartilage, lacked most of the cartilage and mature hypertrophic chondrocytes. These results indicate that BMP signals are essential for cartilage development.

BMP signals are mediated by Smad proteins intracellularly. We attempted to block Smad signaling in developing cartilage by overexpression of Smad6, an inhibitory Smad, in transgenic mice. In those mice, the cartilage grew almost normally until birth, but osteopenia developed after birth. Skeletal abnormalities were much less severe in the present Smad6 transgenic mice than in Noggin transgenic mice examined in a previous study; these two strains of transgenic mice had identical promoter/enhancer sequences. The relatively mild phenotype of Smad6 transgenic mice suggests a mechanism in which Smad6 alone cannot completely inhibit transduction of BMP signals in cartilage.

To analyze the roles of BMPs in the final stage of endochondral bone formation, we generated transgenic mice that expressed BMP4 or Noggin in osteoblasts. Expression of Noggin in osteoblasts inhibited bone formation, as expected. On the contrary, BMP4 overexpression in osteoblasts resulted in disruption of skeletal architecture. Histological examination revealed a reduced mineralized matrix in BMP4 transgenic mice. These results suggest that persistent expression of BMP4 does not cause formation of solid bone.

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Implication of Synoviolin in pathogenesis of arthropathy

T Nakajima

Department of Genome Science, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan
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Rheumatoid arthritis (RA) is one of the common disorders characterized with overgrowth of articular synovial cells, so-called 'pannus', and autoimmune reaction. To understand the pathomechanism of RA, we attempted to characterize the rheumatoid synovial cell and found a novel membranous protein, Synoviolin (synovial cell⁺ protein). Its overexpression causes arthropathy, resembling RA in mice. Moreover, the heterozygote of synoviolin (+/-) is resistant to anticollagen antibody-induced arthritis. These 'gain of function' and 'loss of function' analyses clearly indicate the pathogenetic role of synoviolin in arthropathy.

Topics Symposium (4) Rheumatology (2): B cell and SLE

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Cyclin kinase inhibitors and interferons in lupus

A Theofilopoulos

Scripps Research Institute, Department of Immunology, La Jolla, California, USA

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Many investigators have directly examined the role of deleted/blocked or overexpressed immune-related genes in lupus background mice. The effects of such manipulations have provided important insights into the crucial molecules and pathways involved in this disease and into the development of new therapies. We report here on the effects of two classes of genes: cyclin-dependent kinase inhibitor p21, and interferons (IFNs).

A characteristic feature of lupus in humans and mice is the accumulation of activated/memory T cells and B cells. These G1-arrested cells express high levels of p21, are resistant to proliferation and apoptosis, and express high levels of proinflammatory cytokines. We hypothesized that accumulation of these cells results from repetitive engagements by self-molecules *in vivo*, and that deletion of p21 may lead to their reduction. Indeed, lupus-prone BXSB mice lacking p21 have enhanced T-cell and B-cell proliferation but, importantly, have increased apoptosis, resulting in a net reduction of activated/memory cells and marked inhibition of disease. Increased apoptosis of p21-deleted cells is mediated by engagement of both the extrinsic (Fas/FasL) and intrinsic (Bcl2) related pathways. Modulation of the cell-cycle pathway may be a novel approach to reduce activated/memory-resistant and apoptosis-resistant pathogenic T cells and B cells, and to ameliorate systemic autoimmunity. Another class of molecules to ameliorate lupus is type I and type II IFNs, since considerable evidence exists that these pleiotropic molecules are important effectors in this disease. Our earlier studies showed decreased serologic and histologic disease characteristics and increased survival of MRL-Fas^{lpr} mice deleted of the IFN- γ gene or treated, even at advanced stages, with cDNA encoding IFN- γ -Fc. More recently, we created congenic NZB mice lacking the α -chain of IFN- α/β , the common receptor for the multiple IFN- α/β species. Compared with littermate controls, homozygous-deleted mice had significantly reduced anti-erythrocyte autoantibodies, erythroblastosis, hemolytic anemia, anti-DNA autoantibodies, kidney disease and mortality. These reductions were intermediate in the heterozygous-deleted mice. The disease-ameliorating effects were accompanied by reductions in several immune cell subsets (including B1 cells) and reduced dendritic cell maturation. The cumulative data indicate that both type I and type II IFNs are important mediators in the pathogenesis of murine lupus, and that reducing their activity in the human counterpart may be beneficial.

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Origins of systemic lupus erythematosus: genes, environment, and host immune response

JB Harley¹, JA Kelly², JA James³

¹Department of Medicine, University of Oklahoma, Arthritis and Immunology Program, Oklahoma Medical Research Foundation and US Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma, USA; ²Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA; ³Arthritis and Immunology Program, Oklahoma Medical Research Foundation and Department of Medicine, University of Oklahoma, Oklahoma City, Oklahoma, USA
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Systemic lupus erythematosus (SLE) appears to be a consequence of immune dysfunction mediated by errors in self-recognition that cause autoimmunity. A component of the environmental origin of SLE was suggested by data showing that the earliest autoantibody recognition structure in the anti-Sm response was very similar to and cross-reacted with Epstein-Barr virus nuclear antigen-1 (EBNA-1). Subsequently, an

earliest epitope of the anti-Ro system has been identified and this structure also imitates a structure of EBNA-1. The initial structures bound by anti-Sm and anti-Ro generate SLE-like autoimmunity in animals immunized with these peptides. In pediatric SLE, anti-EBNA-1 is more frequently present and its fine specificity is qualitatively more diverse in SLE than in normals. Anti-EBNA-1 responses also precede the onset of SLE. The well-known association of Epstein-Barr virus infection with SLE may be present because of the particular immunoregulatory details of the anti-EBNA-1 response in SLE patients. The genetic origins of SLE are the other major component of SLE etiology. At this time, to our knowledge, 11 genetic linkages have been both established and independently confirmed. For three of these linkages, an associated candidate gene is known. They are located in the human genome as follows: 1q23 (FcγRIIIA), 1q41, 2q34, 2q37 (PDCD-1), 4p16, 5p15, 6p21 (HLA-DR), 10q22, 11p13, 11q14, and 16q13. Genetic effects tend to concentrate in the major human racial groups (e.g. 1q23, 2q34, 11p13, and 11q14 dominate in African-Americans). Some clinical and laboratory features of SLE have powerful genetic influences and can be used to generate genetic homogeneity (e.g. nephritis with 2q34 and 10q22, hemolytic anemia with 11q14, and pedigrees multiplex for self-reported rheumatoid arthritis with 5p15). The origins of SLE are obviously complicated and involve multiple influences from the environment, the host immune response, perhaps involving EBNA-1, and the genetic constitution of the patient.

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Analysis of B-cell subpopulations in systemic lupus erythematosus

T Dorner¹, A Jacobi¹, M Odendahl¹, GR Burmester¹, A Radbruch¹, G Valet², PE Lipsky³

¹Department of Rheumatology and Clinical Immunology, Charite University Hospital and Deutsches Rheumaforschungszentrum, Berlin, Germany;

²Max-Planck Institute of Biochemistry, Munich-Martinsried, Germany;

³NIAMS, National Institutes of Health, Bethesda, Maryland, USA

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Abnormalities in humoral immunity play a significant role in systemic lupus, mandating further studies in B-cell autoimmunity in this entity. Disease activity in systemic lupus erythematosus (SLE) is usually assessed with complex disease activity scores composed of a variety of different parameters. To determine whether SLE disease activity correlated with abnormal B-lymphocyte activity, B-cell subsets were analyzed and related to clinical measures. The distribution of B-cell subsets was determined by fluorescence-activated cell sorting analysis and compared with the autoantibody profile, with the disease activity measured by the SLE disease activity index (SLEDAI) and the European Consensus Lupus Activity Measurement (ECLAM), disease duration and therapy. The number and frequency of CD27^{high} plasma cells were significantly correlated with the SLE disease activity indices (SLEDAI and ECLAM) and the titer of anti-dsDNA autoantibodies. Circulating B-cell subsets were not influenced by age or gender, but appeared to relate to the duration of disease and the therapeutic regimen, with the numbers and frequencies of CD27^{high} plasma cells increasing and those of CD27-naïve B cells decreasing over time. Patients were divided into those with a SLEDAI score of 0–8 (low activity) and those with a SLEDAI score >8 patients (high activity). High-activity patients were found to have an increased frequency of CD19⁺ B cells as well as CD27^{high} plasma cells. Using a nonparametric data sieving algorithm, these B-cell abnormalities exhibited predictive values for nonactive and active disease of 78.0% and 78.9%, respectively. The predictive value of the B-cell abnormalities was greater than that of the humoral/clinical data pattern, including anti-DNA antibody levels, circulating immune complexes, increased erythrocyte sedimentation rate, mucocutaneous and acute renal involvement, which showed predictive values of 77.8% and 70.0% for active and nonactive disease. Flow cytometric monitoring of B-cell subsets in the peripheral blood provides new insights into the abnormalities in B-cell function in SLE, and may also be a diagnostically valuable option to follow disease activity in this autoimmune disease.

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Antiphospholipid antibodies and thrombosis: pathogenesis of antiphospholipid syndrome

T Koike

Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan

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Antiphospholipid antibodies are present in a wide range of infectious and autoimmune diseases. Antiphospholipid antibodies, in particular anticardiolipin antibodies (aCL), lupus anticoagulants and antiprothrombin antibodies, are of considerable clinical importance because of the close association with predominant clinical features of venous and arterial thrombosis and pregnancy morbidity. The term antiphospholipid syndrome (APS) has been used to define this set of pathologic features. Recognition of this syndrome is now better understood worldwide as related clinical implications are now more well defined.

aCL found in APS patients are directed against phospholipid-binding plasma or serum proteins, in particular, β₂-glycoprotein I (β₂-GPI). Such aCL (anti-β₂-GPI autoantibodies) recognized epitopes appearing on the β₂-GPI molecule when β₂-GPI interacts with a lipid membrane composed of negatively charged phospholipids or when β₂-GPI is adsorbed on a polyoxymethylene polystyrene plate treated with γ-irradiation or electrons.

Anti-β₂-GPI antibodies have been found to activate endothelial cells by inducing a proinflammatory and procoagulant phenotype sustained by the upregulation of adhesion molecule (E-selectin, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1) expression, synthesis and secretion of cytokines, chemokines, endothelin-1 and tissue factor. Anti-β₂-GPI antibody binding has been shown to induce NF-κB translation, leading to proinflammatory cell phenotypes similar to that elicited by interaction with lipopolysaccharide and proinflammatory cytokines (IL-1β, tumor necrosis factor alpha). Very recently, it was reported that anti-β₂-GPI antibodies activate cells through the MyD88-dependent pathway, therefore implicating members of the Toll-like receptors family.

In this lecture, we will discuss the relation between the anti-β₂-GPI antibodies, the Toll-like receptors/IL-1 receptor family on the cell surface and the pathogenesis of APS.

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A Phase I trial of B-cell depletion with anti-CD20 monoclonal antibody (rituximab) in the treatment of systemic lupus erythematosus

R Eisenberg¹, D Albert¹, J Stansberry¹, D Tsai², S Kolasinski¹, S Khan¹

¹Division of Rheumatology, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA; ²Division of Hematology/Oncology, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

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We have tested whether the removal of B cells could suppress the manifestations of systemic lupus erythematosus (SLE). Rituximab is a chimeric monoclonal antibody that targets the pan-B-cell marker CD20. Administration of this biologic causes profound depletion of B cells from the peripheral blood for periods of several months. Rituximab has been shown to be efficacious against B-cell malignancies, particularly non-Hodgkins B-cell lymphoma. To begin to determine whether B-cell depletion with rituximab in SLE could be of benefit, we have initiated a phase I safety trial in 12 patients. Patients with SLE with moderate disease activity who had failed at least one cytotoxic drug were eligible for treatment. Nine patients have been entered to date. The first two patients received a low, subtherapeutic dose, but six of the next seven patients received the full approved regimen of 375 mg/m² once a week for four doses. The first two patients developed human antichimeric antibodies, but the next five patients tested did not. The first patient showed an unusual post-infusion reaction, including bradycardia. The other patients tolerated their infusions well. The first five patients who received the full regimen had >95% depletion of B cells from their peripheral blood 4 weeks after the final dose of rituximab. All patients had lower SLE disease activity index scores after treatment. Three patients with rash and alopecia had improvement. Patient 3 was partic-

ularly striking. She had had a recent history of serious renal, pulmonary and infectious complications, treated with cyclophosphamide and high-dose steroids. After rituximab, patient 3 showed the disappearance of activated T cells from the peripheral blood. One year post rituximab treatment she remains off steroids and cytotoxics, and clinically quiescent. The patient's B cells have now returned, with lower levels of CD27 and CD86 than before treatment, and her T cells are still largely unactivated. These uncontrolled data suggest that rituximab is well tolerated in SLE and that it may have some therapeutic benefit. A controlled trial with appropriate outcomes will be necessary to prove whether this approach is indeed efficacious.

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Failure of receptor revision in the autoreactive B cells in patients with systemic lupus erythematosus

H Yoshikawa¹, H Nagafuchi², M Kurokawa¹, T Sakane¹, N Suzuki¹

¹Department of Immunology and Department of Medicine, St Marianna University School of Medicine, Kawasaki, Japan; ²Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan
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Systemic lupus erythematosus (SLE) is a prototype of autoimmune diseases characterized by B-cell hyper-reactivity and production of various autoantibodies. It has recently been found that the recombination activating gene (RAG) is re-expressed in activated mature B cells, and RAG-expressing B cells revise their surface immunoglobulin or die by failure of productive secondary rearrangement, leading to elimination of the autoreactive B cells. We have investigated the expression of RAG proteins in mature B cells in patients with SLE. Although normal mature B cells did not express RAG proteins spontaneously, activated B cells expressed RAGs. In SLE, whole mature B cells spontaneously expressed RAG proteins excessively. Because we had found that failure of receptor editing of the V_k gene with autoreactive potential (A30-V_k2) is associated with the development of anti-DNA antibody in SLE, we next focused on anti-DNA autoantibody secreting B cells. We found that anti-DNA autoantibody secreting B cells failed to re-express RAGs spontaneously, even after activation with mitogen. Thus, anti-DNA autoantibody secreting B cells did not revise their immunoglobulin gene nor die via apoptosis, leading them to develop and persist with autoantibody secretion. These findings indicate the importance of appropriate RAG re-expression for the elimination of autoreactive B cells even at the mature stage, and the absence of such events in anti-DNA secreting B cells may be important for autoantibody production in SLE.

Topics Symposium (5) Cytokines & Inflammation (1)

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Chemokines in rheumatoid arthritis: novel, potential therapeutic targets of the CCR2 chemokine receptor signaling pathway

K Matsushima, Y Terashima, M Haino

Department of Molecular Preventive Medicine, University of Tokyo, Tokyo, Japan

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Several chemokine receptor antagonists are in phase I/II clinical trials. Macrophages play a pivotal role in rheumatoid arthritis, and the monocyte chemoattractant protein (MCP)-1/CCR2 axis determines the macrophage recruitment into inflamed joints. We have recently discovered molecules specifically associated with CCR2.

We incidentally discovered that an organic germanium, propagermanium (3-oxogermylpropionic acid polymer), which has been used as a therapeutic agent against chronic hepatitis associated with hepatitis B virus in Japan, very specifically inhibits CCR2-mediated monocyte chemotaxis. The effect of propagermanium requires glycosylphosphatidylinositol (GPI)-anchored proteins, as cleavage of GPI anchors by phosphatidylinositol-phospholipase C eliminated the inhibitory activity of propagermanium. In addition, anti-GPI-anchored protein CD55 antibody and anti-CD95 antibody selectively inhibited MCP-1-induced monocyte chemotaxis. Furthermore, GPI-anchored proteins were co-localized with CCR2 on monocytes

under fluorescence microscopy. Moreover, a synthetic peptide corresponding to the N-terminal portion of CCR2 specifically blocked the action of propagermanium, suggesting that propagermanium may bridge GPI-anchored proteins and the N-terminal portion of CCR2, and may interfere with the action of MCP-1 (collaboration with Ishiwata *et al.*, Sanwa Kagaku, Mie, Japan). The biological meaning of the close association of GPI-anchored proteins with CCR2 is unclear at present, but this finding corresponds well with the notion that some of chemokine receptors exist in the lipid raft of leukocyte cell membrane.

Critical roles of C-terminal portion of chemokine receptors in regulating leukocyte chemotaxis have been suggested. To seek CCR2-interacting proteins, we have adopted a yeast two-hybrid system and found a cytoplasmic protein, FROUNT, that specifically interacts with CCR2. Disrupting the interaction through the expression of FROUNT antisense mRNA or a truncated FROUNT mutant specifically abolished the chemotactic response to MCP-1 in human monocytes. FROUNT seems to be critically involved in receptosome formation based on confocal microscopic analysis. Suppression of endogenous FROUNT in a murine model of peritonitis markedly impaired CCR2-dependent recruitment of macrophages into the peritoneal cavity. Those results implicate that FROUNT regulates CCR2-mediated chemotactic signaling and the FROUNT could be a novel therapeutic target for macrophage-mediated chronic inflammatory diseases including rheumatoid arthritis.

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Effect of the combination of the proinflammatory cytokines IL-1, tumor necrosis factor- α and IL-17

P Miossec, C Granet, G Chevreil

Department of Immunology and Rheumatology, Hôpital E Herriot, Lyon, France

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The list of cytokines that may contribute to joint destruction in rheumatoid arthritis is growing extensively. At the same time, inhibition of a single cytokine such as tumor necrosis factor (TNF)- α or IL-1 was able to control disease in a significant proportion of patients. Accordingly, the mode of interactions between these cytokines has to be clarified in order to understand how a single cytokine inhibition can still be effective. These interactions were studied with mesenchymal cells, which are targets of cytokines (synoviocytes, osteoblasts, myoblasts), as well as with the respective organ samples (synovium, bone, muscle) in order to keep intact interactions as seen *in vivo*. Cells and explants were exposed to cytokines used at low concentrations and high concentrations in order to look at their effect alone and a possible additive or synergistic effect when combined. Their respective soluble receptors were used as specific inhibitors alone and in combination.

Effects on AP-1, NF- κ B and Egr-1 activation were explored by RT-PCR and immunocytochemistry. IL-1 and TNF- α induced most of these transcription factors while IL-17 had a weak effect on the different mesenchymal cells. More importantly, when these cytokines were used at low concentrations with no effect alone, their combinations showed a synergistic effect on transcription and nuclear translocation of AP-1 members, Egr-1 and NF- κ B. Moreover, cytokine combinations allowed an enhanced recruitment of factors not expressed by cytokines used alone. Conversely, combination of specific inhibitors (p75 TNF and type II IL-1 soluble receptors) was needed to completely abrogate NF- κ B nuclear translocation in myoblasts stimulated with both IL-1 and TNF- α . Low concentrations of cytokines can have a significant biological effect through their interactions mediated by synergistic mechanisms.

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Human adipose tissue is an important novel source of IL-1Ra: a new connection between immuno-inflammatory diseases and lipid metabolism

J Dayer¹, C Meier²

¹Division of Immunology and Allergy, University Hospital, Geneva, Switzerland; ²Division of Endocrinology and Diabetology, University Hospital, Geneva, Switzerland

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The increased production of IL-1, tumor necrosis factor (TNF)- α and IL-6 is implicated in the pathogenesis of various immuno-inflammatory

diseases (i.e. rheumatoid arthritis), often accompanied by metabolic and cardiovascular complications. Cachexia and obesity may accompany these diseases, and IL-1, TNF- α , IL-6 are said to be increased in white adipose tissue (WAT). Fewer studies devolve on counter-regulatory mechanisms by cytokine inhibitors. Sera of obese patients showed a more than sevenfold increase in IL-1 receptor antagonist (IL-1Ra), which matches levels present in inflammatory autoimmune diseases and sepsis, and correlating with body mass index and insulin resistance. Subcutaneous and visceral human WAT of obese patients contained 0.4 and 0.7 ng IL-1Ra/mg protein, respectively. Thus, in an obese individual weighing 120 kg with 50% body fat, the total WAT is estimated to contain 0.6 mg IL-1Ra protein (i.e. 200 times the amount of IL-1Ra in total serum), thus representing one of the main sources of IL-1Ra production. The increased IL-1Ra expression – not associated with increased IL-1 β – argues for an anti-inflammatory, compensating, mechanism associated with obesity. Our experiments with human WAT explants showed a strong stimulatory effect of phorbol myristate acetate and, more importantly, of IFN- β (as much as fivefold to 10-fold). Since the latter is considered a fibroblast-derived IFN, it is tempting to speculate that stromal cells and adipocytes might be part of a paracrine mechanism regulating IL-1Ra secretion. In fact, newly formed adipose tissue is often found close to inflammatory lesions (i.e. synovial tissue). The functional consequences of the increased production of IL-1Ra by adipose tissue may represent an important counter-regulatory mechanism to inflammation at the local level. Furthermore, IL-1RI and IL-1RacP were also expressed in human WAT. Thus, substances that increase the production ratio of IL-1Ra/IL-1 β by adipose tissue might serve as a novel target for therapeutic strategies in immune disease.

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IFN- β for the treatment of rheumatoid arthritis?

P Tak¹, J van Holten¹, K Reedquist¹, P Sattonet-Roche², T Smeets¹, M Vervoordeldonk¹, C Plater-Zyberk²

¹Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, The Netherlands; ²Serono, Geneva, Switzerland

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IFN- β is emerging as a pivotal molecule involved in synovial inflammation and bone homeostasis. We conducted *in vitro* studies as well as studies using the collagen-induced arthritis model to investigate the effects of IFN- β .

DBA/1 male mice were immunized intradermally with bovine type II collagen in complete Freund's adjuvant. On the first clinical sign of disease, mice were treated for 7 days by daily intraperitoneal injections of recombinant mouse IFN- β or saline. Disease progression was monitored by validated visual clinical scoring and by measurement of paw swelling using calipers. Inflammation and joint destruction were assessed histologically 8 days after the onset of arthritis on decalcified wax-embedded paw sections and quantified. Safranin O staining was performed to determine proteoglycan depletion. In addition, cytokine profiles in the synovium were evaluated by immunohistochemistry. Cytokine expression was also measured in supernatants of rheumatoid arthritis (RA) and osteoarthritis fibroblast-like synoviocytes (FLS) in culture after incubation with IFN- β . We incubated RA FLS, which were transfected with a NF- κ B/luciferase construct, with IFN- β and measured luciferase activity to determine the effects on NF- κ B activity.

In two independent experiments with eight to 10 mice per treatment group in each experiment, IFN- β at doses from 0.25 μ g/injection and higher significantly reduced disease severity. Moreover, IFN- β -treated animals had significantly less cartilage and bone destruction than control animals, demonstrating a protective effect of the treatment. There was a significant reduction in c-Fos expression and osteoclast numbers. The proinflammatory cytokines tumor necrosis factor alpha and IL-6 were significantly reduced, and IL-10 production was increased after IFN- β treatment. *In vitro* studies revealed reduced NF- κ B activity and expression of proinflammatory cytokines in RA FLS after IFN- α treatment.

Taken together, our data show that frequent exogenous administration of IFN- β protein may reduce synovial inflammation and protects against cartilage and bone destruction by inhibition of NF- κ B activity, immunomodulation, and impairment of osteoclastogenesis through inhi-

bition of c-Fos. Continuous IFN- β expression at the site of inflammation may be required to reach these therapeutic effects in RA patients. The exciting biological effects could translate into clinically meaningful improvement if the cytokine is administered frequently, when pegylated IFN- β is used, or when IFN- β gene therapy is used. Therefore, IFN- β gene therapy studies are now underway.

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PPAR γ ligands inhibit catabolic and inflammatory responses in articular joint cells

H Fahmi, J-P Pelletier, F Mineau, J Martel-Pelletier

University of Montreal Hospital Research Center, Notre-Dame Hospital, Montreal, Canada

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Overproduction of inflammatory and catabolic mediators in articular joint tissues is a hallmark of many rheumatic diseases such as osteoarthritis and rheumatoid arthritis. Peroxisome proliferator-activated receptor gamma (PPAR γ) is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. In addition to their roles in lipid and glucid metabolism, PPAR γ ligands have also been shown to modulate inflammatory responses in many cell types. We examined the expression of PPAR γ and the effect of its ligands on inflammatory and catabolic responses in articular joint cells.

We showed that PPAR γ is expressed and transcriptionally active in human chondrocytes and synoviocytes. Pretreatment of human chondrocytes with PPAR γ ligands (15d-PGJ2 and BRL 49653) inhibited IL-1-induced nitric oxide and matrix metalloproteinase (MMP)-13 production. The induction of both inducible nitric oxide synthase and MMP-13 mRNA was inhibited in the presence of 15d-PGJ2. The inhibitory effect of PPAR γ ligands was not restricted to IL-1, since tumor necrosis factor alpha and IL-17-induced nitric oxide and MMP-13 production were also inhibited by 15d-PGJ2. Similarly, pretreatment of synoviocytes with PPAR γ ligands inhibited IL-1-induced MMP-1 at the protein and mRNA levels. The inhibitory effect of 15d-PGJ2 occurred at least in part through a PPAR γ -dependent pathway, probably by interfering with the transcriptional activity of AP-1 and NF- κ B. We also examined the effect of 15d-PGJ2 on cyclooxygenase-2 expression and prostaglandin E₂ production, which are key players in the physiopathology of arthritis. We found that 15d-PGJ2 inhibits IL-1-induced cyclooxygenase-2 expression and prostaglandin E₂ production in both cell types. Taken together, our data indicate that PPAR γ activators may be useful in the development of new anti-arthritic agents.

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The role of mPGES-1 for prostaglandin E₂ production in primary human synovial cells

L Crofford¹, M Qian¹, A Sampey¹, V Lath¹, S Guo¹, M Peters-Golden², M Goldring³

¹Department of Internal Medicine, Division of Rheumatology, University of Michigan, Ann Arbor, Michigan, USA; ²Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan, Ann Arbor, Michigan, USA; ³Rheumatology Division, The Beth Israel Deaconess Medical Center, Harvard Institute of Medicine, Boston, Massachusetts, USA

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The effectiveness of nonsteroidal anti-inflammatory drugs and specific cyclooxygenase (COX)-2 inhibitors for treatment of arthritis provides clinical evidence that increased local prostaglandin (PG) production in joint tissues contributes to symptoms of pain, swelling, and stiffness. Despite improved gastrointestinal safety of specific COX-2 inhibitors, unwanted effects associated with inhibition of COX-2 continue to occur in patients treated with these agents. Since production of stable PGs requires synthase enzymes that function downstream of COX, there are other potential targets for therapeutic intervention in arthritis patients.

PGE₂ is the most abundant prostanoid in synovial fluid and tissues, and its biosynthesis is catalyzed by the coordinate action of COX enzymes and PGE synthases (PGES). There are constitutive forms (COX-1, microsomal PGES-2 [mPGES-2] and cytosolic PGES) and inducible

forms (COX-2 and mPGES-1) of both biosynthetic enzymes, all of which are expressed in human fibroblast-like synoviocytes (FLS). For this reason, FLS are ideal for the study of PGE₂ biosynthetic pathways in a clinically relevant cell.

Both COX-2 and mPGES-1 are increased by the proinflammatory cytokines IL-1 and tumor necrosis factor. However, we have shown that the time frame of their stimulated expression is distinctly different. mPGES-1 mRNA expression lags COX-2 by 2 hours and is sustained up to 48 hours, while mPGES-1 protein lags COX-2 by 4 hours and is sustained to 72 hours. Upregulation of mPGES-1 protein is required for high-level PGE₂ production as measured by enzyme immunoassay and HPLC, and PGE₂ biosynthetic capacity remains robust at 48 hours. At the 48 hour time point, PGE₂ production is blocked by the selective COX-1 inhibitor, suggesting that mPGES-1 is capable of efficiently generating PGE₂ from COX-1-derived substrate.

In contrast to COX-2, IL-induced transcription of mPGES-1 is blocked by the protein synthesis inhibitor cyclohexamide, suggesting a requirement for new protein synthesis. It was recently reported that the transcription factor early growth response gene 1 (Egr-1) binds to the murine mPGES-1 promoter and regulates transcription. Total and nuclear Egr-1 expression is upregulated by IL-1 in FLS. This expression is blocked by the inhibitors of MAPK, as is upregulation of mPGES-1. Two tandem GC boxes are present in the mPGES-1 promoter region, with the proximal GC box overlapping a potential Egr-1 binding site. Under basal conditions, at least three complexes (CI, CII and CIII) bind to this region of the mPGES-1 promoter (-125 to -98) by electrophoretic mobility shift assay. We identified the induced CII complex as Egr-1 by supershift assay. Egr-1 binds to the proximal GC box as determined by competition assays using mutated oligonucleotides. CI and CIII contain Sp3 by supershift assay. It is not yet clear that Sp3 is displaced by Egr-1. We confirmed the functional significance of Egr-1 binding for induced endogenous mPGES-1 expression using plasmids containing wild-type and mutant Egr-1.

In summary, mPGES-1 is a cytokine-inducible enzyme required for production of PGE₂ in FLS. mPGES-1 deficiency in null mice completely blocks PGE₂ production and susceptibility to collagen-induced arthritis. mPGES-1 represents a novel therapeutic target for treatment of the inflammation associated with arthritis.

Topics Symposium (6) Cytokines & Inflammation (2)

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IL-21R mRNA is overexpressed in systemic sclerosis and contributes to the epidermal/dermal cross-talk

O Distler¹, J Distler¹, A Hirth¹, U Müller-Ladner², M Matucci-Cerinic³, H Lorenz⁴, S Gay¹

¹Center of Experimental Rheumatology, Department of Rheumatology, University of Zurich, Switzerland; ²Department of International Medicine I, University of Regensburg, Germany; ³Department of Medicine, Section of Rheumatology, University Hospital, Florence, Italy; ⁴Institute for Clinical Immunology and Rheumatology, Medical Department, University of Erlangen, Germany

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Objectives IL-21R is a class I cytokine receptor associated with the common γ -chain. In mice, IL-21R was detected in fibrotic lungs but not in healthy lungs, suggesting a possible role for IL-21R in fibrotic disorders.

Methods Skin biopsies were obtained from 12 patients with systemic sclerosis (SSc) and 11 healthy controls. Total RNA was isolated from these tissues, reverse transcribed, and IL-21/IL-21R mRNA was quantified using real-time PCR. The expression patterns of IL-21/IL-21R were analyzed by *in situ* hybridization. Stimulation experiments were performed with cultured dermal fibroblasts from SSc patients and healthy controls as well as keratinocytes using IL-1 β , platelet derived growth factor-BB (PDGF-BB), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor

(TGF)- β and IL-21. Human skin biopsies from SSc patients were transplanted onto SCID mice. After 60 days, the expression of IL-21R was determined by *in situ* hybridization. Cultured keratinocytes were stimulated with IL-21 and assessed for differential expression compared with nonstimulated controls using a human Atlas cDNA expression array (Clontech, Palo Alto, CA, USA).

Results IL-21R was detected in all biopsies from SSc patients and controls with a 4.7-fold increase in SSc samples (threshold cycle: SSc patients, 17.9 ± 1.1 ; healthy, 20.1 ± 1.4). *In situ* hybridization showed an upregulation of IL-21R in the epidermis of SSc patients, whereas no signal was detected in healthy skin specimens. These results were confirmed *in vitro*, in that cultured keratinocytes expressed significant levels of IL-21R, whereas no signal was found in fibroblasts. Interestingly, mRNA for IL-21, the proposed ligand for IL-21R, was not detected by real-time PCR and *in situ* hybridization. Various concentrations of IL-1 β , PDGF-BB, MCP-1, TGF- β and IL-21 did not stimulate the expression of IL-21R in cultured keratinocytes and dermal fibroblasts. In the SCID mouse transplantation model, the overexpression of IL-21R in SSc keratinocytes remained unchanged as long as 60 days after transplantation, whereas no signals for IL-21R were detected in fibroblasts. Differentially expressed genes confirmed by SYBR Green real-time PCR after stimulation with IL-21 included vascular endothelial growth factor, vascular endothelial growth factor-C and integrin $\alpha 6$.

Conclusion This is the first human disease-related report regarding IL-21R *in vivo*. These results indicate that, in addition to fibroblasts and endothelial cells, the expression pattern of keratinocytes is altered in patients with SSc. Interestingly, the expression of IL-21R appears to be independent of inflammatory cytokines *in vitro* and independent of both humoral and cellular factors circulating in SSc patients, because the expression of IL-21R mRNA in human epidermal keratinocytes did not change after implantation onto SCID mice.

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Variability of pro-apoptotic properties of cyclooxygenase-2 inhibitors in rheumatoid synovial fibroblasts and cancer cell lines

S Kawai¹, N Kusunoki¹, R Yamazaki²

¹Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Yakult Central Institute for Microbiological Research, Tokyo, Japan

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Recent attention has been focused on selective cyclooxygenase (COX)-2 inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-2 without inhibition of COX-1, resulting in fewer incidences of adverse reactions. Various clinical studies have confirmed that the efficacy of selective COX-2 inhibitors for the patients with rheumatoid arthritis (RA) is similar to that of conventional NSAIDs; however, they cause fewer severe gastrointestinal complications. Recently, we found that some conventional NSAIDs such as indometacin and diclofenac induced apoptosis in association with activation of peroxisome proliferator-activated receptor gamma (PPAR γ) in rheumatoid synovial fibroblasts (RSF). We also found that celecoxib, a selective COX-2 inhibitor, specifically induced apoptosis and inhibited proliferation of RSF in COX-2-independent and PPAR γ -independent manners. Other selective COX-2 inhibitors such as etodolac, meloxicam, nimesulide, NS-398, and rofecoxib, even in very high concentrations, did not induce apoptosis in RSF at all. These results suggested that some NSAIDs including a selective COX-2 inhibitor might be used as disease-modifying antirheumatic drugs if they could be delivered with good efficiency to the affected synovia of patients with RA. It is well known that administration of celecoxib decreases the number of colon polyps in patients with familial adenomatous polyposis. The pro-apoptotic effect of celecoxib was also observed in several kinds of cell lines derived from colon cancer and ovarian cancer, suggesting a common mechanism of action of celecoxib-induced apoptosis among RSF and cancer cell lines. Inactivation of Akt might explain the pro-apoptotic effect of celecoxib on at least colon cancer cell lines.

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39**Complement, its role in innate immunity and autoimmunity****M Daha***Department of Nephrology, Leiden University Medical Center, The Netherlands**Arthritis Res Ther* 2003, **5(Suppl 3):39** (DOI 10.1186/ar840)

The innate immune system provides essential host defense against a wide variety of microbial pathogens. In addition, the innate immune system is responsible for the recognition of necrotic and apoptotic cells and self-antigens that are released from the large number of cells that undergo apoptosis for the maintenance of physiologic homeostasis. The complement system is essential to initiate and drive the acquired immune response.

Until recently, two pathways for complement activation were recognized, namely the classical pathway and the alternative pathway. Recent studies from a large number of laboratories indicate the existence of a third pathway of complement activation, namely the lectin pathway. This third pathway is initiated by the interaction of, for example, mannan binding lectin and a number of other pattern recognition molecules called ficolins, with carbohydrate domains on a wide variety of pathogens such as bacteria and viruses.

Studies in complement-deficient individuals have indicated a strong association between complement deficiencies and infections or with immune complex disease and autoimmunity. Deficiencies in the lectin pathway are associated with serious bacterial infections in immunocompromised individuals, while deficiencies in the early classical pathway components are associated with immune complex disease and autoimmunity. Especially, deficiencies in C1q, the recognition unit of the classical pathway, are associated strongly with systemic lupus erythematosus. Studies in C1q knockout mice also implicate C1q with autoimmunity. Various studies indicate that C1q, but also a number of other molecules of innate immunity, such as C-reactive protein, serum amyloid P-component, pentraxin-3, and possibly mannan binding lectin and ficolins, all in concert, may be involved in the recognition and clearance of harmful potentially pathogenic apoptotic and necrotic cells. It is thought that the deficient removal or the mode of presentation of self-antigens may determine whether autoimmune responses take place or whether tolerance is induced. In this scenario, it is suggested that the method of engagement and subsequent signal induction may ultimately determine the final outcome of the ensuing immune response.

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40**Activation of synovial fibroblasts via Toll-like receptor signaling****D Kyburz¹, M Pierer¹, J Rethage¹, R Seibl¹, RE Gay¹, DA Carson², S Gay¹***¹Center of Experimental Rheumatology, Department of Rheumatology, University Hospital, Zurich, Switzerland ²Department of Medicine, UCSD, La Jolla, California, USA**Arthritis Res Ther* 2003, **5(Suppl 3):40** (DOI 10.1186/ar841)

Toll-like receptors (TLRs) are pattern recognition receptors of the innate immune system. TLR activation has a profound influence on the subsequent adaptive immune responses by influencing T-cell differentiation towards a Th1 phenotype. Whether TLR signaling is involved in autoimmune phenomena is not known. The presence of TLR ligands in the joints of patients with rheumatoid arthritis and the fact that direct injection of bacterial peptidoglycan, a TLR2 agonist, and bacterial DNA, a TLR9 agonist, results in a transient arthritis in mice suggests that TLR signaling is of importance in the development of arthritis. Furthermore, recent evidence indicates that stimulation of TLR9 on B lymphocytes by immune complexes containing chromatin drives production of autoantibodies. We have detected TLR2 expression on synovial fibroblasts of patients with rheumatoid arthritis by immunohistochemistry. Cultured human synovial fibroblasts expressed functional TLR2 *in vitro*. Stimulation of synovial fibroblasts with the TLR2 ligand peptidoglycan induced a significant upregulation of the expression of

matrix metalloproteinases and the cytokine IL-6. Furthermore, chemokine secretion by rheumatoid arthritis synovial fibroblasts was significantly increased. These results argue that the local presence of bacterial products may induce a local inflammatory response by signaling via TLR2. Blockade of TLR signaling pathways may therefore represent a promising new therapeutic option for rheumatoid arthritis.

41**TNF dependency of IL-17-induced joint pathology can be circumvented by Toll-like receptor-2 signaling****W Van den Berg, M Koenders, P van Lent, L Joosten, E Lubberts***Rheumatology Research Laboratory, University Medical Center Nijmegen, The Netherlands**Arthritis Res Ther* 2003, **5(Suppl 3):41** (DOI 10.1186/ar842)

Background T-cell IL-17 is a proinflammatory cytokine present in the synovium of rheumatoid arthritis patients. Through the binding of IL-17 to its receptor (IL-17R) and the subsequent activation of a signaling pathway through TRAF-6 and NF- κ B, IL-17 can induce other cytokines such as IL-1 and tumor necrosis factor (TNF). IL-17 can have both additive and synergistic effects on cytokine induction and tissue destruction with these cytokines, but may have direct pathological effects as well.

Purpose In the present study, we examined the relative dependency of TNF in the IL-17-induced joint inflammation and cartilage damage under naive and various arthritis conditions *in vivo*, including Toll-like receptor-2 (TLR-2)-dependent inflammation.

Methods An adenoviral vector expressing murine IL-17 was used to intra-articularly overexpress IL-17 in the knee joints of control mice or mice deficient for IL-1 or TNF. Experiments were performed under naive conditions and during TLR-2-dependent streptococcal cell wall-induced arthritis (SCW) and passive immune complex-mediated arthritis (ICA) with lysozyme as an antigen.

Results IL-17 overexpression in the knee joint of naive mice resulted in joint inflammation and cartilage proteoglycan depletion, which gradually increased over time. No effects were noted with the same dose of control vector. IL-17 strongly upregulated IL-1 mRNA and protein levels in the synovium compared with the control group. However, no difference in IL-17-induced joint pathology was noted in IL-1-deficient mice ($P=0.39$). Interestingly, when TNF-deficient mice were used, the IL-17-induced joint inflammation and cartilage damage were almost completely absent ($P<0.005$). This indicates that, under naive conditions *in vivo*, the IL-17-induced joint inflammation and cartilage destruction are mediated by synergism with TNF and not with IL-1. Similar experiments were performed under SCW and ICA conditions. SCW arthritis runs through TLR-2, since arthritis was highly reduced in TLR-2-deficient mice, but not in TLR-4^{-/-} mice. In addition, SCW arthritis is fully suppressed in mice deficient in the crucial Toll-like receptor signaling molecule Myd88. Overexpression of the T-cell cytokine IL-17 in the macrophage-mediated SCW model resulted in an elevation of joint inflammation and cartilage proteoglycan depletion compared with the control vector group ($P<0.005$). Furthermore, IL-17 turned this acute model into a more chronic one. Remarkably, IL-17-enhanced SCW arthritis was not reduced in TNF^{-/-} mice, identifying circumvention of TNF dependency in the presence of TLR-2 activation. Intriguingly, IL-17-induced enhancement of Fc γ R-mediated ICA still remained TNF dependent, since joint swelling was significantly reduced in TNF-deficient mice.

Conclusions These data show TNF dependency of IL-17-induced joint pathology under naive conditions and during ICA. However, this is lost during SCW arthritis. TLR-2 shares the same signaling pathway through TRAF-6 and NF- κ B as the IL-17R. It suggests that TNF dependency can be bypassed by IL-17 in combination with TLR-2 triggers.

42**Candidate targets for antibody therapy of rheumatoid arthritis: CD64 and IL-15****J van de Winkel***Department of Immunology, University Medical Center Utrecht, The Netherlands**Arthritis Res Ther* 2003, **5(Suppl 3):42** (DOI 10.1186/ar843)

At present, blockade of cytokines represents one approach to treat rheumatoid arthritis (RA). Anti-tumor necrosis factor (TNF)- α therapy

results in impressive protection against joint inflammation and joint damage in RA patients, although a significant number of patients do not respond. We evaluated blockade of targets more upstream in the inflammation cascade, of activated macrophages and of IL-15.

Inflammatory macrophages exhibit enhanced expression of FcγRI (CD64), the high-affinity receptor for IgG. We observed the presence of CD64 on activated macrophages in RA patients, and their *in vitro* elimination using a CD64-targeted immunotoxin. In addition, we demonstrated *in vivo* elimination of CD64-expressing activated macrophages, resulting in significant inhibition of disease activity in an adjuvant arthritis model, in newly generated human CD64 transgenic rats. IL-15 triggers inflammatory cell recruitment, angiogenesis and production of other inflammatory cytokines, including IFN-γ, TNF-α and IL-17, which are all upregulated in inflammation. We generated monoclonal antibodies using human immunoglobulin-transgenic mice. One of the IL-15-specific antibodies, HuMax IL-15, did not compete with IL-15 for binding to its receptor, but potently interfered with the assembly of the IL-15 receptor α,β,γ complex. This antibody blocked IL-15-induced T-cell proliferation, and monocyte TNF-α release *in vitro*. HuMax IL-15 effectively inhibited inflammation in SCID-RA models and is currently clinically evaluated in human RA.

Topics Symposium (7) Cell Biology

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Mechanisms of resistance against Fas-induced apoptosis in rheumatoid arthritis synovial fibroblasts

T Pap¹, A Baier², I Meinecke², A Drynda¹, R Gay³, W Neumann², S Gay³

¹Division of Experimental Rheumatology, Otto-von-Guericke-University, Magdeburg, Germany; ²Department of Orthopedic Surgery, Otto-von-Guericke-University, Magdeburg, Germany; ³Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zürich, Switzerland

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The activation of synovial fibroblasts (SF) is a hallmark of rheumatoid arthritis, and the resistance of rheumatoid arthritis (RA)-SF against Fas-induced cell death is one of their characteristic features. However, the mechanisms that are responsible for the low susceptibility of RA-SF to programmed cell death and, specifically, the pathways that link altered apoptosis to their aggressive behavior are only incompletely understood. In our present studies, we have compared the susceptibility of synovial fibroblasts from RA and osteoarthritis (OA) patients to Fas-induced apoptosis, and have studied mechanisms that contribute to both the invasiveness of RA-SF and their resistance against apoptosis.

As determined by different techniques (measurement of cytoplasmic mononucleosomes and oligonucleosomes, FACS analysis), RA-SF were significantly less susceptible to Fas-induced cell death than OA-SF despite their abundant expression of Fas. Stimulation of RA-SF with tumor necrosis factor alpha (TNF-α) did not induce apoptosis, but in a dose-dependent manner reduced Fas-mediated cell death. This was accompanied by the activation of NF-κB and the upregulation of disease-relevant matrix metalloproteinases. Of interest, adenoviral gene transfer of TIMP-3 reduced the invasiveness of RA-SF in the SCID-mouse *in vivo* model of RA, and completely reversed the apoptosis-inhibiting effect of TNF-α, in part by reducing the activation of NF-κB. The effects of TNF-α on apoptosis were similar in OA-SF. However, RA-SF differed from OA-SF by their elevated expression of the small ubiquitin-like modifier sentrin-1/SUMO-1. Retroviral gene transfer of antisense and expression constructs of sentrin-1/SUMO-1 confirmed its involvement in the altered susceptibility of RA-SF to apoptosis. Analysis of the subcellular localization of sentrin-1/SUMO-1 and gene transfer of SUMO-1-specific proteases revealed that modifications of transcriptionally active nuclear proteins by sentrin-1/SUMO-1 contribute to the regulation of apoptosis and the production of matrix-degrading enzymes.

Collectively, our data suggest that in RA-SF there is a close functional association between pathways that confer the resistance against apoptosis and that mediate the progressive destruction of cartilage. Both cytokine-dependent and cytokine-independent mechanisms contribute to these processes. While TNF-α-mediated prevention of cell death is not specific for RA and is seen in a variety of fibroblast-like cells, activation of signaling pathways involving sentrin-1/SUMO-1 appears to be a characteristic feature of RA-SF.

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Immunologic reactants in the pathogenesis of atherosclerosis in rheumatic diseases

B Cronstein, A Reiss

New York University School of Medicine, New York, USA

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It is increasingly clear that patients suffering from systemic lupus erythematosus and rheumatoid arthritis are at significantly greater risk of developing atherosclerotic cardiovascular disease than otherwise unaffected individuals. Recent studies in our laboratory demonstrate that immunologic reactants such as immune complexes that have fixed C1q and IFN-γ diminish the capacity of macrophages to appropriately metabolize and transport lipoproteins. The effect of these agents on macrophage function suggests a role for these reactants in the premature development of atherosclerotic cardiovascular disease in rheumatic diseases. It was recently observed that methotrexate, unlike any other disease-modifying antirheumatic drugs studied, diminished the risk for development of atherosclerotic cardiovascular disease. Although the explanation for this phenomenon may be that methotrexate is simply a more effective anti-inflammatory agent, recent work in our laboratory suggests an alternative explanation. We have demonstrated that many, if not most, of the anti-inflammatory effects of methotrexate are due its capacity to increase release of adenosine, which interacts with its receptors on the cell surface to modulate inflammation. Adenosine, acting at its receptors on the surface of macrophages, increases the expression of enzymes involved in metabolizing cholesterol and of transporters involved in export of cholesterol from the vessel wall to the liver for elimination. The adenosine receptor-mediated effect on expression of these molecules is associated with diminished foam cell formation in an *in vitro* assay. These results suggest an explanation for the effect of methotrexate therapy on the development of atherosclerotic cardiovascular disease and, more importantly, indicates a novel target for the development of new anti-atherosclerotic agents.

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Cell biology of synovial inflammation and secondary osteoporosis in rheumatoid arthritis

Y Tanaka, Y Okada, S Nakayamada, K Nakano, K Fujii, K Saito

First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, Kitakyushu, Japan

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Rheumatoid arthritis (RA) is a representative autoimmune disease characterized by lymphocyte accumulation and synovial proliferation, which are induced by inflammatory cytokines and adhesion molecules. Although periarticular as well as systemic osteoporosis and subsequent joint destruction are major complications of RA, the precise mechanisms remain unclear. Osteoblasts not only play a central role in bone formation by synthesizing bone matrix proteins, but they regulate osteoclast maturation by cognate interaction, resulting in bone resorption. RANKL expressed on osteoblasts provides essential signals to osteoclast progenitors for their maturation. We have proposed that the LFA-1/ICAM-1-mediated adhesive pathway of osteoblasts is required for juxtacrine stimulation of osteoclast maturation by membrane-bound RANKL on osteoblasts. Moreover, proinflammatory cytokines such as IL-1 produced abundantly in rheumatoid synovium induce both RANKL and ICAM-1 on osteoblasts, leading to an efficient juxtacrine stimulation for the osteoclastogenesis. However, ICAM-1-positive osteoblasts, induced by IL-1, arrest at the G₀/G₁ phase of the cell cycle, which is involved by upregulation of p21 and reduced activities of cdk6. IL-1-

induced ICAM-1-positive osteoblasts can bias bone turnover to bone resorption, committing their growth arrest, in the context of the balance between survival and apoptosis of the cells. Such a regulation of the cell cycle is regulated by H-Ras, a small G-protein. H-Ras signals followed by Raf-1/MAPK induce cell-cycle arrest of osteoblasts via Fas upregulation and bcl-2 downregulation, whereas H-Ras followed by PI3K induces their proliferation. Taken together, during bone remodeling processes, proinflammatory cytokines cause an imbalance in bone metabolism by favouring bone resorption via the expression of RANKL and ICAM-1, as well as apoptosis of osteoblasts, which are differentially regulated by intracellular signals via H-Ras. Such osteoclast maturation mediated by the adhesion with osteoblasts is coordinated with immune signaling, and thereby is relevant to pathological events such as secondary osteoporosis observed in RA.

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New strategies for the *in vivo* regulation of metalloprotease gene expression in osteoarthritis chondrocytes by inflammatory mediators

JP Pelletier¹, C Boileau¹, D Schrier², C Flory², J Brunet¹, F Mineau¹, G Tardif¹, M Boily¹, J Martel-Pelletier¹

¹Osteoarthritis Research Unit, University of Montreal Hospital Centre, Notre-Dame Hospital, Montreal, Quebec, Canada; ²Pfizer Global Research and Development, Ann Arbor, Michigan, USA
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Objective To study the effect of oral treatment with PD200347, a gabapentinoid (GBP), on osteoarthritis (OA) progression and OA mediators, matrix metalloproteases (MMPs) and the inducible form of nitric oxide synthase (iNOS) expression in a dog experimental model of OA.

Methods OA was surgically induced in dogs by sectioning the anterior cruciate ligament. OA dogs were divided into three groups after surgery: group 1, placebo-treated (OA); group 2, oral treatment with 15 mg/kg/day GBP; and group 3, oral treatment with 90 mg/kg/day GBP. Dogs were killed 8 weeks after surgery. The severity of lesions was scored macroscopically and histologically. Cartilage specimens from femoral condyles and tibial plateaus were processed for RNA extraction and quantitative RT-PCR or immunohistochemistry. Specific probes and specific antibodies were used to study IL-1 β , iNOS, MMP-1, MMP-3 and MMP-13 mRNA and protein levels, respectively.

Results GBP treatment at both dosages tested (15 or 90 mg/kg/day) dose-dependently reduced the development of cartilage lesions. Quantitative RT-PCR and immunohistochemical analysis showed that GBP treatment also significantly reduced key OA mediator (IL-1 β , iNOS, MMP-1, MMP-3 and MMP-13) gene expression and synthesis.

Conclusion This study demonstrated for the first time the effectiveness of a GBP on the reduction of the development of structural changes in the dog OA model. The effect of GBP is mediated through the inhibition, at the transcriptional level, of major mediators of pathophysiological pathways.

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Regulation of collagenase-3 (MMP-13) gene and protein expression in human cartilage: when simple things get complicated

J Martel-Pelletier, G Tardif, JP Pelletier

Osteoarthritis Research Centre, University of Montreal Hospital Centre, Notre-Dame Hospital, Montreal, Quebec, Canada
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In cartilage, collagen type II is of particular importance as its breakdown results in the irreversible loss of structural integrity of the tissue. Within the protease, the collagenases have a major involvement in this collagen network degradation. Evidence demonstrates that collagenase-3 is the major enzyme accounting for collagen degradation in osteoarthritic (OA) cartilage.

Collagenase-3 has a greater effect (five to 10 times) on type II collagen than collagenase-1, and, in OA, is localized predominantly in the lower intermediate and deep layers of the cartilage, where type II collagen fibers are of the largest size and chondrocytes possess the most efficient capacity to reconstitute the extracellular matrix. Collagenase is

upregulated in OA cartilage and is suggested to be implicated in cartilage remodeling in pathological conditions. Various factors induce its transcription, including proinflammatory cytokines and growth factors such as IL-1 β , IL-17, tumor necrosis factor alpha, transforming growth factor beta and hepatocyte growth factor. Interestingly, we recently reported that transforming growth factor beta, but not IL-1 β , treatment of normal cartilage mimicked the *in situ* collagenase-3 distribution in OA cartilage.

The proximal promoter sequence contains a TATA box, as well as AP-1, Ets/PEA-3, and OSE-2 binding sites. The AP-1 site is essential for both basal and proinflammatory cytokine inducible transcription, and the PEA-3 site exerts a cooperative effect. We also demonstrated that some AP-1 proteins play a different role in terms of collagenase-3 production according to the stimulator. Indeed, IL-17-induced collagenase-3 resulted in FosB activation, whereas IL-1 β stimulated c-Fos, which may explain the different capacities of these cytokines in producing this enzyme. Our data also suggest that JunB protein plays a rate-limiting step in cytokine-induced collagenase-3 production in OA chondrocytes. Moreover, we recently identified a novel protein-binding site on the collagenase-3 promoter that appears to be directly implicated in the repression of its basal transcription. This site was designated AGRE for AG-rich element. This site was not found in other human metalloprotease genes or in the mouse collagenase.

Contrary to the other human collagenase genes that are transcribed into one mature mRNA, human cells expressed collagenase-3 transcripts of 3.0, 2.5 and 2.2/2.0 kb as demonstrated by northern blot. We recently identified five different collagenase-3 RNA species in humans; each could be translated in a cellular environment, indicating that they could be synthesized in response to specific cellular events. For two of the RNA species, the enzyme synthesized would differ from the original collagenase-3 and will have potentially different function/activity. Moreover, one of the transcripts appears to be an alternative transcription start site. Start sites are known to regulate gene expression by affecting the level of transcription initiation, the translation efficiency of the mRNA produced, and the generation of protein isoforms differing at their amino termini, or may respond differently to the cellular environment.

At present, a therapeutic intervention based on the inhibition of metalloproteases is under intensive investigation, and collagenase-3 appears to be an attractive target for the development of disease-modifying OA drugs. However, the human collagenase-3 is subjected to different levels of regulation and constitutes a more complex system than originally thought. It would be interesting to verify whether compounds that block only one pathway, such as the synthesis/activity of the original collagenase-3, are sufficient to block all the effects of this enzyme, or whether all the enzyme transcripts should rather be targeted in order to achieve maximum therapeutic efficacy.

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TIMP-3 inhibits aggrecan breakdown in pig articular cartilage stimulated with IL-1

H Nagase, C Gendron, M Kashiwagi

Matrix Biology Department, Kennedy Institute of Rheumatology, Imperial College London, UK

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Degradation of articular cartilage seriously impairs the function of joints and it is a hallmark of various types of arthritides. The primary cause of this process is due to elevated proteolytic enzyme activities that degrade aggrecan proteoglycan and type II collagen fibrils, major components of the extracellular matrix in cartilage. While the network of collagen fibrils is degraded primarily by collagenases and possibly by other matrix metalloproteinases (MMPs), such as MMP-2 and MMP-14, aggrecan is degraded by MMPs and the more recently discovered 'aggrecanases' that belong to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family.

To investigate the relative contribution of MMPs and ADAMTSs in cartilage aggrecan degradation during the progression of joint destruction, we have used the pig articular cartilage explants treated with IL-1 as an *in vitro* cartilage degradation model and have tested the ability of tissue inhibitors of metalloproteinases (TIMPs), TIMP-1, TIMP-2 and TIMP-3, to block the release of aggrecan. MMPs are inhibited by all three

TIMPs, but aggrecanases (ADAMTS-1, ADAMTS-4 and ADAMTS-5) are inhibited only by TIMP-3.

Treatment of pig articular cartilage in culture with IL-1 for 3 days resulted in approximately 70% of aggrecan degradation, and the fragments were released into the medium. The degradation of aggrecan was completely inhibited by the addition of recombinant N-terminal inhibitory domain of TIMP-3 (N-TIMP-3) at the concentration of 0.1 mM, but not by TIMP-1 or TIMP-2. This indicates that aggrecan degradation in this model is due to aggrecanases, but not due to MMPs. TIMP-3 is known to cause apoptosis of several cell types. N-TIMP-3 caused apoptosis of pig chondrocytes only at concentrations above 0.75 μ M. Our mutagenesis studies of N-TIMP-3 also revealed that N-TIMP-3 contains the metalloproteinase reactive site common among TIMPs and the unique sites that interact with aggrecanases, suggesting that it may be possible to design TIMP variants that selectively inhibit aggrecanases.

Our studies suggest that TIMP-3 and the related molecules may be potential therapeutics to prevent articular cartilage from degradation during the progression of osteoarthritis and rheumatoid arthritis.

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Poster Discussion (1) Bone & Cartilage Group

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Bone cell differentiation and the role of Fos/AP-1 proteins

E Wagner¹, R Eferl¹, A Hoebertz¹, L Kenner¹, K Matsuo²

¹IMP, Vienna, Austria; ²Keio University School of Medicine, Tokyo, Japan
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Fos proteins such as Fos, FosB, Fra-1 and Fra-2 are key regulators of bone development. Transgenic mice expressing Fos develop osteoblastic bone tumors, whereas mice lacking Fos (*Fos*^{-/-}) are osteopetrotic and lack bone-resorbing osteoclasts [1]. The Fos-related protein Fra-1, itself a Fos target gene, is essential for mouse development, whereas transgenic mice overexpressing Fra-1 develop an osteoblastic bone disease, osteosclerosis [2]. Interestingly, gene replacement of *Fos* by *Fra-1* showed functional equivalence of these two proteins [3]. To better understand how Fos and Fra-1 control osteoblast and osteoclast differentiation, we generated conditional alleles of *Fos* and *Fra-1*. The embryonic lethality of the *Fra-1* knockout mice was rescued with a conditional allele of *Fra-1* using *MORE-cre* mice. The mutant mice are viable but are osteopenic. Interestingly, conditional deletion of *JunB* with the same *MORE-cre* line also rescued the lethality, and the mutant mice developed severe osteoporosis. Finally, inactivation of *Fra-2* gives rise to pups that also exhibit severe osteoporosis and die at birth, probably due to heart failure. The signal transduction pathways operating in osteoclastogenesis have been extensively studied and the events downstream of RANKL signalling are well described [1]. Positive and negative regulatory loops are in place, which involve the activation NF- κ B and JNK, eventually leading to the expression of Fos, an essential gene for osteoclast differentiation [1]. Micro-array analysis revealed that the expression of NFATc1 is abolished in *Fos*^{-/-} precursors [4]. However, introduction of an activated form of NFATc into *Fos*^{-/-} precursor cells rescued the bone resorptive function and restored transcription of osteoclast marker genes. Recent experiments addressing the molecular events during osteoclast formation will be discussed.

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Bone sparing effect of osteoprotegerin and anti-inflammatory cytokines in collagen-induced arthritis

M de Vernejoul¹, N Saidenberg², M Boissier², M Cohen-Solal¹

¹INSERM U349 Lariboisière Hospital, Paris, France; ²UPRES EA-3408 Avicenne Hospital, Bobigny, France

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Rheumatoid arthritis (RA) is associated with focal and systemic bone loss involving RANKL, which is secreted by both osteoblasts and activated lymphocytes, whereas osteoprotegerin (OPG) inhibits bone resorption in osteoclast precursors. RANKL expression is increased by inflammatory cytokines. The aim of our studies is to evaluate the respective effect of OPG, on one hand, and either IL-4 or antibody, on the other, on inflammation and on bone tumor necrosis factor alpha (TNF- α) remodelling. Indeed, we hypothesised that inhibiting inflammation could be sufficient to inhibit bone loss. Moreover, we tested whether the combination of OPG with either IL-4 or TNF- α antibody had an additive effect on bone loss. IL-4 and TNF- α antibodies were tested in two independent experiments. We used a model of collagen-induced arthritis (CIA) in DBA/1 mice by immunisation (bovine type II collagen). Bone mineral density (BMD) was measured at the total body (Piximus Lunar) at baseline before immunization and at sacrifice, allowing one to measure the bone gain (Δ BMD). Deoxypyridinolin changes (Δ D-Pyr) were measured in urine. Histomorphometric parameters were measured at the femur metaphysis. OPG had no effect on clinical arthritis score in any experiment. By contrast, OPG was able to increase the Δ BMD and to decrease the Δ D-Pyr and trabecular spacing evaluated by histomorphometry. By contrast, both IL-4 and TNF- α antibody decreased the arthritis score but did not induce a change in Δ BMD and decrease in bone resorption evaluated by Δ D-Pyr and trabecular spacing. There was no additive effect of OPG and TNF- α antibody on any parameter related to bone, whereas we observed a significant additive effect of IL-4 and OPG on the inhibition of bone resorption and the increase in Δ BMD.

In conclusion, these data show that, in this model of inflammatory arthritis, systemic bone loss is prevented only by treatment directly decreasing osteoclast differentiation. Furthermore, treatment with one anti-inflammatory cytokine, IL-4, but not with TNF- α antibody had an additive bone-sparing effect with OPG.

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The JAK/STAT pathway but not the Erk1/2 pathway mediates IL-6/sIL-6R downregulation of type II collagen, aggrecan core and link protein transcription in articular chondrocytes

P Bogdanowicz¹, F Legendre¹, J Dudhia², J-P Pujol¹

¹Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médecine, Caen, France; ²Department of Veterinary Basic Sciences, Royal Veterinary College, London, UK

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Introduction IL-6 regulates several functions, including immunological reactions in host defense or inflammation. Accumulating evidence suggests that IL-6 is implicated in rheumatoid arthritis and in osteoarthritis. IL-6 may contribute to the destructive changes of cartilage accompanying joint diseases, but its mechanism of action on the cartilage and the pathways whereby it controls their gene expression are unknown. However, until recently, the IL-6 signaling pathway for these cells has not been identified nor has the functional significance of their effects on the major extracellular matrix components been investigated.

Objectives The aim of this study was to analyze the signaling pathways of IL-6/sIL-6R in articular chondrocytes, and their involvement in the control of matrix gene expression.

Methods Bovine articular chondrocytes were treated by IL-6 (100–500 ng/ml) with or without IL-6sR (200–500 ng/ml) for increasing

periods. Western blotting and gel retardation assays were performed with or without mitogen-activated protein kinase inhibitor (PD 98059) and STAT inhibitor (parthenolide). Collagen type II and aggrecan core and link protein mRNA levels were determined by northern blots.

Results In the absence of sIL-6R, IL-6 (500 ng/ml) had a slight effect on JAK-1 and JAK-2 phosphorylation. Addition of sIL-6R at 500 ng/ml dramatically increased these effects, with a peak at 15 min. STAT1 and STAT3 were also found to be phosphorylated and translocated to the nucleus. Increased binding of protein factors to specific STAT responsive elements was observed. ERK1 and ERK2 were activated by IL-6/sIL-6R treatment, with a maximum at 30 min. Exposure of cells to IL-6/sIL-6R induced an inhibition of collagen type II, aggrecan core and link protein expression. This downregulation was abolished in the presence of STAT inhibitor but not in the presence of mitogen-activated protein kinase inhibitor, indicating that the STAT signaling pathway is implicated in the control of these two cartilage-specific matrix genes.

Conclusions To exert a significant effect on chondrocytes, IL-6 requires the presence of its soluble receptor. The cytokine plays a role in the cartilage breakdown through a STAT-induced inhibition of collagen type II, aggrecan core and link protein expression.

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Induction of heme oxygenase-1 inhibits IL-1b nitrite oxide production in articular chondrocytes

F Rannou

Division of Biomedical Sciences, University of California, Riverside, California, USA and Division of Rehabilitation, Cochin Hospital, University of Paris V, France

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IL-1b is one of the main cytokines leading to the degradation of articular cartilage. One mechanism through which this cytokine exerts its effects is by articular chondrocytes being induced to produce nitrite oxide (NO). These findings are supported by clinical studies revealing a significant increase of NO production in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis. Modulating IL-1b-induced NO in articular chondrocytes appears to be an interesting challenge for reducing or inhibiting the cartilage destruction.

Heme oxygenase-1 (HO-1) is an inducible enzyme catalyzing the degradation of heme. The beneficial effects of HO-1 have been described for several diseases. This enzyme confers protection mainly through its antioxidant and anti-inflammatory functions.

The objectives of this work were to investigate the expression and activity of HO-1 in IL-1b-treated articular chondrocytes, and the corresponding molecular mechanism.

We used high-density primary cultures of rabbit articular chondrocytes. HO-1 expression was evaluated by Western blotting. HO-1 activity on IL-1b-induced NO was evaluated with use of the Griess reaction. Transient transfection of and Western blotting for IκBα, NF-κB, inducible nitrite oxide synthase and HO-1 elucidated the molecular mechanism of the HO-1 activity on IL-1b-induced NO.

We found evidence, for the first time, of the inducible expression of HO-1 in articular chondrocytes. Overproduction or overexpression of HO-1 is able to decrease dramatically in a dose-dependent manner the IL-1b-induced NO release in the culture medium from articular chondrocyte culture. The inhibition of NO production occurs at the transcriptional level through an NF-κB-dependent pathway.

These results demonstrate the critical role of HO-1 in inhibiting the IL-1b-induced NO. *In vivo* experiments are under investigation.

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Overexpression and induction of heat shock protein 70 protect chondrocytes from cell death *in vitro* and *in vivo*

L Grossin, C Cournil-Henrionnet, A Watrin-Pinzano, B Terlain, JY Jouzeau, P Netter, P Gillet

UMR 7561, CNRS Nancy-I, Faculté de Médecine, Vandoeuvre-les-Nancy, France

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Introduction Osteoarthritis (OA) is characterized by fibrillation and erosion of hyaline cartilage, subchondral bone sclerosis and osteophyte

formation at the joint margins. These changes, resulting from poorly understood events, lead to both cartilage matrix degradation and inhibition of matrix component synthesis. Moreover, cartilage hypocellularity due to cell death (apoptosis or necrosis) contributes to the development of OA. Heat shock protein 70 (Hsp70) plays a protective role as a molecular chaperone in cells by facilitating the folding, intracellular transport, assembly, and disassembly of proteins. An increase in the expression of Hsp70 in chondrocytes has been associated with the severity of OA lesions and could be an indicator of the early stages of OA. Some experiments recently demonstrated that Hsp70 protects cells from death induced by stresses, mechanical and biological factors.

Objectives To determine whether Hsp70 overexpression is able to protect rat chondrocytes towards mono-iodoacetate (MIA) cytotoxicity, *in vitro* and *in vivo*.

Methods Induction of Hsp70 was performed with MG132, a proteasome inhibitor, and overexpression of Hsp70 was realized by the non-viral gene transfer method. The chondroprotective effect of Hsp70 was assessed *in vitro* by exposing cells to MIA and the protection was determined by MTT/lactate dehydrogenase analyses. An antisense strategy was used to confirm that Hsp70 played the major role in protecting chondrocytes from MIA toxicity. In a second step, the relevance of a preventive induction or overexpression of Hsp70 was assessed *in vivo*, during experimental OA in the rat induced by intra-articular injection of MIA.

Results *In vitro*, the increase of Hsp70 by proteasome inhibitor exposure or by gene transfer was able to efficiently protect chondrocytes from MIA toxicity. The antisense strategy confirmed that Hsp70 mainly mediated this chondroprotective effect. *In vivo*, the induction (intra-articular injection of MG132) or overexpression of Hsp70 (electric gene transfer) was sufficient to decrease the severity of the OA-lesions induced by MIA exposure, as demonstrated by histological and biochemical analyses.

Conclusions Overexpression of Hsp70 in cartilage could be an interesting way to protect *in vitro* chondrocytes from cell death and to reduce *in vivo* the progression of OA-related chondral erosions.

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Role of HC-gp39 in chondrogenesis: upregulation of SOX-9 expression by HC-gp39 in mouse chondrocytes

C Jacques¹, A Recklies², A Levy¹, A Labat¹, F Berenbaum¹

¹Department of Physiology and Physiopathology, UMR 7079 CNRS, University of Paris, France; ²Shriners Hospital for Children, Montreal, PQ, Canada

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Introduction Increased levels of the chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) have been demonstrated in synovial fluids of patients with rheumatoid or osteoarthritis. Recently, this protein has emerged as a potential growth factor for synovial cells and chondrocytes, but little is known about its role in differentiation. The transcription factor SOX-9 has been demonstrated to be a master regulator of the differentiation of mesenchymal cells into chondrocytes.

Aim We investigate the effects of two growth factors, insulin-like growth factor-I (IGF-I) and HC-gp39, on SOX-9 expression, and we sought to identify transduction signalling pathways involved in SOX-9 expression.

Methods Expressions of SOX-9 and type II collagen were analysed in primary culture of mouse costochondral chondrocytes using Western blot analysis. Chondrocytes were stimulated by IGF-I and/or HC-gp39 during 24 hours. The kinetics with IGF-I and/or HC-gp39 (up to 60 min) was assessed with different antibodies against phosphorylated and nonphosphorylated forms of p38 MAPK, ERK1/2 MAPK, SAPK/JNK and AKT.

Results IGF-I (25 ng/ml) and HC-gp39 (1 µg/ml) were found to markedly upregulate SOX-9 protein expression at 24 hours, which was in parallel with an increased expression of type II collagen protein. An IGF-I and HC-gp39 cotreatment showed an additive effect on SOX-9 protein expression. IGF-I and HC-gp39 were found to rapidly activate the phosphorylation of ERK1/2 MAPK and AKT (peak at 2 min), whereas p38 MAPK and SAPK/JNK pathways were not involved.

Conclusions These results indicate that the expression of the gene for the master chondrogenic factor SOX-9 is stimulated by IGF-I and HC-

gp39 in chondrocytes, and strongly suggest that this regulation is mediated by the ERK1/2 MAPK and AKT pathways. Because SOX-9 is essential for chondrocyte differentiation, we propose that HC-gp39 could be considered a novel actor involved in chondrogenesis.

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Evidence that rosiglitazone acts as an antidegradative agent on cartilage cells *in vitro*

M Francois¹, L Tsagris¹, C Forest¹, P Richette¹, M Raymondjean², MT Corvol¹

¹UMR-S 530, Inserm-Universite Paris V, Paris, France; ²UMR 7079, CNRS-Paris VI, Paris, France

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Recent studies have documented the role of the natural ligand (15d-PGJ2) of peroxisome proliferator-activated receptor gamma (PPAR γ) as an anti-inflammatory agent in IL-1-treated cartilage cells and synovial fibroblasts in culture. Similar but less pronounced effects have been reported with pharmacological concentrations of synthetic PPAR γ ligands, such as thiazolidinediones. These anti-IL-1 effects were attributed to the transcriptional inhibition of NF- κ B and/or AP-1-dependent genes, while PPAR γ implication remained questionable. We studied the effects of rosiglitazone, a thiazolidinedione with high affinity for PPAR γ , in IL-1-treated rabbit articular chondrocytes (ARC) in culture. When used at 10 μ M, rosiglitazone inhibited IL-1-induced nitric oxide production, COX-2 mRNA, decreased the degradative effect of IL-1 on ³⁵S-sulfated proteoglycans and the 55 kDa gelatinolytic activity secreted by ARC, and downregulated MMP-1 mRNA. By contrast, when used at 0.1–1 μ M, rosiglitazone decreased proteoglycan degradation and MMP-1 gene expression, whereas it did not modify chondrocyte nitric oxide production nor COX-2 mRNA expression. Transient transfection of ARC with MMP-1-Luc showed that IL-1 stimulation was inhibited in a dose-dependent manner by rosiglitazone from 0.1 μ M to 1 μ M, indicating that rosiglitazone was acting at the transcriptional level. The electrophoretic mobility shift assay showed that IL-1-induced NF- κ B binding activity was not changed by rosiglitazone, while IL-1-induced AP-1 binding activity was reduced. We analysed rosiglitazone's effect on MMP-1 promoter activation in cells transiently cotransfected with MMP-1-Luc vector. We showed that the inhibitory effect of rosiglitazone was significantly more pronounced in cells cotransfected with wild-type PPAR γ than without, while this effect was completely suppressed by cotransfecting cells with the dominant-negative PPAR γ . Altogether, these data show for the first time that rosiglitazone has a selective inhibitory effect on IL-1-induced MMP-1 in chondrocytes, which involves a mechanism whereby PPAR γ and AP-1 are implicated.

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The effect of cyclooxygenase-2 selective inhibitors on the proliferation and apoptosis of human articular chondrocytes

YW Song, EB Lee, EM Park, JC Lee, YJ Lee

Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea

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Aim Cyclooxygenase-2 (COX-2) selective inhibitors are being widely used in the treatment of osteoarthritis, with their favorable efficacy and safe profiles. The proliferative role of COX-2 has been recognized in several human cancers and its inhibitors were found to induce apoptosis in these cancer cells. Since the apoptosis of chondrocytes is an important contributor to the development of the osteoarthritis, we determined to investigate the effect of selective COX-2 inhibitors on the proliferation and apoptosis of human chondrocytes.

Methods Human chondrocytes from osteoarthritis patients were treated with selective COX-2 inhibitors (celecoxib, MF-tricyclic) and nonselective inhibitors (sulindac and indomethacin) under various conditions. Cell proliferation was assessed by MTT assay and the apoptosis was quantified by flow cytometry analysis after propidium iodide staining.

Results The COX-2 selective inhibitor celecoxib strongly inhibited the proliferation of chondrocytes in a dose-dependent and time-dependent manner. When compared with dimethylsulfoxide controls, chondrocytes proliferated 49.1 \pm 9.7% under 40 μ g/ml celecoxib, 80.4 \pm 28.3% under 40 μ g/ml sulindac sulfide, 93.1 \pm 13.3% under 40 μ g/ml

indomethacin and 113.7 \pm 8.9% under another COX-2 selective inhibitor, MF-tricyclic. Incubation with 80 μ g/ml celecoxib for 12 hours lead to apoptosis in 6.6% of the chondrocytes, while incubation with 80 μ g/ml MF-tricyclic lead to apoptosis in 4.8% of the cells.

Conclusions In this study, we found that the COX-2 selective inhibitor celecoxib could inhibit proliferation of chondrocytes from osteoarthritis patients, while another COX-2 selective inhibitor, MF-tricyclic, does not affect the proliferation of the cells. Further study will be warranted on the clinical effects of different COX-2 selective inhibitors in osteoarthritis patients. Furthermore, our results suggest that celecoxib may induce apoptosis of chondrocytes via other pathways than COX-2 inhibition.

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The role of IL-4 in the control of mechanical stress-induced inflammatory mediators by rat chondrocytes

K Nishida¹, H Doi¹, A Shimizu³, M Yorimitsu¹, M Takigawa², H Inoue¹

¹Science of Functional Recovery and Reconstruction, Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Okayama, Japan; ²Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Okayama, Japan; ³Department of Orthopaedic Surgery, Ehime University Medical School, Ehime, Japan

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A complex environment controls the metabolism of chondrocytes in the inflammatory joint. Although mechanical stimuli are essential for the chondrocyte metabolism, excessive mechanical force contributes to the release of inflammatory mediators leading the cartilage destruction under the arthritic condition. IL-4 is a chondroprotective cytokine, which is also involved in the integrin-mediated chondrocyte mechanotransduction. We examined the effects of cyclic mechanical stress on the gene expression profile of rat chondrocytes, and tested the *in vitro* effects of recombinant IL-4. Chondrocytes were obtained from the knee joints of 7-day-old Wister rats. Cyclic mechanical stress was applied on the cultured chondrocytes for 24 hours using Flexercell strain unit (Flexcell International Co., Hillsboro, NC, USA) with 0.5 Hz, 7% elongation, with or without treatment by recombinant IL-4. The cDNA microarray analysis revealed that, of 1080 genes, 37 genes were upregulated and 46 genes were downregulated after mechanical stress. RT-PCR and real-time PCR analysis confirmed that treatment by IL-4 resulted in the significant downregulation of mRNA expression of cysteine proteinase cathepsin B, and the inducible form of nitric oxide synthase. Next, an osteoarthritis model was created in the knee joints of Wistar rats (200 g) with anterior cruciate ligament and medial collateral ligament transection. The rats were killed 2, 4, or 6 weeks after the surgery, and the gross morphology and histology of the knee joint cartilage were examined. The preliminary results showed that intra-articular administration of IL-4 exerted a protective effect on the development of osteophytes, and cartilage lesions in this model of osteoarthritis. These results suggested the possible role of IL-4 in the control of mechanical stress-induced inflammatory mediators *in vitro* and *in vivo*.

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TNF-alpha induced chondrocyte apoptosis in NF-kB suppression is augmented by inhibition of p38 mitogen-activated protein kinase or phosphatidylinositol 3-kinase

H Kim

Internal Medicine, Hallym University College of Medicine, Chunchun, Korea

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Background Therapeutics employing knowledge on various signaling pathway are being developed, with NF- κ B one of the most promising targets. NF- κ B has been suggested to play a role not only in the induction of inflammatory mediators, but also in the protection of apoptosis.

Objectives This study pursued the role of the NF- κ B pathway in the regulation of chondrocyte death induced by tumor necrosis factor alpha (TNF- α) and the pertinent target molecules involved.

Methods NF- κ B activation was specifically inhibited using an adenoviral vector encoding the transgene for I κ B super-repressor (ad-I κ B-SR). The human juvenile costal chondrocyte cell line (C28/I2) was used for the experiment. Infection with ad-I κ B-SR was performed for 360 min using a multiple of infection of 1:200, after which the cells were incubated for 18 hours in medium containing 10% FCS. The cultures were then changed to serum-free medium, and treated with 10 ng/ml TNF- α . The proportion of cell death was analyzed by MTT assay. Activation of p38 mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3K) was inhibited by preincubation for 1 hour with SB202190 and Ly 294002, respectively. The expression of apoptosis-related protein was analyzed with Western blot assay. The activation of c-JUN N-terminal kinase was analyzed by gel kinase assay.

Results Despite complete inhibition of NF- κ B activation, treatment with TNF- α led to cell death in only about 23% of ad-I κ B-SR-infected chondrocytes after 24 hours. Preincubation with SB202190 and Ly 294002 led to a significant increase in cell death, resulting in 53% and 30% cell death after 24 hours, respectively. The expression of Bcl-XL and XIAP significantly decreased, and activation of c-Jun N-terminal kinase was prolonged up to 4 hours in infected cells treated with TNF- α .

Conclusion In our experimental system, specific inhibition of NF- κ B activation rendered chondrocytes susceptible to cell death induced by TNF- α . However, completion of cell death required inhibition of another signaling pathway such as p38 MAP kinase and PI3K. The expression of Bcl-XL and XIAP, and activation of c-Jun N-terminal kinase was affected by ad-I κ B-SR transduction, implying its role in the NF- κ B-regulated cell survival signaling in human chondrocytes.

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Cell biology and functional genomics of osteoarthritic chondrocytes

T Aigner¹, E Bartnik², R Zimmer³

¹Department of Pathology, University of Erlangen, Erlangen, Germany; ²Aventis Pharma Deutschland, Disease Group Osteoarthritis, Frankfurt, Germany; ³Practical Informatics and Bioinformatics, Computer Science Department, University of Munich, Germany

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Introduction Functional genomics represents a new challenging approach in order to analyze complex diseases such as osteoarthritis on a molecular level. In particular, the identification and characterization of new target molecules for therapeutic intervention is of interest. Also, potential molecular markers for diagnosis and monitoring of osteoarthritis will contribute to a more appropriate patient management.

Objectives In our analyses, we attempt to establish a broader gene expression profile of osteoarthritic chondrocytes by modern screening technologies in order to characterize more properly the cellular events and regulatory pathways directly involved in cartilage destruction.

Methods Cartilage from human femoral condyles of the normal knee joints were obtained from donors at autopsy. Late-stage osteoarthritic cartilage was obtained from patients undergoing total knee replacement surgery. Gene expression analysis was performed by various array technologies and by quantitative PCR. For normalization we applied a novel computational normalization method [1].

Results Our first focus in looking at chondrocyte gene expression is always linked to the main function of this cell type, the preservation and turnover of the cartilage matrix. Thus, for a start, matrix components and matrix-degrading proteases were the focus of our interest [2]; the analysis of the extracellular matrix proteins showed the largely absent expression of cartilage collagens in normal cartilage and very much increased mRNA levels of several collagen genes in advanced osteoarthritis. With regard to the cartilage-matrix-degrading metalloproteinases, a characteristic pattern was observed in osteoarthritic cartilage versus normal articular cartilage. Future studies will focus on the investigation of pathogenetic pathways based on bioinformatic modelling of their involved genes.

Conclusions Overall, cDNA technology offers cartilage research a powerful tool for investigating gene expression patterns of a high number of different genes simultaneously. Despite constitutive drawbacks of gene expression technologies, still existing technical problems, and the lack of broadly applicable tools in biostatistics and bioinformatics, even now gene arraying and subsequent technologies initiate new research strategies for understanding pathogenesis and development of drug targets.

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CCAAT/enhancer-binding proteins mediate the repression of transcription of cartilage-derived retinoic acid-sensitive protein induced by IL-1 β

K Okazaki¹, Y Iwamoto¹, L Sandell²

¹Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; ²Department of Orthopaedic Surgery, Washington University School of Medicine, St Louis, Missouri, USA

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Introduction IL-1 β is one of the major proinflammatory cytokines involved in arthritis joints. IL-1 β promotes the arachidonic acid cascade, resulting in production of prostaglandin. In addition, IL-1 β represses the expression of matrix proteins in cartilage, such as type II, type IX and type XI collagen and cartilage-derived retinoic acid-sensitive protein (CD-RAP), leading to degradation of the cartilage structures.

Objective To investigate the transcriptional mechanism by which CD-RAP expression is repressed by IL-1 β .

Methods and results Deletion constructs of the CD-RAP promoter were transfected into rat chondrocytes and incubated in the absence or presence of IL-1 β . The results revealed an IL-1 β -responsive element located between -2138 and -2068 bp. As this element contains the CCAAT/enhancer-binding protein (C/EBP) motif, the function of C/EBP- β was examined. IL-1 β stimulated the expression of C/EBP- β . The direct binding of C/EBP- β to the C/EBP motif was confirmed by electrophoretic mobility shift assay. Expression of the -2251 bp CD-RAP promoter construct was downregulated by cotransfection with C/EBP- β expression vectors in a dose-dependent manner. Mutation of the C/EBP motif within the -2251 bp construct abolished the inhibitory response to IL-1 β . These results suggest that C/EBP- β is a critical factor mediating IL-1 β -induced repression of CD-RAP transcription. C/EBP- β expression vectors were also found to downregulate the reporter construct containing the promoter and enhancer of the type II collagen gene. The IL-1 β -induced repression of CD-RAP and type II collagen genes via stimulation of C/EBP- β were confirmed in chondrocytes from normal human articular cartilage. Finally, the enhancer factor, Sox9, known to be downregulated by IL-1, was shown to bind adjacent to the C/EBP site competing with C/EBP binding.

Discussion C/EBP- β is known to mediate the arachidonic acid cascade induced by IL-1 β . These results suggest that C/EBP- β play an important role in the distinct effects of IL-1 β : the promotion of inflammatory reaction and the repression of cartilage-specific proteins in joint disease. Expression of matrix proteins are influenced by availability of both positive and negative trans-acting factors.

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Tissue-engineered cartilage using thermoresponsive gelatin as an *in situ* forming and moldable scaffold with chondrocytes: *in vitro* and *in vivo* performances

S Ibusuki¹, T Matsuda², Y Iwamoto¹

¹Department of Orthopaedic Surgery, Graduate School of Medicine, University of Kyushu, Fukuoka, Japan; ²Department of Biomedical Engineering, Graduate School of Medicine, University of Kyushu, Fukuoka, Japan

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We devised a tissue-engineered cartilage using thermoresponsive gelatin, poly(*N*-isopropylacrylamide)-grafted gelatin (PNIPAAm-gelatin).

An aqueous solution of the PNIPAAm-gelatin can spontaneously gel above 34°C. This character of the PNIPAAm-gelatin might fit a gel to a given shaped chondral defect.

For *in vitro* study, chondrocytes isolated from the articular cartilage of Japanese White Rabbits were three-dimensionally cultured in PNIPAAm-gelatin gel for up to 12 weeks. The chondrocyte–PNIPAAm-gelatin constructs were evaluated at cell and tissue levels. At the cell level, cellular viability and the degree of cellular differentiation were assessed. At the tissue level, the appearance of the tissue, the amount of extracellular matrices (ECMs), and the mechanical properties were assessed by comparison with those of native hyaline cartilage. During 3 weeks of culture, the cellular viability was more than 95%. Confocal laser scanning microscopic images demonstrated that round-shaped cells, which are found in hyaline cartilage, were predominant in the construct. With an increase in culture time, the population of the cells arrested in the G₁/G₀ phase of the cell cycle in a three-dimensional condition was significantly higher than that in a monolayer condition. As type II collagen and sulfated-glycosaminoglycan specific to hyaline cartilage were detected in sections of the construct, little type I collagen, which is a marker of dedifferentiated chondrocytes, was detected. These results indicate that the inoculated cells could express their differentiated phenotype. Moreover, the amounts of ECMs increased and closed to that of native hyaline cartilage with time. Mechanical properties of the constructs tended to close towards those of native cartilage with culture time. These results indicate that cartilaginous tissue using PNIPAAm-gelatin can be reconstructed in *in vitro* conditions. In animal studies, the combination of chondrocyte–PNIPAAm-gelatin constructs precultured for 2 weeks and the cell-incorporated PNIPAAm-gelatin solution were used as implants for chondral defects in the patellae of rabbits. Macroscopic evaluation of the implant harvested at 24 weeks postoperatively showed that, at the rough surface of the implants, tissue continuity to adjacent cartilage with minimal concave deformation was acquired. Tissue sections showed a homogeneous distribution of ECMs in the implanted tissue and no inflammatory cells. Moreover, mechanical properties of the constructs became closer to those of native cartilage. These results indicate that PNIPAAm-gelatin should serve as an adequate scaffold for articular cartilage regeneration.

Poster Discussion (2) Cytokines, Growth Factors & Mediators Group

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Cartilage-derived morphogenetic protein-1 and cartilage-derived morphogenetic protein-2 are present in ligament fibroblasts and lead to chondrogenic differentiation

K Bobacz, L Erlacher, J Smolen, W Graninger

Department of Rheumatology, Internal Medicine III, University of Vienna, Austria

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Objective The enhancement of tissue repair by growth factor stimulation is of potential therapeutic interest. Given the importance of cartilage-derived morphogenetic protein (CDMP)-1 and CDMP-2 in ligament formation and repair, we studied the effects of these growth factors on the proliferation and metabolism of ligament fibroblasts as well as their osteogenic/chondrogenic differentiation potential.

Methods Ligament fibroblasts were obtained from 3-month-old calves, plated as monolayers or micromass cultures and stimulated with CDMPs. The expression of the indicated growth factors was assessed either by RT-PCR or by Western immunoblotting. The presence of their respective type I and type II receptors, as well as lineage-related markers were investigated in stimulated and unstimulated cells by RT-PCR and by northern blotting. The biosynthesis of matrix proteoglycans was assessed by [³⁵S]sulfate incorporation in monolayers. Alcian-blue staining as well as toluidin-blue was performed in micromass cultures. The cell proliferation rate was measured by the use of [³H]thymidine incorporation.

Results The presence of CDMP-1 and CDMP-2 was detected on mRNA as well as on the protein level. Type I and type II receptors were endogenously expressed in unstimulated ligament fibroblasts. The growth factors stimulated cell proliferation as measured by [³H]thymidine incorporation and total proteoglycan synthesis as assessed by [³⁵S] incorporation. Alcian-blue and toluidin-blue staining showed the differentiation into chondrocytes in the growth-factor-treated ligament fibroblasts. Moreover, transcription analysis of stimulated ligament fibroblasts demonstrated an upregulation of chondrogenic markers (aggrecan, collagen type II, type IX and type X) but not of osteogenic markers.

Conclusion CDMP-1 and CDMP-2 induce matrix synthesis and cell proliferation in cells derived from bovine ligament. The expression of chondrocyte markers suggests that adult cells from ligaments and tendons have the capability of differentiation into chondrocytes under the influence of CDMPs. Given our current dilemma in the treatment of osteoarthritis, CDMP-1 and CDMP-2 could therefore contribute an interesting therapeutic molecule for osteoarthritis.

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Altered phosphorylation of Syp may be responsible for abnormal insulin-like growth factor-1 signaling in human osteoarthritic osteoblasts

D Lajeunesse, F Massicotte, I Aubry, J Martel-Pelletier, J Pelletier

Rheumatic Disease Unit, Centre Hospitalier de l'Université de Montréal, Hôpital Notre-Dame, Montréal, Québec, Canada

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Introduction The subchondral bone plays a prominent role in the pathophysiology of osteoarthritis (OA), possibly related to abnormal osteoblast metabolism. In particular, abnormal responses to insulin-like growth factor-1 (IGF-1) were noted in OA osteoblasts.

Objectives To investigate whether IGF-1 signaling is abnormal in OA osteoblasts compared with normal osteoblasts.

Methods We used primary human subchondral osteoblasts from normal and OA individuals. Cells were stimulated, or not, with 100 ng/ml IGF-1 for up to 5 min. Proteins were separated by SDS-PAGE or immunoprecipitated with anti-insulin receptor substrate-1 (anti-IRS-1) antibodies followed by SDS-PAGE, and detected with selective antibodies.

Results Upon binding to its receptor (IGF-1R), IGF-1 activates the p42/44 mitogen-activated protein kinase (MAPK) pathway using SHC or phosphorylation of IRS-1 via Grb2. The interaction of IRS-1 with IGF-1R is also modulated via the binding of a tyrosine phosphatase, Syp. IGF-1R α -subunits, SHC, IRS-1, Syp, Grb2 and p42/44 MAPK levels, by Western blot analysis, were similar in normal and OA osteoblasts under basal condition and following IGF-1 treatment. IGF-1 stimulated IGF-1R autophosphorylation in normal and OA osteoblasts similarly. However, IRS-1 phosphorylation was reduced whereas p42/44 MAPK phosphorylation was higher in OA osteoblasts than normal osteoblasts in response to IGF-1. In normal osteoblasts, Syp was poorly phosphorylated under basal conditions and rapidly became phosphorylated upon IGF-1 stimulation, while Syp was already highly phosphorylated under basal conditions in OA osteoblasts and was dephosphorylated upon IGF-1 stimulation. Co-immunoprecipitation of Syp using IRS-1 antibodies showed that this interaction is poor under basal conditions in normal cells yet increases following IGF-1 treatment, while in OA osteoblasts this interaction was very strong and rapidly dropped with IGF-1 treatments, indicating that phospho-Syp is the trigger. Co-immunoprecipitation of Grb2 using IRS-1 antibodies showed that Grb2 interaction with IRS-1 was increased in OA osteoblasts compared with normal osteoblasts under basal conditions and following IGF-1 stimulation.

Conclusion These results suggest that an abnormal interaction of phospho-Syp with IRS-1 in OA osteoblasts leads to a reduced activity of IRS-1-dependent pathways. In addition, the increased interaction of Grb2 with IRS-1 suggests that the SHC–Grb2 interaction is reduced in OA osteoblasts, and hence could not explain the stimulation of p42/44 MAPK.

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15-deoxy-delta^{12,14}-prostaglandin J₂ induces apoptosis in human articular chondrocytes**K Masuko-Hongo, Z-Z Shang, T Kato, H Nakamura, K Nishioka***Department of Bioregulation, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan**Arthritis Res Ther* 2003, **5(Suppl 3):64** (DOI 10.1186/ar865)

A derivative of prostaglandin D₂, 15-deoxy-delta^{12,14}-prostaglandin J₂ (15d-PGJ₂) is one of the cyclooxygenase (COX) products in the arachidonic acid cascade. PGJ₂ series have been reported to exert various bioactivities, including modulation of viability of a wide array of cells, and are known to act as an active ligand for the nuclear receptor peroxisome proliferator-activated proteins gamma. 15d-PGJ₂ is also suggested to play a role in cartilage metabolism; however, this issue is still not fully understood. We investigated the potential role of the 15d-PGJ₂ in the induction of apoptotic death in human articular chondrocytes. Cartilage samples were obtained from patients who underwent joint surgery. Articular chondrocytes were isolated from the cartilage by mincing and enzymatic treatment, and the cells were further cultured in a monolayer system *in vitro*. Confluent cells at the first passage were stimulated with the 15d-PGJ₂, and the effect of the 15d-PGJ₂ in the viability of chondrocytes were assessed using Hoechst staining, flow cytometric analysis and the caspase-3 activity assay. The results showed that the 15d-PGJ₂ was potent to induce chondrocyte apoptosis characterized by cellular shrinkage and nuclear condensation, along with an increased activity of caspase-3 in the treated cells. Involvement of the 15d-PGJ₂ in chondrocyte apoptosis may have an important implication in the pathogenesis of inflammatory and degenerative joint diseases.

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Prostaglandin E₂ is an enhancer for IL-1β-induced expression of membrane-associated prostaglandin E synthase in rheumatoid synovial fibroblasts**F Kojima¹, H Naraba², S Kawai¹***¹Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²National Cardiovascular Center Research Institute, Osaka, Japan**Arthritis Res Ther* 2003, **5(Suppl 3):65** (DOI 10.1186/ar866)

Introduction Membrane-associated prostaglandin E synthase (mPGES) is a recently identified terminal enzyme of arachidonic acid cascade that catalyzes prostaglandin (PG)H₂ to PGE₂. We recently reported that expression of mPGES in rheumatoid synovial fibroblasts (RSF) was upregulated by IL-1β, and that the time-course of its expression was delayed and prolonged when compared with that of cyclooxygenase-2 (COX-2).

Objective To identify the regulating factors of mPGES expression in RSF.

Methods RSF from patients with rheumatoid arthritis (RA) on surgery, who gave written consent to the use of tissues for this research, were treated with IL-1β, rofecoxib, NS-398, and meloxicam (selective COX-2 inhibitors). The effects of PGE₂ and selective EP1, EP2, EP3, and EP4 receptor agonists (DI-004, AE1-259-01, AE-243, and AE1-329, respectively; Ono Pharmaceutical Co., Osaka, Japan) were also studied in various conditions. Expression of the mRNA and protein of mPGES and COX-2 were analyzed by northern and Western blot analyses, respectively. Expression of PGE₂ receptor mRNA in RSF was determined by RT-PCR. PGE₂ and cAMP production was measured by an ELISA.

Results Enhanced expression of mPGES mRNA and protein in IL-1β-stimulated RSF was attenuated by rofecoxib, NS-398, or meloxicam. This attenuation was restored by addition of PGE₂. Exogenous PGE₂ only (without IL-1β stimulation) did not induce expression of mPGES in RSF, indicating that PGE₂ was an enhancer of mPGES expression in the IL-1β-stimulated condition.

We then examined which PGE₂ receptor was important in the enhancing action of PGE₂ in RSF. EP2 and EP4 receptor mRNA was detected in RSF; however, EP1 and EP3 were negative. Addition of AE1-259-01 (EP2 agonist) or AE1-329 (EP4 agonist) as well as PGE₂ restored the inhibitory effects of mPGES expression by rofecoxib. In addition, the restoration by PGE₂ was mimicked by forskolin, a direct

activator of adenylate cyclase. Intracellular cAMP was increased by IL-1β and it was inhibited by rofecoxib.

Conclusions The enhancing property of PGE₂ via EP2/EP4 receptors on mPGES expression may play an important role in the cytokine-stimulated articular inflammation in RA patients. It also seems that COX-2 inhibitors effectively decrease PGE₂ production not only by COX-2 inhibition, but also by reduction of mPGES expression.

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IL-1 but not IL-18 induces osteoprotegerin and TRAIL in rheumatoid arthritis synovial fibroblasts**J Morel, R Audo, B Combe***Service d'Immuno-Rhumatologie, CHU Lapeyronie, Montpellier, France and Inserm U454, Montpellier, France**Arthritis Res Ther* 2003, **5(Suppl 3):66** (DOI 10.1186/ar867)

Introduction IL-18, a member of the IL-1 family, seems to be involved in bone destruction observed in rheumatoid arthritis (RA). Synovial proliferation secondary to an apoptosis defect and the RANK/RANKL system may be implicated in IL-18-induced bone destruction. Therefore, we examined IL-18, IL-1β and tumor necrosis factor (TNF)-α effects on RANKL, the pro-apoptotic factor TRAIL, and osteoprotegerin (OPG), the soluble receptor of RANKL and TRAIL in synovial fibroblasts.

Materials and methods Synovial fibroblasts (SFB) isolated from RA patients were stimulated with IL-18, IL-1β or TNF-α for 24 hours. Conditioned media was collected and mRNA was then extracted. Expression of OPG and soluble RANKL (RANKLs) were assessed by quantitative RT-PCR (Sybr Green) and by ELISA. IL-18, IL-1β and TNF-α did not induce RANKLs mRNA and protein expression. TRAIL expression was examined by semi-quantitative RT-PCR.

Results Unstimulated fibroblasts constitutively expressed OPG 2±0.364 ng/ml. IL-18 did not increase mRNA and protein OPG expression. IL-1β induces OPG mRNA expression and a 14-fold increase of OPG protein (*P*<0.05; *n*=4). TNF-α also increases OPG mRNA and protein levels, but not significantly as compared with unstimulated RA SFBs (*P*=0.21; *n*=4). For TRAIL, IL-18 again does not induce TRAIL mRNA, whereas TNF-α-stimulated and IL-1β-stimulated synovial fibroblasts produce TRAIL mRNA.

Conclusion IL-18 does not regulate the RANKL/OPG system and TRAIL in RA SFBs. IL-1β and TNF-α induce expression of OPG and TRAIL but not of RANKL. These results contrast with the known implication of these cytokines in bone destruction. Since OPG can also bind to TRAIL, a cytokine involved in apoptosis, OPG may block the pro-apoptotic function of TRAIL.

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Negative regulation of the hypoxia-inducible transcription factors by IPAS defines a novel anti-angiogenesis therapeutic strategy**Y Makino¹, L Poellinger², H Nakamura¹, C Morimoto¹, H Tanaka^{1,3}***¹Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Japan;**²Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden; ³Rheumatology Clinic, Research Hospital,**Institute of Medical Science, The University of Tokyo, Japan**Arthritis Res Ther* 2003, **5(Suppl 3):67** (DOI 10.1186/ar868)

Angiogenesis is an essential component of proliferative synovitis leading to joint destruction in rheumatoid arthritis (RA). Recent studies have demonstrated the presence of hypoxia and the accumulation of pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF), in the synovium from patients with RA, indicating a possible involvement of those factors in synovial angiogenesis. The hypoxia-inducible transcription factor-1α (HIF-1α) is a key regulator of VEGF gene expression and angiogenesis under hypoxic conditions [1]. We have recently demonstrated abnormal expression of HIF-1α and

VEGF in the synovium from patients with RA, indicating a potential contribution of the HIF-1 α -VEGF system to angiogenic processes of synovitis in RA. The precise mechanism of HIF-1 α -mediated control of VEGF expression and angiogenesis, however, is largely unknown.

Given this background, we aimed to elucidate the molecular mechanism underlying hypoxia-inducible VEGF expression via HIF-1 α , and identified a novel basic helix-loop-helix/Per, Arnt, Sim protein (inhibitory Per, Arnt, Sim domain protein [IPAS]) that has high structural similarity to HIF-1 α [2]. In sharp contrast to HIF-1 α , IPAS was not capable of activating hypoxia-inducible gene expression. Co-expression of IPAS and HIF-1 α resulted in repression of hypoxia-inducible VEGF gene expression, demonstrating a dominant-negative regulatory activity of IPAS on HIF-1 α -mediated control of gene expression. *In situ* hybridization analysis of various mouse tissues showed predominant IPAS expression in the avascular corneal epithelium, correlating with low levels of VEGF gene expression under hypoxic conditions. Strikingly, application of an IPAS antisense oligonucleotide to the mouse cornea induced angiogenesis under normoxic conditions, and unmasked hypoxia-dependent induction of VEGF gene expression in hypoxic cornea cells. Moreover, ectopic expression of IPAS in hepatoma cells impaired induction of genes involved in adaptation to a hypoxic environment, and resulted in retarded tumor growth and tumor vascular density *in vivo*. Taken together, IPAS, a dominant-negative regulator of HIF-1 α function, would define a novel anti-angiogenic mechanism under hypoxic conditions, providing a possible therapeutic strategy for angiogenic diseases including RA.

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Innate response cytokines in inflammatory synovitis: novel targets and strategies

I McInnes¹, BP Leung², JAG Gracie¹, D Xu², XQ Wei², J Brewer², FY Liew²

¹Centre for Rheumatic Diseases, University of Glasgow, UK; ²Division of Immunology, Infection and Inflammation, University of Glasgow, UK *Arthritis Res Ther* 2003, **5**(Suppl 3):68 (DOI 10.1186/ar869)

Host immune and inflammatory factors contribute to progression of inflammatory synovitis – targeting specific factors such as tumour necrosis factor brings about substantial clinical benefit. We have focused on the effector function of innate immune response cytokines in synovitis including IL-15 and members of the IL-1/IL-1 receptor superfamily, particularly IL-18. Our strategy has been to identify mediators with plausible bioactivity, to establish that they or their target ligands are present in inflammatory synovial tissues and, thereafter, to target them *in vivo* using appropriate inflammatory rodent models. Thus, IL-15 is a 4 α -helix inflammatory cytokine with plausible biologic effects on synovial cell subsets, expressed in synovial tissues in rheumatoid arthritis (RA) and psoriatic arthritis, and when targeted *in vivo* leads to suppression of collagen-induced arthritis in DBA/1 mice. A recent phase I/II study in patients with RA in which HuMaxIL-15 was administered weekly for 4 weeks suggested that IL-15 blockade was well tolerated and provided preliminary evidence for efficacy, suggesting our approach is valid. We recently gathered a similar evidence base for IL-18, demonstrating widespread IL-18 expression in synovial tissues in RA and psoriatic arthritis effector function in T cells, macrophages, fibroblasts and neutrophils in synovial tissues, and finally providing *in vivo* evidence that IL-18 has an effector function *in vivo* by showing that IL-18-deficient mice have reduced the severity of collagen-induced arthritis. A striking feature is the synergy exhibited by IL-18 with IL-12 and IL-15. Studies in which IL-18 is targeted in human inflammatory arthropathies are now awaited. Finally, since IL-15 and IL-18 are both key mediators of dendritic cell (DC) effector function, and are expressed in synovial DCs, we have focused on the means whereby DCs could promote or sustain inflammatory synovitis. To this end, we have shown that collagen-pulsed DCs adoptively transferred into naïve DBA/1 recipients initiate a remitting relapsing arthritis, medi-

ated in part via innate response cytokines, including tumour necrosis factor. By facilitating transfer of gene-targeted cell donors, or administration of cytokine inhibitors, this model provides a novel system in which to test the relative role of innate response cytokines during initiation of but also during chronic phases of inflammatory arthritis.

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Role of lipoxins, antflammins and serum amyloid A signaling via the common ALXR receptor in joint tissue inflammation

S Fiore¹, S Sodin-Semrl¹, G Antico², A Spagnolo¹, B Barbaro¹, J Varga¹, L Miele²

¹Section of Rheumatology, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois, USA; ²Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, Illinois, USA *Arthritis Res Ther* 2003, **5**(Suppl 3):69 (DOI 10.1186/ar870)

Serum amyloid A (SAA) is an acute-phase protein and inflammatory marker of rheumatoid arthritis, which has recently become recognized as a cytokine-like reactant. SAA has been shown to bind to the ALXR (FPRL1/LXA₄R) receptor and to elicit proinflammatory activities. Indeed, we have determined that SAA elicits the proinflammatory activation of human fibroblast-like synoviocytes, with a profile of activity similar to that of IL-1 β . Uteroglobin is a ubiquitously expressed secretory protein with anti-inflammatory properties that shares specific sequence motifs with annexin-1, a steroid-induced anti-inflammatory peptide that is also capable of interacting with ALXR. Data obtained by flow cytometry, ELISA and plasmon resonance, using ALXR stably transfected CHO cells, suggest that uteroglobin can downmodulate SAA proinflammatory effects by specifically interfering at the ALXR receptor level. In agreement with the ability to bind to the same receptor used by SAA, a functional antagonism between uteroglobin and SAA was observed, with uteroglobin causing inhibition of SAA-induced release of both IL-8 and IL-6, and blockage of SAA-induced PLA₂ activation. Beside the receptor antagonism between proinflammatory ALXR ligands (SAA, urokinase proteolytic plasminogen peptides [uPA], MK1, etc.) and anti-inflammatory ALXR ligands (LXA₄, annexin-1, uteroglobin) so far identified, results also suggest that each of these mediators can modify ALXR expression levels as well as other genes. In fact, present studies suggest that SAA acts in a positive autoregulatory feedback mechanism by increasing SAA 1 + 2 expression, while maintaining unchanged levels of its constitutively expressed isoform SAA4, as demonstrated by RT-PCR. Following SAA treatment, upregulation is extended to the expression of the ALXR mRNA, a response common to LXA₄ and IL-1 β . The antagonistic activities of SAA, uPA, uteroglobin, annexin-1 and LXA₄ might constitute a novel complex signaling mechanism by which these mediators sharing a common receptor could achieve opposing roles in the regulation of the joint inflammatory processes.

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IL-6 as a therapeutic target in systemic-onset juvenile idiopathic arthritis

S Yokota, T Miyamae, T Imagawa, M Mori

Department of Pediatrics, Yokohama City University, Yokohama, Japan *Arthritis Res Ther* 2003, **5**(Suppl 3):70 (DOI 10.1186/ar871)

Background To investigate the safety and efficacy of a recombinant human anti-IL-6R monoclonal antibody (MRA) for children with systemic-onset juvenile idiopathic arthritis (So-JIA), a long-term administration of MRA was performed in three children with So-JIA.

Case report Two boys and one girl with recurrent spiking fever, rash, and arthritis diagnosed as So-JIA (case 1, 7 years old; case 2, 8 years old; case 3, 13 years old) have been treated for 2.6 years, 2.5 years, and 1.9 years, respectively, with MRA after long-term treatment with prednisone and cyclosporin A, being complicated with macrophage activation syndrome during the course of the disease, and resulting in short stature, obesity and destruction of vertebrae. Serum levels of C-reactive protein were persistently high, The white blood cell count was over 15,000/ μ l with marked neutrophilia, and increased levels of IL-6 were detected. Intravenous administration of MRA was initiated, and prompt responses were obtained in both clinical and laboratory findings. Before accumulation of

MRA in serum, fluctuations of C-reactive protein, the erythrocyte sedimentation rate and serum amyloid A levels were observed, but the clinical status of spiking fever, rash, and arthritis became stable. MRA was administered once every other week, and therapeutic effects were persisted during the observation period in all three cases. Around 100 days after the initiation of therapy, the white blood cell count was decreased to a normal number, and they started to grow in height (+18 cm, +13 cm, and +11 cm, respectively). Cyclosporin A was discontinued, and prednisone was tapered and ceased. Increased bone density was demonstrated, and an uptake of the marker isotope in an epiphyseal plate by Ga-scintigram gradually emerged, suggesting that IL-6 or IL-6/sIL-6R complexes had previously interrupted the bone growth at epiphysis. Three episodes of infection were clearly assessed by increased serum IL-6 levels. JIA core set and C-HAQ scores were remarkably improved. No definite findings of tuberculosis was determined.

Conclusion For patients with So-JIA, administration of MRA dramatically improved inflammatory disease within 1 week, which indicated a pathogenic role of serum IL-6 and IL-6/sIL-6R complexes in So-JIA. This demonstrated MRA to be the first-line drug for the treatment of the disease.

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Synergistic interactions of proinflammatory cytokines with oncostatin M: implications for joint destruction

T Cawston¹, H Wang¹, C Richards², A Rowan¹

¹Rheumatology, University of Newcastle, Newcastle upon Tyne, UK;

²Department of Pathology and Molecular Medicine, McMaster University, Ontario, Canada

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Oncostatin M (OSM) is an IL-6 family cytokine that we have previously shown to synergise with IL-1 to induce cartilage proteoglycan and collagen degradation in a cartilage explant culture system, and these observations now extend to IL-6 [1,2]. A significant finding of these studies was the synergistic induction of the collagenase, matrix metalloproteinase (MMP)-1, which occurs via interplay between the JAK/STAT, AP-1 and MAPK pathways [3]. These studies have important implications for inflammatory joint disease since OSM (and, indeed, IL-6) have been proposed to be protective in rheumatoid arthritis. Recently, we also demonstrated that OSM can exacerbate the effects of another important proinflammatory mediator, tumour necrosis factor (TNF)- α [4]. In order to assess the effects of these cytokine combinations *in vivo*, we have assessed the effects of intra-articular gene transfer of OSM in combination with either IL-1 or TNF- α on murine knee joints using recombinant adenovirus.

Engineered adenoviruses were administered for only 7 days, after which time joints were fixed, decalcified and sectioned. Histological analyses indicated marked synovial hyperplasia and inflammatory cell infiltration for IL-1-treated, TNF- α -treated and OSM-treated joints, but not in controls (joints treated with an 'empty' adenovirus). The inflammation was more pronounced for both the OSM+IL-1 and OSM+TNF- α combinations with evidence of cartilage and bone destruction. Significant loss of both proteoglycan and collagen was also seen for these combinations, and immunohistochemistry revealed an increased expression of MMPs with decreased tissue inhibitors of metalloproteinases in both articular cartilage and synovium. The effects of these combinations were significantly greater than those seen with any of the cytokines alone. Taken together, these data confirm that, *in vivo*, OSM can significantly exacerbate the effects of both IL-1 and TNF- α , resulting in inflammation and tissue destruction characteristic of that seen in rheumatoid arthritis. This study provides further evidence to implicate the upregulation of MMPs as a key factor in joint pathology, and further supports the concept of combinatorial therapeutic approaches for the treatment of inflammatory joint diseases.

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Novel intracellular function of IL-1 receptor antagonist type 1 in endothelial cells

C Gabay, F Soussi, M Berti

Division of Rheumatology, University Hospital of Geneva, Switzerland

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IL-1 receptor antagonist (IL-1Ra) is a member of the IL-1 family that competitively inhibits the binding of IL-1 to its cell surface receptors, and thus acts as a natural IL-1 inhibitor. Four different IL-1Ra peptides are produced from the same gene by the use of alternative first exons, mRNA splicing, and alternative translation. One isoform is secreted (sIL-1Ra), while the three other isoforms remain intracellular (icIL-1Ra1, icIL-1Ra2, icIL-1Ra3). In a previous study, we observed that icIL-1Ra1 is produced in high amounts in the joints of mice with collagen-induced arthritis (CIA) and that the expression of icIL-1Ra1 coincided with the resolution of articular inflammation. Recently, we showed that mice transgenic for icIL-1Ra1 were protected from CIA in a similar manner to sIL-1Ra transgenic mice. However, as icIL-1Ra1 was also detected in the circulation, it was impossible to determine whether icIL-1Ra1 exerted its anti-inflammatory effect inside cells or through its interaction with cell surface receptors. To address this question, we examined the intracellular effects of icIL-1Ra1 on endothelial cell migration. IL-1 α is produced as a 31 kDa protein (pre-IL-1 α) that is cleaved to generate C-terminal mature IL-1 α . The N-terminal region of pre-IL-1 α contains a nuclear localization domain, and both pre-IL-1 α and the 16 kDa N-terminal IL-1 α are able to migrate into the nuclei and to modulate endothelial cell migration. The migration of the endothelial cell line ECV was significantly increased in cells transfected with pre-IL-1 α or N-terminal IL-1 α propiece. In contrast, the addition of mature IL-1 α in culture medium was devoid of any effect on cell migration, indicating that the effect of pre-IL-1 α and 16 kDa N-terminal IL-1 α was independent of their interaction with cell surface receptors. Most interestingly, expression of icIL-1Ra1 in ECV cells completely reversed the effects of pre-IL-1 α and N-terminal IL-1 α . Immunofluorescence studies indicated that the N-terminal IL-1 α was located in the nuclei, icIL-1Ra1 was located both in the cytoplasm and in the nuclei, and co-expression of icIL-1Ra1 modified the intracellular localization of the N-terminal IL-1 α . In conclusion, this study demonstrates for the first time that icIL-1Ra1 may carry out important intracellular regulatory functions in endothelial cells.

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Unbalanced levels of bone resorption promoting factors in bone marrow from patients with rheumatoid arthritis in comparison to osteoarthritis

W Maslinski¹, J Jaworski³, M Ziolkowska¹, J Kowalczewski², J Pazdur³

¹Department of Pathophysiology and Immunology, Institute of Rheumatology, Warsaw, Poland; ²Clinic of Orthopaedy, Institute of Rheumatology, Warsaw, Poland; ³Clinic of Rheumatic Diseases, Institute of Rheumatology, Warsaw, Poland

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Introduction RANKL and IL-6 participate in osteoclast differentiation and activation, and therefore regulate bone resorption. The biological activity of RANKL is inhibited by its natural decoy receptor osteoprote-

Table 1

Levels of RANKL, osteoprotegerin (OPG), IL-6, soluble IL-6 receptor (sIL-6R), and soluble glycoprotein 130 (sgp130) in the bone marrow of rheumatoid arthritis and osteoarthritis patients

Patients	RANKL (pg/ml)	OPG (ng/ml)	OPG:RANKL ratio	IL-6 (pg/ml)	sIL-6R (ng/ml)	sgp130 (ng/ml)	sgp130:sIL-6R ratio	sIL-6R:IL-6 ratio
Rheumatoid arthritis	61 ± 50	9.9 ± 3.3	163:1	284 ± 370	33.7 ± 19	195 ± 111	5.8:1	118:1
Osteoarthritis	40 ± 34	12.4 ± 3.2	311:1	132 ± 76	16.7 ± 6.9	123 ± 55	7.3:1	126:1
Rheumatoid arthritis vs osteoarthritis	NS	$P < 0.05$		Not significant	$P < 0.0002$	$P < 0.009$		

NS, not significant.

gerin (OPG), while IL-6 activity is either enhanced by soluble IL-6 receptor (sIL-6R) or inhibited by soluble glycoprotein 130 (sgp130), an extracellular domain of the signaling chain of the IL-6 receptor complex.

Objectives To compare the levels of RANKL, OPG, IL-6, sIL-6R and sgp130 in bone marrow plasma isolated from rheumatoid arthritis (RA) and osteoarthritis (OA) patients.

Methods Bone marrow samples isolated from 29 RA patients and 12 OA patients (mean age 51 ± 14.3 years and 56.3 ± 10.5 years, respectively) undergoing hip replacement surgery were diluted twice in heparinized PBS. The levels of cytokines were measured using specific ELISAs.

Results The levels of tested cytokines, the ratio of respective ligands to their receptors and the statistical significance of the data are presented in Table 1.

The levels of RANKL in RA patients were elevated by 53% in comparison with those in OA patients. In contrast, the levels of OPG were diminished by 20%. Thus, the ratio of OPG/RANKL, thought to better reflect environmental signals promoting bone resorption than levels of individual cytokines, is diminished in RA patients in comparison with OA patients. Similarly, the 115% increase of IL-6 and the 102% increase of sIL-6R is not compensated for by only the 59% increase of sgp130 in RA patients in comparison with OA patients.

Conclusion The present data indicate that the bone marrow microenvironment of RA patients in comparison with OA patients is enriched in factors promoting osteoclastogenesis, osteoclast activation and bone resorption. At the same time, the levels of corresponding natural decoy receptors is diminished. Thus, RA-associated osteoporosis and bone erosions may, at least partially, be related to unbalanced production of RANKL, IL-6 and sIL-6R in the bone marrow.

74**Molecular pain signalling in joints**

H Sprott¹, H Shen¹, RE Gay¹, BA Michel¹, S Gay¹, A Aeschlimann²

¹Center for Experimental Rheumatology, Department of Rheumatology and Physical Medicine, University Hospital Zurich, Switzerland;

²RehaClinic Zurzach, Switzerland

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Joint pain in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) is only in part explained by inflammatory processes. Neuropeptides and opioids play an important role in the pathogenesis of pain besides proinflammatory cytokines. Molecules like serotonin, histamin, bradykinin and prostaglandins and their receptors are of particular interest. Several research groups have found that these mediators can also be produced from non-neuronal cells. Nerve fibers in RA appear to undergo degenerative processes that lead to a reduction of these fibers [1]. Our group reported the expression of secretoneurin in synovial tissue of patients with RA and OA [2]. In a randomized, double-blind trial, Stein and colleagues demonstrated the efficacy of intra-articular opioid application in chronic arthritis [3]. Other investigators confirmed the antinociceptive and anti-inflammatory effect of opioids [4]. Other neuropeptides, such as the pituitary adenyl cyclase activating polypeptide, also show anti-inflammatory actions [5]. Evaluated by Taqman real-time PCR, we found a significant reduction of the

δ -opioid receptor and the κ -opioid receptor mRNA in synovial fibroblasts in seven patients with RA (κ -opioid receptor, 0.09-fold \pm 0.0276, $P=0.037$; δ -opioid receptor, 0.05-fold \pm 0.0134, $P=0.040$) and in seven patients with OA (κ -opioid receptor, 0.15-fold \pm 0.0315, $P=0.040$; δ -opioid receptor, 0.18-fold \pm 0.0561, $P=0.040$) compared with in healthy controls.

The investigation of pain-modulating molecules is an important task in pain research. The search for novel pathogenetic mechanisms involving neuropeptides and opioids/opioid receptors should improve the management of pain in patients with RA and OA.

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Poster Discussion (3) Development & Genomics Group**75****Functional haplotypes in citrullinating enzyme peptidylarginine deiminase 4 are associated with rheumatoid arthritis**

K Yamamoto¹, A Suzuki², X Chang², S Tokuhiro², T Sawada¹, Y Nakamura², R Yamada²

¹Department of Allergy and Rheumatology, University of Tokyo, Japan;

²Laboratory for Rheumatic Diseases, SNP Research Center, The Institute of Physical and Chemical Research, Kanagawa, Japan

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Introduction Previous studies of rheumatoid arthritis (RA) indicated that the risk of disease in the siblings of affected individuals (sib) is increased twofold to 17-fold, suggesting that genetic factors are important. Recently, five sib-paired linkage studies from Europe, North America and Japan were published. Although there was no locus suggested by all the studies in common, some loci were suggested by multiple studies. Chromosome 1p36 was one such loci. Within 10 Mb centromeric from these microsatellite markers, there was a region containing clusters of enzymes that were very likely to be functionally relevant to RA-specific autoantibody production. These enzymes were peptidyl-arginine deiminases (PADIs). PADIs are enzymes that post-translationally convert arginine residues to citrulline. Citrullination and citrullinated peptides have been recognized to be one of the most RA-specific phenomena because citrullination was revealed to be related to the most RA-specific autoantibodies. The clinical importance of the measurement of anti-citrullinated peptide antibodies and the specificity of the autoantibodies suggest a specific role of citrullination and PADIs in RA pathophysiology.

Methods and results We performed a case-control association study, using single nucleotide polymorphisms discovered by the Japanese

Millennium Genome Project in the 1p36 region, containing the genes for PADI1, PADI2, PADI3 and PADI4. We identified a RA-susceptibility haplotype in the PADI4 gene, but no RA association in the neighboring PADI genes. Expression of the PADI4 gene was confirmed in hematological cells by northern blot hybridization and in synovial tissue of RA patients by *in situ* RT-PCR and immunohistochemistry. Moreover, the susceptibility haplotype of PADI4 was related to anticitrullinated filaggrin antibody levels in sera of RA patients. We then found a difference in mRNA stability between nonsusceptibility and susceptibility variants. **Conclusions** Our results imply that the RA-susceptibility haplotype of PADI4 increases production of the citrullinated peptides that act as autoantigens, resulting in an increase of the risk of developing RA.

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Analysis of IL-10 gene variants in rheumatoid arthritis

T Huizinga, J Schonkeren, F Kureeman, F van Gaalen, R Westendorp, FC Breedveld, R Toes

Department of Rheumatology, Leiden University Medical Center, The Netherlands

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IL-10 is known for its anti-inflammatory and regulatory function on different cell types involved in the pathogenesis of rheumatoid arthritis. Taking advantage of a stable intermediate phenotype (IL-10 induced by lipopolysaccharide-stimulated blood cultures), large interindividual differences in IL-10 production were found. In two separate large studies including a total of >50 twins, 70% of the variation between individuals could be explained by genetic factors. Differences in IL-10 protein production were present at the level of mRNA production, and analysis of the half-life of mRNA indicated that differences in transcription accounted for the interindividual differences. To test whether the different haplotypes were transcribed at a different rate, allele-specific transcript quantification was performed, and preliminary data indicated that some donors exhibited a 1:1 ratio of the respective mRNA species, and some donors a 1:1.2 ratio. To determine the length of the genomic region associated with different production of IL-10, eight single nucleotide polymorphisms (SNPs) and two CA repeats in the IL-10 gene, and six SNPs in the IL-10 homologues IL-19, IL-20 and IL-24 were analysed in 53 families in relation to IL-10 production. The preliminary data suggest that the region associated with differences in IL-10 production is a region of limited size around the IL-10 gene. Transient transfection assays of the -1413 to +31 fragments of the different haplotypes of the 5' region of the IL-10 gene in the myeloid cell lines U937 and Monomac6, in the B-cell line RAJI and in the T-cell line Jurkat showed no difference in promoter activity in response to stimulation for any of the haplotypes. However, a SNP outside this region, the -A2849G SNP, was associated with differences in IL-10 production. Next, a higher rate of joint destruction was observed in patients with the genotype associated with high IL-10 production (mean joint damage score of 20 vs 7; $P < 0.001$), most probably due to higher autoantibody titers in patients with the high IL-10 genotype.

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OLF1/EBF-associated zinc finger protein gene: a novel lupus susceptibility locus on chromosome 16q identified in a Chinese cohort

N Shen¹, XB Fong¹, SL Chen¹, Y Wang¹, BH Hahn², BP Tsao²

¹Department of Rheumatology, Renji Hospital, Shanghai Second Medical University, Shanghai, China; ²Division of Rheumatology, University of California Los Angeles, Los Angeles, California, USA
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Objective Three independent genome scans have established the presence of a significant systemic lupus erythematosus (SLE)-linked locus on chromosome 16q12, which overlaps with the intervals identified in several other autoimmune diseases. To identify the putative SLE susceptibility gene within this interval, we fine-mapped a 10 cM interval

of 16q12 containing nine densely spaced microsatellite markers (D16S419, D16S3044, D16S409, D16S540, D16S517, D16S3136, D16S416, D16S3034, and D16S415) using a cohort of 240 Chinese SLE patients and their parents.

Methods Evidence for linkage disequilibrium was assessed using the ETDT and GeneHunter programs. TaqMan real-time quantitative PCR was used for detecting mRNA expression of a positional candidate gene. Five single nucleotide polymorphisms (SNPs) located within the candidate gene were genotyped by allelic discrimination-PCR using a 5'-nuclease assay.

Results Our data showed overall skewing of the transmission (t) of alleles of maker D16S517 ($P = 0.000008$, $P_c = 0.00001$) from heterozygous parents to affected offspring, and showed preferential transmission of one of the alleles of D16S517 to affected offspring (transmission:nontransmission=68:21, $P = 0.000001$, $P_c = 0.00001$). The marker D16S517 is located within an intron of a positional candidate gene, OLF1/EBF-associated zinc finger protein (OAZ), which may be involved in lymphocyte early development and regulation. Elevated levels of the OAZ gene expression were found in SLE patients (mean $\Delta C_T \pm SEM = 5.21 \pm 0.85$, $n = 42$) compared with in normal controls (mean $\Delta C_T \pm SEM = 6.22 \pm 0.95$, $n = 36$; $P < 0.001$). The gene expression level of OAZ was not correlated with SLE disease activity index scores and the treatment status. Genotyping of five SNPs within OAZ gene introns in an extended Chinese cohort of 325 families also indicated preferential transmission of a certain four SNP haplotypes (haplotype T-A-G-G, transmission:nontransmission=43:26, $P = 0.04$). Haplotypes combining SNPs and the SLE-associated D16S517 allele showed significant association with SLE susceptibility (transmission:nontransmission=58:15, $P = 0.000001$; transmission:nontransmission=54:15, $P = 0.000003$; transmission:nontransmission= 41:13, $P = 0.000139$, respectively).

Conclusion These data suggest the presence of a SLE susceptibility gene physically close to marker D16S517 in a Chinese cohort. The high expression of OAZ and the significant association of OAZ haplotypes with SLE susceptibility suggested OAZ might be a novel candidate susceptibility gene within the 16q interval.

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Transcriptional co-activator CBP/p300 regulates chondrocyte-specific gene expression via association with Sox9

S Takahashi, M Tsuda, Y Takahashi, H Asahara

Scripps Research Institute, La Jolla, California, USA

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Chondrocytes are critical components for the precise patterning of a developing skeletal framework and articular joint formation. Sox9 is a key transcription factor that is essential for chondrocyte differentiation and chondrocyte-specific gene expressions; however, the precise transcriptional activation mechanism of Sox9 is not fully understood. Here we demonstrate that Sox9 utilizes CBP/p300 to exert its effects. Sox9 associates with CBP/p300 in chondrocyte cell line SW1353 via its carboxyl terminus activation domain in a cell type-specific manner. In promoter assays, CBP/p300 enhances Col2a1, which encodes cartilage-specific type II collagen gene, the promoter activity via Sox9. Chromatin immunoprecipitation shows that p300 is bound to the Col2a1 promoter region. Furthermore, the CBP/Sox9 complex disrupter peptide suppresses Col2a1 gene expression and chondrogenesis from mesenchymal stem cells. These data demonstrate CBP/p300 functions as a coactivator of Sox9 for cartilage tissue-specific gene expression and chondrocyte differentiation.

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Sox9 represses gene expression via histone deacetylase activity

M Tsuda, S Takahashi, N Taniguchi, T Furumatsu, H Asahara

Scripps Research Institute, La Jolla, California, USA

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Sox9 is a transcription factor that is essential for chondrocyte development. Previously we have shown that p300, a ubiquitously expressed co-activator, interacted with Sox9 to activate gene expression. Even though

both Sox9 and p300 are expressed in some other tissue, it is only chondrocyte that expresses type II collagen.

To clarify the mechanism that prevents Sox9 from activating chondrocyte-specific gene expression in those tissues, we examined the interaction between co-repressors and Sox9. Among the co-repressors, some HDACs interacted with Sox9 both *in vitro* and *in vivo*. Overexpression of those HDACs reduced the transcriptional activity of Gal4-fused Sox9. TSA, but not TPX-B, caused de-repression of transcription regulated by Sox9. These data identify Sox9 as the first transcription factor to interact with HDACs, and suggest that HDAC may bind to Sox9 to regulate tissue-specific gene expression.

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Novel function of GATA-3, revealed by conditional deficient mice

IC Ho¹, SY Pai², ML Truitt³

¹Division of Rheumatology, Immunology, and Allergy, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts, USA; ²Dana Farber Cancer Institute, Boston, Massachusetts, USA; ³Brigham and Women's Hospital, Boston, Massachusetts, USA
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GATA-3 is a zinc finger protein that is preferentially expressed in T cells in adult animals. Previous studies have shown that GATA-3 is essential for the transition from common lymphoid precursors to the most immature thymocytes. In addition, GATA-3 is selectively expressed in Th2 cells. Overexpression of GATA-3 forced developing Th1 cells to produce Th2 cytokines, including IL-4, IL-5, and IL-13. However, it remains unclear whether GATA-3 is also important at the later stages of T-cell development. Nor do we understand whether GATA-3 is essential for the differentiation of Th2 cells or for the maintenance of the Th2 phenotype. The lack of GATA-3-deficient mice or T cells has precluded further studies to address these important questions. We have recently generated conditional GATA-3-deficient mice. Studies on the conditional GATA-3-deficient mice has uncovered several novel roles of GATA-3 in regulating the development and function of T cells.

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Novel tools for molecular analysis in synovium

U Mueller-Ladner¹, E Neumann¹, I Tarner¹, J Grifka², O Distler³, A Wunder⁴, S Gay³

¹Department of Internal Medicine I, University of Regensburg, Germany; ²Department of Orthopedics, University of Regensburg, Germany; ³Center for Experimental Rheumatology, Department of Rheumatology, University Hospital Zürich, Switzerland; ⁴Center for Molecular Imaging, Harvard University, Boston, Massachusetts, USA
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Objective Current approaches to analyze and track disease-related genes and proteins in synovium or in animal models for arthritis including gene transfer models are based on RNA isolated either from cultured synovial cells or from synovial biopsies. This strategy does neither allow one to distinguish between specific gene expression of cells originating from different synovial compartments due to a potential mixture of different expression profiles nor to track these genes or gene products in the synovium. Therefore, we established laser-mediated microdissection (LMM) and differential display for analysis of gene expression profiles of histologically defined areas in rheumatoid synovium, and established novel molecular imaging techniques to track the molecules associated with pathogenic synovial processes.

Methods Synovial cryosections derived from distinct human arthritides were used to obtain cell samples from distinct synovial areas using LMM. RNA was isolated and analyzed using differential display fingerprinting. Differential expression of identified sequences was confirmed by *in situ* hybridization and immunohistochemistry. Molecular imaging using different fluorescence-based techniques was performed to track molecules of interest such as human serum albumin-coupled molecules and circulating cells such as T-cell hybridomas and dendritic cells following adoptive cellular gene transfer within the synovium.

Results Microdissected synovial tissue sections containing about 600 cells yield enough RNA for a stable, reproducible RNA fingerprint. Several genes – known and unknown ones with regard to rheumatoid arthritis pathophysiology – could be identified as being expressed differentially between the synovial lining, the sublining, and the microvasculature (e.g. thrombospondin-4 in the lining layer, ciz-1 in the sublining, and CD82 around the microvasculature). In addition, molecular bioluminescence-based imaging revealed that T-cell hybridomas and dendritic cells home to the synovium. It was found that the arthritic joint is the primary target of adoptive cellular gene transfer and that the synovial fibroblasts are the target of albumin-coupled molecules.

Conclusion High-sensitivity molecular analysis methods such as LMM and differential display, in combination with molecular imaging of 'vehicle' cells, genes and proteins of interest, present a valuable tool to obtain novel insights into compartment-dependent synovial pathways. In addition, the results also demonstrate the potential of these novel analytic strategies in a nonmalignant multifactorial inflammatory disease.

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Modulation of the gene expression pattern in peripheral blood mononuclear cells by biologicals in rheumatoid arthritis

J Kekow¹, D Koczan², S Drynda¹, A Drynda¹, H Thiesen²

¹Clinic of Rheumatology at Vogelsang, University of Magdeburg, Germany ²Institute for Immunology, University of Rostock, Germany
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Tumor necrosis factor (TNF)- α and IL-1 β belong to the key mediators in the pathogenesis of rheumatoid arthritis (RA). Neutralization of these cytokines with biologicals like anti-TNF (etanercept) and IL-1 receptor antagonist (IL-1ra) (anakinra) induces a rapid and sustained decline in disease activity in RA patients. However, about 30% of patients receiving these therapies are nonresponders.

Here we report the application of DNA array technology (Affymetrix) in monitoring the modulation of gene expression of cells from peripheral blood under anti-TNF (etanercept) and IL-1ra (anakinra) treatment. Blood samples were taken from 14 RA patients before and 72 hours after initiation of etanercept ($n=10$) or anakinra ($n=4$) therapy. Total RNA from mononuclear cells (peripheral blood mononuclear cells) was prepared with the RNeasy kit (Qiagen). Affymetrix chip technology was used to analyse the expression levels. The therapy response was determined by changes in the disease activity score (DAS28) 3 months after the start of treatment.

Under anti-TNF therapy, the expression of genes for cytokines such as TNF- α , IL-1 β , IL-1ra and PBEF, and for chemokines like IL-8, MIP-1 α and MIP-1 β , as well as for other disease-associated proteins like cyclooxygenase-2, intracellular adhesion molecule-1 and manganese superoxide dismutase, changed differentially. Interestingly, changes in the expression profile detected 3 days after the start of treatment were found to be closely associated with the outcome of therapy as reflected 3 months later by the disease activity score. Using real-time PCR, Affymetrix data were confirmed and expression of low abundant transcripts, which were not detected on microarrays such as IL-6, were observed. The application of anakinra influenced the expression levels of L-1 β , IL-8, and cyclooxygenase-2 in a similar fashion, this being in contrast to anti-TNF treatment expression levels of TNF- α , MIP-1 α and MIP-1 β , which remained unchanged.

Our data give new insights into the effects of biologicals used in RA treatment on the transcriptional level. Early expression profiling of biological treatment can be a useful tool for monitoring changes at the mRNA level after neutralization of TNF- α and IL-1 β , and thus for the predicting the outcome of therapy at an early stage of treatment. The data indicate the presence of genetic heterogeneities within the group of RA patients, suggesting the presence of genetic polymorphisms in the identified genes.

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The new application of chip technology to transcriptome and proteome analysis of rheumatoid disease

T Nakanishi¹, K Nishida², K Ohyama¹, M Takigawa¹, H Inoue²

¹Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; ²Department of Orthopedics, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan
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Rheumatoid arthritis (RA) is one of the complex diseases that showed multiple progression stages. At first, inflammatory response occurs by self-antibodies, and then the proliferation of synoviocytes is abnormally promoted. These synoviocytes invade into articular structures and, finally, articular cartilage is destroyed. To analyze growth-promoting factors in synovial cells from articular tissues of RA, we performed DNA chip analysis and compared the expression pattern of mRNA with that of synoviocytes from osteoarthritis (OA) tissue or normal tissue. The results showed that the expression of many genes, including some novel genes, was upregulated in RA synoviocytes compared with OA synoviocytes or normal synoviocytes. RT-PCR analysis and immunohistochemical analysis showed that some genes and their products were highly distributed in active synoviocytes of articular tissues of RA. Furthermore, we compared the protein chip patterns of RA and OA, and found several markers of RA. These results show the utility of chip technology for analysis of rheumatoid disease.

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Gene hunting in primary osteoarthritis

J Loughlin

Institute of Musculoskeletal Sciences, University of Oxford, UK
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Primary osteoarthritis (OA) has a large genetic component, with heritability estimates of at least 50% for most joint sites. Identifying the genes encoding for OA susceptibility will shed considerable light on the causes of this common debilitating disease and will suggest new avenues for the development of novel therapeutics. My group is actively hunting for OA susceptibility genes. We conducted the first and so far the largest OA genome-wide linkage scan, on over 500 affected sibling pairs ascertained by large-joint replacement surgery. We identified a number of regions of the human genome that harbour OA susceptibility. We have been conducting extensive gene-based association studies within these linkage intervals on a case-control cohort containing more than 2000 individuals, using single nucleotide polymorphisms and microsatellite repeats. We are now beginning to identify the genes that are mutated in OA. Mutations include common missense changes and effects on gene expression. Some of the mutated genes are regulators of chondrocyte development, which implies that an inability of the articular chondrocyte to maintain a prehypertrophic phenotype may be a critical factor in the disease. I report our latest findings within the context of the results from other OA genetic studies.

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New approach to chemical biology for drug discovery: construction of new affinity beads and their application

H Handa

Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Kanagawa, Japan
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Affinity purification is an established technique used to identify ligand-binding proteins; however, its widespread use has been limited by the inefficiency and instability of conventional matrices. The unstable nature of conventional matrices narrows the spectrum of ligands that can be used. Moreover, nonspecific binding of proteins to the solid support has complicated identification of target proteins. Another difficulty is frequent low purification efficiency, which requires partial purification of the source material prior to affinity chromatography to improve results.

The present article describes the preparation and use of affinity beads for identification of drug receptors. The drugs of interest are immobilized to the matrix, which consists of latex beads covalently coupled with spacers. The latex beads are composed of glycidylmethacrylate-covered glycidylmethacrylate-styrene copolymer cores (SG beads) that were developed originally for the affinity purification of DNA-binding proteins. To reduce steric hindrance, divalent epoxide, ethylene glycol diglycidylether molecules are introduced to the SG beads (SGE beads) as spacers after aminolysis of epoxy groups on the surfaces of the beads.

The new SGE beads offer several distinct advantages compared with commonly used supports such as agarose beads. These advantages have enabled us to identify drug receptors directly from crude cell extracts within a few hours. Drug derivatives with a reactive group are then covalently coupled to the SGE beads. Crude cell extracts are incubated with the drug affinity beads. The desired drug-binding proteins are then purified in a batchwise manner. The desired drug-binding proteins bind to the drug on the affinity beads, while other proteins flow through the beads. The drug-binding proteins are eluted from the affinity beads with a high salt solution or a detergent containing solution following brief centrifugation. A typical target protein is purified more than 1000-fold with a yield of 70%. If necessary, the batchwise step can be repeated, enabling further purification. This procedure is not only effective, but is also simple and straightforward to perform. Using the affinity beads, we have identified multiple drug receptors, which could be utilized for drug screening, drug design, evaluation of drug efficiency for a given individual, and so on.

Poster Discussion (4) Immunology Group (1)

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Repertoire analysis of anergic B cells from patients with systemic lupus erythematosus

NS Longo, R Fischer, J Daruwalla, AC Grammer, PE Lipsky

Autoimmunity Branch, NIAMS, National Institutes of Health, Bethesda, Maryland, USA
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Patients with active systemic lupus erythematosus (SLE) have atypical CD19⁺ peripheral blood B cells that require 100-fold more anti-immunoglobulin or recombinant CD154 to induce *in vitro* proliferation. Additional phenotypic and *in vitro* functional analyses strongly suggested that the CD19⁺ cells are anergic. Since anergy is one of the mechanisms by which the function of autoreactive B cells can be downregulated, a comparison of the productive B-cell repertoires of CD19⁺ and CD19⁺ subsets from the same lupus patient was undertaken to determine whether the anergic B cells were oligoclonal or otherwise enriched in cells expressing genes known to encode autoantibodies. To accomplish this, the usage of V_HDJ_H, V_KJ_K and V_λJ_λ gene elements by individual B cells was determined for each subset. The two subsets exhibited low mutational frequencies (CD19⁺, V_H=0.6%, V_K=0.05%, V_λ=0.1%; CD19⁺, V_H=0.2%, V_K=0.05%, V_λ=0.2%) and involved substitutions that rarely increased the basic amino acid content. The immunoglobulin heavy and light chain repertoires of each subset were polyclonal and very similar. However, some differences from the normal repertoire were noted. For example, the V_K1 family was under-represented and V_K4 B3 and V_K5 B2 were over-represented in both subsets. Multiple small clones (average size 3.5) were present in the V_H, V_K and V_λ repertoires of both subsets. A large (n=32) clone using V_H4-34, D3-22 and J_H6 gene elements, containing FR mutations, was found. Notably, it was split two-thirds within the CD19⁺ subset and one-third within the CD19⁺ subset. In spite of the overwhelming similarities between subsets, there were three distinct differences. The V_H4 family was significantly (37% versus 17%) more abundant in the CD19⁺ subset. Second, CD19⁺ V_λJ_λ clones were two-fold more numerous and more diverse in gene utilization than the CD19⁺ subset. Third, B cells utilizing V_K5 B2 genes were significantly (16% versus 7%) more abundant in the CD19⁺ subset. Because of the polyclonal distribution of immunoglobulin heavy and light chain genes, and the overall similarity between the two subsets, it is likely that anergy in the CD19⁺ subset is not antigen specific.

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Transplant-tolerance induction by CTLA-4Ig in liver-transplanted rats: a lesson for the treatment of autoimmune diseases?

U Fischer, S Boas-Knoop, U Neumann

Department of General and Transplantation Surgery, Charité, Humboldt University, Berlin, Germany

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Introduction The immunology of transplantation is of general interest, because "it offers one of the few negotiable pathways into the central regions of biology" (Medawar, 1957). The holy grail of transplantation immunology is tolerance induction. This might be achieved in the clinic by blocking the T-cell costimulation using CTLA-4Ig, a fusion protein consisting of the extracellular domain of CTLA-4 linked to the constant region of IgG₁. CTLA-4Ig proved to be effective in prolonging the transplant survival in various transplantation models [1] and in the treatment of autoimmune diseases. CTLA-4Ig is currently under investigation in a clinical trial to determine its safety and efficacy for patients suffering from rheumatoid arthritis [2].

Experiment CTLA-4Ig was administered on days 3 and 4 after rat liver transplantation in a MHC-mismatched donor-recipient combination. We also studied the therapeutic effect of donor-specific splenocyte transfusions on day 4 alone and in combination with CTLA-4Ig on days 3 and 4. We achieved a prolonged transplant acceptance in the animals receiving the combination of cells and CTLA-4Ig, and investigated whether a Th1/Th2 immune deviation might be responsible for this phenomenon.

Methods A rat liver transplantation model in a high-responder rat strain combination was used. The Th1 cytokines IL-2 and IFN- γ and the Th2 cytokines IL-4 and IL-10 were analysed by RT-PCR.

Results CTLA-4Ig prolonged the survival of the recipients from 10 days median in the untreated control group to 30 days median. Donor-specific splenocytes alone had no influence on the survival, but the combination of CTLA-4Ig and donor-specific splenocytes resulted in a survival of >150 days in all animals. A Th1/Th2 immune deviation was not observed in the animals with long-term graft acceptance. The Th1 cytokines IL-2 and IFN- γ and the Th2 cytokines IL-4 and IL-10 were equally expressed in the control group and in the group with prolonged transplant acceptance.

Conclusions The combination of CTLA-4Ig and donor-specific splenocytes results in a long-term transplant acceptance (>150 days), which cannot be achieved by CTLA-4Ig or a splenocyte transfusion alone. The Th2 cytokines are not dominant protective in preventing the transplant rejection.

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The Th2 cytokines IL-4 and IL-10 are internal controllers of human Th1-biased immunity *in vivo*

A Skapenko¹, GU Niedobitek², JR Kalden¹, PE Lipsky³, H Schulze-Koops¹

¹Nikolaus Fiebiger Center for Molecular Medicine, Clinical Research Group III, University of Erlangen-Nuremberg, Erlangen, Germany;

²Department of Pathology, University of Erlangen-Nuremberg, Erlangen, Germany; ³NIAMS, National Institutes of Health, Bethesda, Maryland, USA
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Chronic inflammation in several human autoimmune diseases, such as rheumatoid arthritis, is driven by activated Th1 cells. The delineation of the mechanisms controlling the evolution of Th1-mediated immune responses in autodestructive immune reactions is therefore crucial for the understanding of the pathogenesis of autoimmune diseases. However, analysis of such mechanisms in humans is hampered by the inability to address these questions directly *in vivo*. We developed a novel *in vivo* model for human Th1-mediated inflammation and analyzed the regulation of human Th1 inflammation *in vivo*. Peripheral blood mononuclear cells (PBMC) from healthy human individuals were transferred by intraperitoneal injection into female NOD/SCID mice that lack

T cells and B cells and that express reduced natural killer cell activity. At different time points, human PBMC were recovered from the intraperitoneal cavity and analyzed phenotypically and functionally. Human PBMC developed a strongly Th1-biased immune response, as indicated by spontaneous proliferation of CD4 and CD8 T cells, by increased expression of the T-cell activation markers HLA-DR and CD25, and by massive expansion of IFN- γ -producing T cells. In contrast, Th2 cells were absent in the recovered human T cells. Histopathological analysis revealed infiltration of activated human lymphocytes into the portal tracts of the liver and into the perigastric and perirenal fatty tissues, which was frequently organized in granuloma-like structures resembling Th1-mediated granulomas in sarkoidosis or Wegener's disease. The development of the human Th1-biased immune response in the mice could be blocked by cyclosporine and was dependent on antigen-presenting cells. Further analysis of the regulation of the human Th1 immune response *in vivo* revealed that the development of the Th1 immune response was tightly controlled by the endogenously produced cytokines IL-4 and IL-10, as neutralization of these cytokines markedly exaggerated the Th1 response. Interestingly, treatment of established inflammation with daily intraperitoneal injections of recombinant IL-4 or IL-10 markedly decreased T-cell activation and IFN- γ production, indicating that the anti-inflammatory cytokines IL-4 and IL-10 are able to interrupt established human Th1-mediated inflammation. These data suggest that potent regulatory mechanisms involving IL-4 and/or IL-10 control the development of Th1-mediated human inflammation *in vivo*, which might have an important implication for future therapies of chronic autoimmune inflammatory diseases.

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Post-translational modification of type II collagen influences T-cell tolerance to self-type II collagen

H Yamada¹, R Holmdahl², T Shuto¹, Y Nakajima¹, J Shida¹, T Mawatari¹, Y Iwamoto²

¹Department of Orthopaedic Surgery, Kyushu University, Fukuoka, Japan; ²Section for Medical Inflammation research, BMC, Lund, Sweden
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T-cell response to type II collagen (CII) is essential in collagen-induced arthritis, a murine model of autoimmune arthritis. Collagen-induced arthritis is induced in mice by immunization with heterologous CII. However, T cells in these animals respond only to heterologous CII but not to homologous CII, suggesting T-cell tolerance to self-CII. In addition, we have found that the T-cell response to heterologous CII is also reduced in heterologous CII transgenic mice. In this study, we crossed the heterologous CII transgenic mice with anti-heterologous CII-specific TCR transgenic mice to verify the mechanism of T-cell tolerance to self-CII. Surprisingly, T cells in the TCR and heterologous CII double transgenic mice showed no evidence of tolerance. We found that the transgenic TCR recognizes the heterologous CII epitope only when lysine at position 266 is post-translationally hydroxylated. We also found CII prepared from the joint cartilage is dominated by the glycosylated form of lysine at position 266, which may result in the lack of T-cell tolerance in the TCR and heterologous CII double transgenic mice. These data imply the importance of post-translational modification of CII in the induction of T-cell tolerance to self-CII.

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Does sustained downregulation of the TCRzeta chain define the transition from antigen mode to inflammation mode in effector T lymphocytes?

A Cope, Z Zhang, J Clark, N Panesar, P Amjadi

The Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, UK

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The molecular events that define the early phase of activation and differentiation of effector T cells have been well characterized. Those events promoting the effector function of chronically activated T cells at sites of inflammation are less well understood. Using *in vitro* and *in vivo* models, we have explored the effects of the chronic inflammatory process on T-cell differentiation by studying the effects of tumor necrosis factor (TNF)

on T-cell activation and effector responses. These studies have revealed that TNF-stimulated T cells resemble rheumatoid arthritis synovial T cells since they express cell surface activation antigens, but are profoundly hyporesponsive to T-cell receptor (TCR) engagement. This may be explained, at least in part, by the observation that TNF selectively targets the expression of the TCRzeta chain. Loss of TCRzeta expression perturbs the assembly, expression and stability of the TCR/CD3 complex, leading to attenuation of membrane proximal tyrosine phosphorylation, intracellular calcium mobilisation and the transcription of cytokine genes upon TCR engagement, when compared with untreated T cells.

We set out to explore whether TCRzeta expression could be used to identify chronically activated, hyporesponsive T cells *in vivo*. Using a FACS-based assay, we have identified subsets of CD3⁺ T cells expressing low levels of TCRzeta (hereafter termed TCRzetadim cells) in the peripheral blood of healthy donors, as well as patients with inflammatory arthritis. Subsequent experiments revealed that, when compared with TCRzetabright cells, the TCRzetadim population is enriched for cells expressing effector memory cell surface markers. By staining with HLA class I/peptide tetramer complexes, CD8⁺TCRzetadim cells are enriched for antigen-specific T cells after stimulation *in vitro* with specific peptide. While TCRzetadim cells are hyporesponsive to TCR engagement, they retain effector potential, since a significant proportion are TNF- α and IFN- γ producers upon stimulation with phorbol ester and calcium ionophore. In contrast, fewer produce IL-10 when compared with the TCRzetabright subset. These data suggest that TCRzetadim T cells, which are abundant at sites of inflammation, may represent a subset of circulating antigen experienced effector memory cells. Sustained downregulation of TCRzeta may define a checkpoint where the intracellular signals driving T-cell differentiation switch from antigen mode to inflammation mode.

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Role of $\beta 1$ integrin and its signaling molecule, Cas-L, in pathophysiology and therapeutic interventional targets of rheumatoid arthritis

C Morimoto

Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Japan
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Accumulating evidences suggest that $\beta 1$ integrin-dependent cell activation and migration pathways are critical points of intervention in several inflammatory and autoimmune diseases, such as rheumatoid arthritis (RA). Indeed, in RA patients, increased expression of $\beta 1$ -integrins and their ligands have been reported on the surface of synovial fluid lymphocytes and synovium cells, respectively, suggesting that $\beta 1$ integrins play an important role in triggering and maintaining the inflammatory response of the disease.

It is reported that tyrosine phosphorylation of cellular proteins is an early obligatory event in cell activation and signal transduction. Our study showed that PLC- γ , focal adhesion kinase (FAK), paxillin, Fyn, Lck, ERK1/2, and pp105 are phosphorylated on their tyrosine residues upon engagement of $\beta 1$ integrins in T cells. Moreover, we have demonstrated that the pp105 has been shown to associate with FAK that is autophosphorylated and activated upon the engagement of $\beta 1$ integrins. In addition, isolation of cDNA encoding pp105 has revealed that this protein belongs to the Crk-associated substrate (Cas) family, hence designated the Cas-lymphocyte type (Cas-L). Cas-L is a 105 kDa docking protein that is heavily tyrosine phosphorylated by FAK and Src family kinases upon the engagement of $\beta 1$ integrins in T cells. Transfection of Cas-L into Jurkat T cells markedly enhances cell motility and IL-2 production upon the engagement of $\beta 1$ integrins through its tyrosine phosphorylation. These results clearly indicate the involvement of Cas-L in $\beta 1$ integrin-mediated costimulation of signal transduction and cell migration. Our current study on Yeast-Two-hybrid analysis identified HTLV-I Tax and Smad-7 as new candidates for the Cas-L binding partner. I would now like to review $\beta 1$ integrin-mediated signal transduction through Cas-L, a novel docking protein, and its role in the pathophysiology and therapeutic intervention of RA.

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Human cartilage glycoprotein-39 directed T-cell responses in health and arthritic diseases

R Toes¹, J Van Bilsen¹, L Lard¹, A Miltenburg², F Breedveld¹, T Huizinga¹, R De Vries³

¹Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; ²Organon B.V., Oss, The Netherlands; ³Department of Immunohematology and Bloodtransfusion, Leiden University Medical Center, Leiden, The Netherlands
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Objective Although (self)antigen-directed T cells are thought to be key mediators of many autoimmune diseases, a functionally distinct population of CD4⁺ T cells, T regulatory (T_{reg}) cells, dominantly inhibit induction and progression of autoimmunity as shown in several autoimmune models. In humans, the presence of T_{reg} cells has been shown, but their role in autoimmune disease is not known. Likewise, no (auto)antigens recognized by T_{reg} cells have yet been identified at the molecular level. The aim of these studies is to gain a better understanding of the identity of (auto)antigens recognized by T_{reg} cells.

Results When analyzing the natural T-cell response against a candidate autoantigen in rheumatoid arthritis, human cartilage glycoprotein-39 (HC gp-39), we found that healthy donors, although displaying a typical Th1 reaction against a mixture of recall antigens, reacted against HC gp-39 by production of IL-10. The IL-10 production was mediated by CD4⁺ T cells. When HC gp-39-directed immunity of RA patients was analyzed, a marked contrast was observed as Th1-like reactivity was observed in a substantial number of patients as determined by the production of IFN- γ . More importantly, we found that HC gp-39-directed immunity in healthy donors inhibits the T-cell response against a mixture of recall antigens. Likewise, HC gp-39-specific immunity as well as HC gp-39-directed, IL-10-producing CD4⁺ T-cell lines were able to suppress MHC class I-restricted cytotoxic T lymphocyte reactivity.

Conclusion Together, these data point to a disease-associated bias in the type of T-cell response against HC Gp-39, and identify HC gp-39 as a naturally occurring autoantigen recognised by T_{reg} cells in humans.

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IL-12 priming during initial antigen contact increases generation of effector and memory CD8 T cells by changing properties

J Chang, J-H Cho, S-Y Choi, S-J Ha, Y-C Sung

Department of Life Science, Pohang University of Science and Technology, Pohang, Republic of Korea
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Initial antigen contact is known to trigger an instructive developmental program by which naive CD8 T cells differentiate into effector and memory cells. However, it remains to be determined how initial cytokine signals act on the generation of effector and memory CD8 T cells. Here, we demonstrate that IL-12 treatment during initial antigen contact results in the significant increase of both primary and memory CD8 T-cell populations. These quantitative differences were associated with qualitative changes in CD8 T cells directly caused by IL-12. Initial IL-12 priming also improved the intrinsic properties of memory CD8 T cells, leading to better protective immunity and vaccine-induced memory CD8 T-cell responses. Our results suggest that IL-12 is an important regulatory factor for developmental programming of CD8 T cells. Future application of IL-12 priming will be discussed.

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Establishment of juvenile and tolerant immune systems in patients with autoimmune diseases treated with immunoablation and autologous stem cell therapy

A Radbruch¹, A Thiel¹, S Kimmig¹, T Alexander¹, E Gromnica-Ihle², F Hiepe³, R Arnold⁴

¹Deutsches Rheuma-Forschungszentrum Berlin (DRFZ), Berlin, Germany; ²Rheumaklinik Berlin-Buch, Berlin, Germany; ³Department of Rheumatology, Osteology and Clinical Immunology, Charite, Humboldt University, Berlin, Germany; ⁴Department of Hematology and Oncology, Charite, Humboldt University, Berlin, Germany
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Immunoablation (i.e. complete destruction of a patient's autoreactive immune system) in combination with autologous stem cell transplantation (ASCT) is used for treatment of severe autoimmune diseases. If successful, the patients will undergo long-term remission. We have analysed reconstitution of the immune system in such patients, to determine whether it recovers by expansion of surviving lymphocytes or develops from stem cells *de novo*. During a follow-up period of up to 60 months after ASCT, one polychondritis patient and two systemic lupus erythematosus patients in clinical remission were analysed for reappearance of naive, activated and memory B lymphocytes and T lymphocytes, for reactivity against pathogens and autoantigens, and for presence of autoantibodies. Titers of disease-specific autoantibodies decreased after ASCT with the half-life of secreted antibodies, and did not reappear. Naive T cells and naive B cells reappeared and reached normal levels within several months after ASCT. The repertoire of the naive T cells was not restricted. The numbers and frequencies of recent thymic emigrants (CD31⁺/CD45RA⁺/CD4⁺), marked by expression of T-cell receptor recombination excision circles, equalled those of cord blood and were considerably higher than in normal probands of the age of the patients. Thus, the thymus is reactivated and the reconstituted immune system rejuvenated and tolerized in those patients. The immunological memory has to be and is indeed generated *de novo*.

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The physiological immunosuppressive network and its putative role in the pathogenesis of autoimmunity: inhibition through signaling, cellular contact, anergy, apoptosis

H Lorenz, N Blank, M Kriegel, M Schiller, E Scherb, S Winkler, JR Kalden

Department of Medicine III, Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany
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A complex cellular system like the human immune system only functions in a tightly regulated balance between activation and inhibition. We therefore hypothesise that defects in immunosuppression might contribute to the pathogenesis of autoimmunity in man. In our work we established a broad basis for testing the function of various immunosuppressive mechanisms in health and autoimmune disease. Signals through certain CD45 epitopes are strongly immunosuppressive *in vitro*. We therefore hypothesised that we will be able to induce anergy in CD45-treated lymphocytes. As control we used a human leukocyte antigen DR (HLA DR) antibody with equal inhibitory capacities on cell proliferation. We found that HLA DR signals, but not CD45 signals, could induce lymphocytic anergy, which was monocyte dependent. Moreover, these anergic lymphocytes were extremely sensitive for protein kinase C-mediated apoptosis, indicating that immunosuppressive mechanisms are inter-related. We are currently studying the exact prerequisites for this new mode of anergy and apoptosis in cells from normal donors and autoimmune patients. This is especially interesting as we have shown in our previous work that regulation of apoptosis might be disturbed, especially in systemic lupus erythematosus. Shedding of activating surface molecules represents another physiological way to prevent hyperactivation. We have recently identified a family with a mutation close to the protease docking site of tumor necrosis factor alpha converting enzyme in the tumor necrosis factor receptor p55 molecule, leading to a periodic fever syndrome with

autoimmune phenomena. Based on our working hypothesis, we postulated that chronically activated lymphocytes need to acquire modulated signaling complexes with proinhibitory capacities. To test this hypothesis we purified lipid microdomains of chronically superantigen-activated lymphocytes. First data seem to confirm this as we have found altered protein composition of raft-associated proteins with inhibitory capacity. Depletion of CD4⁺CD25⁺ regulatory T cells (T_{reg}) in mice leads to a polyglandular autoimmune syndrome (PGAIS). We have tested the function of T_{reg} in normal donors, and in patients with PGAIS or only type I diabetes mellitus. The data suggest that the function of T_{reg} might be compromised in patients with PGAIS. This is currently being further analysed in more detail. In conclusion, these data clearly provide evidence that a disturbance of physiological immunosuppressive mechanisms might contribute to the pathogenesis of autoimmune diseases.

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Circulating anergic B cells in the periphery of active systemic lupus erythematosus patients: functional and phenotypic characterization

A Grammer¹, R Fischer¹, R Slota¹, N Longo², H Sohn³, C Yarboro², G Illei², S Pierce³, P Lipsky²

¹B cell Biology Group, Autoimmunity Branch, NIAMS, National Institutes of Health, Bethesda, Maryland, USA; ²Repertoire Analysis Group/Autoimmunity Branch and Office of the Clinical Director, NIAMS, National Institutes of Health, Bethesda, Maryland, USA; ³Laboratory of Immunogenetics, NIAID, National Institutes of Health, Bethesda, Maryland, USA

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A CD19^{low}IgD⁺ B-cell population that was observed in the periphery of active systemic lupus erythematosus (SLE) patients, but not in nonautoimmune normal individuals, was examined to determine its functional and phenotypic characteristics. Whereas CD19^{low}IgD⁻ cells in the periphery of SLE patients are intracytoplasmic (IC) Ig⁺ plasma cells, CD19^{low}IgD⁺ cells are IC Ig⁻. In contrast to CD19^{high}IgD⁺ B cells, CD19^{low}IgD⁺ B cells are small, in the G₀/G₁ phase with very few cells in the S/G₂/M phase or undergoing apoptosis, remain viable in the G₀/G₁ phase in short-term culture without additional stimulation, and are positive for the anti-apoptotic gene A20. Additionally, in contrast to CD19^{high}IgD⁺ B cells, CD19^{low}IgD⁺ B cells are less activated, as assessed by lower expression of activation markers such as CD69, CD154, CD38, CD27, CD86 and CD11c. Moreover, CD19^{low}IgD⁺ B cells express a higher level of CD5, CD21 and CD23 when compared with CD19^{high}IgD⁺ B cells. Furthermore, CD19^{low}IgD⁺ B cells have less mutated heavy chain Ig genes as assessed by single-cell PCR of genomic DNA, and do not express AID when compared with CD19^{high}IgD⁺ B cells. Whereas CD19^{high}IgD⁺ B cells were rescued from apoptosis and driven into the S/G₂/M phase following stimulation with a low amount of anti-IgM antibody or rCD154, CD19^{low}IgD⁺ B cells required 100-fold more stimulation to rescue them from apoptosis and drive them into cell cycle. Of interest, CD19^{low}IgD⁺ B cells exhibit higher amounts of BCR, phosphorylated CD19 and phosphorylated lyn in cytoskeletal-associated membrane fractions compared with CD19^{high}IgD⁺ B cells. Clinically, the number of circulating CD19^{low}IgD⁺ B cells in SLE patients is greater in those with normal complement levels. Treatment of SLE patients with a blocking anti-CD154 antibody diminishes the presence of circulating CD19^{low}IgD⁺ B cells. Together, these results indicate that CD19^{low}IgD⁺ cells in the periphery of SLE patients may be a functionally anergic population of *in vivo* stimulated B cells that disappear when the disease becomes quiescent.

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Novel B-lymphocyte-deleting agents for the treatment of autoimmune diseases: induction of the mitochondrial pathway of apoptosis

G Silverman, CS Goodyear

Department of Medicine, University of California San Diego, La Jolla, California, USA

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Background To develop new approaches for the targeted deletion of pathogenic autoimmune B lymphocytes, we have studied a bacterial

product, protein A of *Staphylococcus aureus* (SpA). We have previously discovered that SpA has the properties of a B-cell superantigen, enabling interactions with conserved variable region framework sub-domains of the antigen receptors of lymphocytes rather than the complementarity determining region involved in the binding of conventional antigens [1]. Exposure to SpA can induce immune tolerance affecting a supraclonal set of lymphocytes [2].

Objective To evaluate the outcome of *in vivo* SpA treatment.

Results Within hours, a sequence of events is initiated only in targeted B cells, with rapid downregulation of B-cell antigen receptor and the coreceptors CD19 and CD21, the induction of an activation phenotype, and limited rounds of proliferation. Massive B-cell-specific apoptosis then occurs through a process heralded by the dissipation of mitochondrial membrane potential, followed later by the induction of the caspase pathway and DNA fragmentation. Post exposure, B-cell apoptotic bodies are deposited in the spleen, the lymph nodes and Peyer patches. While *in vivo* apoptosis does not require a death receptor, such as Fas, recent studies have demonstrated that apoptosis is linked to an induced imbalance in the members of the Bcl-2 family. By microfluorimetry and deconvoluted confocal microscopy, SpA induced a rapid depletion of Bcl-XL. There was also increased expression and translocation of a pro-apoptotic BH3-only member, which can form heterodimers resulting in Bax/Bak-mediated death.

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Impact of altered peptide derived from collagen II on T-cell activation and collagen-induced arthritis

Z Li, Q Zhou, Y Cheng, H Lu

Department of Rheumatology and Immunology, People's Hospital, Arthritis Institute, Beijing University Medical School, Beijing 100044, China
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Background It has been shown that collagen II (CII) peptides complexed with human leukocyte antigen-DR4/1 and recognized by a specific $\alpha\beta$ T-cell receptor (TCR) lead to T-cell activation. This is probably the initial event in pathogenesis of collagen-induced arthritis (CIA). Substitution of the TCR binding residues of CII peptides gives rise to altered peptides that were unable to bind to the TCR. The altered peptides may abolish CII-mediated T-cell activation and CIA.

Objective To evaluate the inhibitory effect of a panel of seven peptides with single or combined substitutions of TCR binding residues (Glu₂₆₇-Lys₂₇₀) of CII on T-cell activation, and to examine the role of the altered CII peptides in CIA.

Methods IL-2 production by T cells cocultured with native CII peptide and the seven altered peptides was measured and analyzed. One of the altered peptides (P₂₆₈₋₂₇₀) with the best inhibitory effect on IL-2 secretion was injected subcutaneously (50 μ g, 3 day interval) into the CIA Wistar rats after onset of arthritis. Subsequently the swollen joint score and severity of arthritis were evaluated.

Results All seven altered peptides significantly inhibited IL-2 production to a different extent, compared with control peptide ($P < 0.05$ or $P < 0.001$, respectively). In the CIA model study, the swollen joints score was much lower in rats receiving the altered P₂₆₈₋₂₇₀ peptide, compared with control rats ($P < 0.01$). The swollen joints of CIA rats waned 1-3 days earlier in the altered peptide group than in the control rats.

Conclusion Altered CII peptide with substitution of TCR binding residues inhibited T-cell activation and CIA. This might potentially be a novel therapeutic approach in rheumatoid arthritis.

Poster Discussion (5) Immunology Group (2)

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Multiplex autoantibody profiling using 'synovial proteome' microarrays identifies citrulline-modified peptides as major targets of the autoimmune response in rheumatoid arthritis

W Hueber¹, BJ Lee¹, M Genovese¹, W van Venrooij², L Steinman³, PJ Utz¹, WH Robinson¹

¹Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, California, USA; ²Department of Biochemistry, University of Nijmegen, The Netherlands; ³Department of Neurology, Stanford University School of Medicine, Stanford, California, USA
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Introduction Proteomics approaches to comprehensively characterize the spectrum of B-cell autoreactivity in autoimmune diseases are an exciting new area of research. We have developed autoantigen microarrays for multiplex autoantibody profiling, and have previously demonstrated specific and sensitive detection of autoantibodies in a variety of autoimmune diseases, including systemic lupus erythematosus, Sjögren's disease and mixed connective tissue disease [1]. Rheumatoid arthritis (RA) is an autoimmune disease affecting 0.8% of the world population, driven by as yet unidentified autoantigens. Recent research suggests that deimination of certain proteins gives rise to immunogenic epitopes that may play a key role in RA autoimmunity. This post-translational modification is characterized by the conversion of the amino acid arginine to citrulline, catalyzed by peptidyl arginine deiminase.

Objective We use our 1344-feature 'synovial proteome' antigen microarrays to test the hypothesis that microarray-based specific detection of autoantibodies against candidate antigens enables diagnosis and classification of RA.

Methods Our 1472-feature synovial proteome microarrays contain more than 300 distinct proteins and peptides, representing putative autoantigens in RA. Antigens include native and citrullinated fibrinogen, vimentin and keratin, citrulline-modified filaggrin and fibrinogen peptides, heterogeneous nuclear ribonucleoprotein (hnRNP)-B1, hnRNP-D, and overlapping hnRNP-A2 peptides, collagen type I, type II, type III, type IV and type V, overlapping human cartilage glycoprotein-39 peptides, several heat shock proteins and the endoplasmic reticulum molecular chaperone BiP, glucose-6-phosphate isomerase, acetyl-calpastatin, and many other antigens.

Results Statistical algorithms demonstrated specific reactivity in RA sera against combinations of citrulline-modified filaggrin and fibrinogen peptides as well as deiminated proteins, but not against corresponding control peptides and proteins containing native arginine residues. Array analysis of two separate cohorts of RA patients and control patients for reactivity against panels of deiminated proteins and peptides provided a sensitivity of 45% at a specificity level of 95% for the diagnosis of RA. Reactivity against other antigens was observed but not specific.

Summary The data suggest that multiplex autoantibody profiling allows for the specific and sensitive detection of autoantibodies directed against citrulline-modified filaggrin peptides and other deiminated polypeptides in RA. Array-based studies are underway to determine the fine specificity of autoimmune responses against panels of citrulline-modified peptides.

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Antileukoproteinase: modulation of neutrophil function and therapeutic effects in anticollagen II antibody-induced arthritis

H Burkhardt¹, B Sehnert¹, A Cavcic¹, B Boehm¹, K Nandakumar², R Holmdahl²

¹Department of Internal Medicine III, University of Erlangen-Nuernberg, Germany; ²Section for Medical Inflammation Research, CMB, Lund University, Sweden

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Background Secretory leukocyte protease inhibitor, or antileukoproteinase (ALP), is a physiological inhibitor of granulocytic serine proteases.

Objectives The aim of the present study was to investigate the therapeutic effects of ALP in an arthritis model induced by the transfer of monoclonal antibodies (mAbs) to collagen type II (CII). Upon binding to the intact cartilage matrix, the intraperitoneally injected CII-specific mAbs provoke a massive infiltration of neutrophils into the joints and induce the development of an acute polyarthritis, leading to cartilage and bone destruction.

Methods For arthritis induction, the combination of two mAbs to CII (CIIc1 and J1) were injected intraperitoneally into QB mice. After 3–4 days, untreated mice developed a polyarthritic disease. Arthritis severity was quantified by clinical scoring. The therapeutic effects of ALP upon systemic therapy with recombinant ALP (daily doses of 0.1 mg for 1 week, starting immediately after disease induction) were analyzed in a cohort of 12 mice in comparison with untreated controls ($n=12$).

Results First, ALP treatment resulted in a 50% reduction of arthritis incidence. Also, a marked amelioration of arthritis severity resulting in the reduction of clinical scores below 50% of untreated controls was observed in ALP-treated mice ($P<0.05$). Finally, histopathological analysis of the joints revealed a protective effect of ALP treatment on cartilage and bone.

A concomitant functional analysis was performed in an *in vitro* system of Fc-receptor-stimulated neutrophils to elucidate immune-complex-dependent pathways that might be modulated by ALP treatment. In this model of neutrophil stimulation by IgG-coated Latex beads, a marked inhibitory effect of ALP on the induction of the conformational change of the leukocyte integrin LFA-1 into its active form could be demonstrated. Beyond this modulatory effect of ALP, affecting neutrophil adhesion to cytokine-activated endothelium, additional inhibitory effects could be demonstrated on the phagocytosis of immune complexes and the oxidative burst.

Conclusions Taken together, the data indicate the therapeutic potential of ALP in the model of CII mAb-induced arthritis. Besides its antiprotease activity ALP seems to exert a variety of additional blocking effects on neutrophil functions such as adhesion, phagocytosis and respiratory burst, suggesting that it might be an important multifunctional regulator of inflammatory responses.

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Natural antibodies and antiphospholipid antibodies bind to injured tissues, activate complement and cause tissue damage

G Tsokos, S Fleming

Department of Cell Injury, Walter Reed Army Institute of Research and Department of Internal Medicine, Uniformed Services University, Bethesda, Maryland, USA

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Complement traditionally represents the first line of defense against infections. More recently, complement was shown to play a role in shaping the repertoire of the immune response. However, it is well established that uncontrolled and excessive complement activation significantly contributes to diverse pathologies ranging from inflammation to autoimmune tissue injury and injury that follows ischemia, hypoperfusion and reperfusion. In addressing the role of complement in tissue injury, two central questions arise: how complement is activated in each type of injury, and to what extent is complement activation responsible for tissue injury? To obtain insight into these questions, we have adopted a mesenteric ischemia/reperfusion (IR) model of tissue injury because the local intestinal damage is associated with complement activation and recruitment and activation of neutrophils. Because immunoglobulin-deficient, *RAG-1^{-/-}* mice are protected from IR injury, and because some *Cr2^{-/-}* mice have limited numbers of B1 B cells, we asked whether *Cr2^{-/-}* mice have an altered response to IR injury. *Cr2^{-/-}* mice are protected from IR injury that is completely restored after injection of normal IgG and IgM. In addition, antibodies with specificities against negatively charged phospholipids and DNA attach to injured tissue and activate complement, resulting in damage. Finally, transfer of normal B cells into the *Cr2^{-/-}* mouse also restores IR-induced tissue damage. We propose that certain autoantibodies, produced by B1 cells and constituting part of the natural antibody repertoire, bind to cryptic antigens expressed by injured cells and tissues, activate complement and effect tissue damage.

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Search for the precursor of ectopic follicular dendritic cells

C de Groot¹, C de Groot¹, F Muller¹, J Haringman², P Tak²

¹Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands; ²Department of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, The Netherlands

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Introduction Chronic inflammation is often accompanied by the formation of ectopic lymphoid tissue, including infiltrations of T lymphocytes and B lymphocytes, and formation of follicular structures around a network of follicular dendritic cells (FDCs). Ectopic follicular structures may serve as survival niches for undesirable B-cell clones, and FDCs provide powerful survival signals to B lymphocytes [1]. There is broad consensus that FDCs show phenotypic overlap with fibroblasts and are of mesenchymal origin, but the formation of FDCs is poorly understood and a FDC-precursor cell has not been identified [2]. It has been shown in mice that the expression of members of the tumor necrosis factor receptor family, especially of the receptor for lymphotoxin-beta (LTβR), on FDC precursors is a condition for FDC maturation.

Objectives This study aims to identify precursor cells for FDCs, and to elucidate the crucial steps that induce FDC networks both under physiological and pathological conditions.

Materials and methods Fibroblast-like synoviocytes (FLS) derived from synovial biopsies of the knee joint of patients with rheumatoid arthritis (RA) or control arthritides, and fibroblasts from human skin and tonsils were cultured *in vitro* as described previously [1]. Mesenchymal stem cells (MSC) were cultured from human bone marrow biopsies. The expression of LTβR (as a minimal characteristic of FDC precursors), and of DRC-1 and CD21L (FDC-specific markers) was studied with both immunocytochemical staining (LTβR and DRC-1) and RT-PCR (CD21L).

Results None of the FLS or fibroblasts expressed LTβR, DRC-1, or CD21L, either spontaneously or after coculture with IFN-γ, IL-1-β, tumor necrosis factor alpha, LTα1β2 (gift from Dr Jeff Browning), or combinations thereof. These data suggest that resident tissue fibroblasts or FLS do not contain significant numbers of FDC precursors. In contrast, preliminary experiments with cultured MSC indicate that these cells express the LTβR. Further studies are underway to see whether and under what conditions MSC can be induced to develop the FDC phenotype.

Conclusions Precursors of FDCs could neither be found in resident tonsillar or dermal fibroblasts, nor in FLS cultured from patients with RA or other types of arthritis. Of interest, *in vitro* cultured MSC do express the LTβR, and hence fulfill a basic criterion of a putative FDC precursor.

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Dendritic cells present a tissue-specific autoantigen under steady state and autoimmune conditions in the draining lymph node

C Scheinecker¹, R McHugh², E Shevach², R Germain³

¹Department of Rheumatology, Internal Medicine III, University of Vienna, Austria; ²Cellular Immunology Section, Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland, USA; ³Lymphocyte Biology Section, Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland, USA

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The MHC-dependent presentation of processed tissue-specific self-antigens maintains peripheral (extra-thymic) tolerance but also can lead to activation of autoreactive T cells. Although hematopoietic antigen-presenting cells have been shown to participate in these processes,

the isolation and characterization of the specific cells responsible for mediating such diverse events has not yet been accomplished. We have analyzed the presentation of an endogenous, gastric-specific H⁺/K⁺-ATPase peptide by dendritic cells (DC) in the context of the MHC class II molecule I-Ad. DC (CD11c⁺, MHC class IIbright, CD80low, CD86^{+/−}, CD40^{+/−}) were isolated from the draining gastric lymph node (gDC) and from the peripheral lymph node (pDC) or mesenteric lymph node (mDC) of unmanipulated healthy BALB/c mice. Isolated DC were then assessed for their capacity to induce IFN- γ synthesis by the H⁺/K⁺-ATPase-specific CD4⁺ T-cell clone TXA-23. In contrast to pDC or mDC, gDC significantly increased IFN- γ production by TXA-23. Visual evidence for the uptake of H⁺/K⁺-ATPase by DC in the stomach and their apparent migration to the draining lymph node (LN) was obtained by immunofluorescence staining and confocal microscopy analysis. Using a monoclonal antibody (2G11) against the H⁺/K⁺-ATPase beta-subunit, H⁺/K⁺-ATPase was consistently detected within CD11c⁺ DC in gastric LN sections but not in peripheral LN sections. In addition, we were able to detect CD11c⁺ DC in close proximity to H⁺/K⁺-ATPase-positive parietal cells in the stomach mucosa. Upon the induction of experimental autoimmune gastritis, DC were found among the first cells to infiltrate the stomach mucosa. Moreover, proportions as well as absolute numbers of DC, along with B cells, were found to be substantially increased in the gastric LN but not in the peripheral LN at later timepoints. In line with this, the frequency of H⁺/K⁺-ATPase staining in CD11c⁺ cells selectively increased in the gLN. Analysis of DC function further suggests that overall gDC presentation of H⁺/K⁺-ATPase increases in diseased animals. These results provide the first clear identification of DC as the cells involved in the uptake and presentation of a tissue-specific antigen in normal animals, and the augmentation of such presentation during the development of overt autoimmune disease.

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Induction of type II collagen-reactive IL-10-producing CD11b⁺ dendritic cells in Payer's patch of orally tolerized animals

H Kim¹, M So-Youn¹, C Mi-La², L Jung-Eun², P Sung-Hwan², L Kang-Eun², P Kyung-Soo²

¹Rheumatism Research Center, Department of Internal Medicine, School of Medicine, The Catholic University of Korea, Kang-Nam St Mary's Hospital, Seoul, Korea; ²The Catholic University of Korea, Catholic Research Institutes of Medical Science, Seoul, Korea
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To further understand the role of dendritic cells (DCs) in the immune tolerance mechanism, we examined the phenotypic distribution and cytokine profiles of DCs in Payer's patch, separate from an orally tolerized collagen-induced arthritis (CIA) model.

Oral tolerance was successfully generated at 5 weeks after first immunization with type II collagen (CII), showing that the joint inflammations were significantly subdued in the CIA mice model after six times repeated administration of soluble CII (100 μ g) at 2 day intervals. The distribution of DC subtypes was evaluated by confocal microscopy, labeled with fluorescent-tagged antibodies of CD8 α (lymphoid DC) and CD11b (myeloid DC) markers. The production and expression of IL-10 and IL-12 cytokines in each DC subsets were examined by FACS analysis and confocal microscopy.

In tolerized mice, CD11c(pan DC)⁺CD11b DCs were clearly present within the subepithelial dome region of Peyer's patch and CD11c⁺CD8 α DCs were not significantly localized in the interfollicular region. These finding is quite comparable with normal controls or CIA animals.

When the DCs were cocultured with T cells for 3 days in the presence of CII, IL-10-producing CD11b⁺ DCs were increased and IL-12-producing DCs were decreased in tolerized mice. CD4⁺CD25⁺ T cells in Peyer's patch were also induced by consecutive CII stimulation in tolerized mice, not in control and CIA model mice. We found that the IL-10-producing DCs and CD4⁺CD25⁺ T cells in tolerized mice were successfully generated by *in vitro* culture with CII antigen stimulation. In conclusion, the data suggest that induction of antigen-specific myeloid DCs and CD4⁺CD25⁺ T cells in Peyer's patch plays a very important role in initiation of the oral tolerance mechanism.

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Immunosuppressive effect of mesenchymal stem cells in collagen-induced arthritis

C Jorgensen, F Djaoud, F Apparilly, J Sany, D Noel

Lapeyronie Hospital, Immunorheumatology Department, Inserm, University of Montpellier 1, Montpellier, France

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Background and objective Mesenchymal stem cells (MSCs) are bone marrow-derived progenitor cells, widely investigated for their potential for differentiation towards multiple lineages, such as osteocytes and chondrocytes. Recently, we and other workers showed that MSCs exhibit immunosuppressive properties inducing *in vivo* tolerance of T lymphocytes towards allogeneic cells. Moreover, these cells can be easily genetically modified to express ectopic molecules, such as anti-inflammatory cytokines. Here, we investigate whether naive and IL-10-expressing MSCs could display an inhibitory effect towards self-reactive T lymphocytes *in vitro*, and could have a biological effect in the murine model of collagen-induced arthritis (CIA).

Methods We used the murine C3H10T1/2 cell line (C3 MSCs) and we derived cell clones stably expressing vIL10 (C3-IL10 MSCs). Secretion of IL-10 was measured in cell culture supernatants by ELISA, and its immunosuppressive effect was evaluated on the proliferation of T lymphocytes in a mixed lymphocyte reaction. In the CIA model, C3 MSCs or C3-IL10 MSCs (106 cells) were intravenously injected on the day of immunization with collagen, and secretion of vIL10 was monitored in the mouse sera during the overall experimental period.

Results First, we tested *in vitro* the functionality of C3-IL10 MSC clones. Secretion of vIL10 by C3-IL10 MSCs was up to 500 pg/106 cells per 24 hours, and the antiproliferative activity of these MSCs on alloreactive T lymphocytes was twofold higher than C3-MSCs. Second, we evaluated the potential immunosuppressive role of MSCs in the CIA murine model. Using both clinical, radiological and histological analysis, we showed that disease progression was reduced when MSCs were injected.

Conclusion In this study, we demonstrate that the immunosuppressive role of MSCs might be of therapeutic value in CIA, and that MSCs may be used to systemically express anti-inflammatory cytokines

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Immunosuppression and immunological tolerance induction: lessons from a transplant model

A Filatenkov, S Tullius, H Schmidt, A Selke, H Volk

Department of Surgery, Charite Virchow Klinikum, Humboldt University, Berlin, Germany

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Introduction Patients who receive organ transplants need life-long immunosuppression to prevent rejection of their grafts. Induction of immunological tolerance to the transplanted organs can solve this problem. However, according to the current opinion in clinical immunosuppression, administration of cyclosporin A (CyA) prevents tolerance induction.

In our study, we attempted to find out whether immunosuppression with CyA is really so harmful for tolerance induction.

Methods We established a high-responder (DA-Lew) kidney transplant model in rats.

DA kidneys were grafted into unilaterally nephrectomized LEW receiving no immunosuppression or low (1.5 mg/kg), medium (3.0 mg/kg) or high (15 mg/kg) CyA for 10 days.

To rule out nephrotoxic effect of CyA, the original DA grafts were replaced by secondary DA kidneys 30 days after first transplantation; secondary grafts were followed for 90 days. During the second engraftment, no immunosuppression was administered.

Results Surprisingly, we observed a dramatic difference in secondary graft survival depending on the dose of immunosuppression. Animals that had received an initial graft with low-dose CyA survived indefinitely. Almost no alloantibodies were detectable. By contrast, increased (3.0 mg/kg \times 10 days) or high-dose (15 mg/kg \times 10 days) immunosuppression after first engraftment did not result in acceptance of secondary grafts. Animals in those groups developed acute rejection of secondary grafts. High levels of alloreactive antibodies were also detected.

Discussion Immunosuppression with CyA does not prevent immunological tolerance by itself. Rather, higher doses of CyA have a detrimental effect on tolerance induction. CyA in low doses acts as tolerance inducer. Whether this sharp dose-dependent effect of CyA on tolerance induction is a general effect of immunosuppressive agents or is restricted only to CyA is still to be examined.

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Antigen-specific suppression of inflammatory arthritis by dendritic cells

R Thomas, E Martin, B O'Sullivan

Centre for Immunology and Cancer Research, University of Queensland, Brisbane, Australia

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Purpose Antigen-specific suppression of a previously primed immune response is a major challenge for immunotherapy of autoimmune disease. We have shown that NF- κ B inactivation in dendritic cells (modified DC) converts them into cells that tolerize rather than immunize to specific antigen [1]. Antigen-exposed modified DC prevent priming of immunity, and they suppress previously primed immune responses. Regulatory CD4⁺ T cells, which can transfer antigen-specific tolerance in an IL-10-dependent fashion, mediate the tolerance. We hypothesized that modified DC exposed to arthritogenic antigen would suppress clinical arthritis after disease onset.

Methods Antigen-induced arthritis was induced in C57/Bl6 mice by priming to methylated bovine serum albumin (mBSA) antigen followed by challenge injection of mBSA to one knee. Knee swelling was apparent within 2 days, with peak clinical signs apparent at 5 days. Mice were treated with antigen-exposed modified DC between 2 and 6 days after mBSA challenge to the knee joint.

Results Clinical arthritis was suppressed in each group receiving mBSA-exposed modified DC within 4 days compared with mice that received either no DC or keyhole limpet hemocyanin-exposed modified DC. Clinical improvement was associated with mBSA-specific tolerance in mice receiving mBSA-exposed modified DC. Tolerance induction was not impaired by concomitant administration of anti-tumor necrosis factor alpha monoclonal antibody. Subsequent rechallenge with intra-articular IL-1 induced flare of arthritis in all groups, which could be effectively suppressed by a second administration of mBSA-exposed modified DC.

Conclusions The data indicate that modified DC induce antigen-specific immune suppression in this model of inflammatory arthritis, even after full clinical expression of the disease. These observations have important implications for antigen-specific therapy of autoimmunity.

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Cell death and immune regulation: *in vivo* application of a novel agonistic monoclonal antibody for death receptor Fas

S Yonehara

Graduate School of Biostudies and Institute for Virus Research, Kyoto University, Japan

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Fas (CD95/Apo-1) is a cell surface death receptor belonging to the tumor necrosis factor receptor super family. Fas can transduce an apoptosis-inducing signal in cells when stimulated by Fas ligand (FasL) or by agonistic anti-Fas monoclonal antibodies. Mice with Fas-defective lymphoproliferation and with FasL-defective generalized lymphoproliferative disease (gld) mutations develop autoimmune disease and lymphadenopathy, indicating that the death receptor Fas is a functional molecule in eliminating either autoreactive peripheral T lymphocytes and B lymphocytes or tumor cells. On the contrary, Fas has been also regarded to work as an inducer of tissue damage in fulminant hepatitis, graft-versus-host disease and tissue-specific autoimmune disease. *In vivo* stimulation of Fas by administration of different agonistic anti-mouse Fas monoclonal antibodies (mAbs) also induces opposite biological effects in mice. Administration of anti-mouse Fas mAb Jo2 killed

mice within 5 hours by causing fulminant hepatitis with hemorrhage, while administration of anti-mouse Fas mAb RK8 to FasL-deficient MRL-gld/gld mice, which never kills mice, not only reduced the autoimmune symptoms including nephritis, arthritis and vasculitis, but also reduced lymphadenopathy.

I shall propose a strategy for therapeutic use of a novel agonistic anti-Fas monoclonal antibody HFE-7A for autoimmune disease including rheumatoid arthritis and other diseases including cancer. Anti-mouse Fas mAb HFE7A, which cross-reacts with Fas of various mammals, ranging from humans to mice, can induce apoptosis *in vitro* both in human and mouse Fas-expressing T-cell lines. Moreover, HFE7A shows very interesting *in vivo* effects of inducing Fas-mediated apoptosis in lymphocytes including mouse thymocytes and abnormal gld T cells and for inhibiting Fas-mediated fulminant hepatitis induced by Jo2. Not only mice, but also monkeys given a high dose of HFE7A never showed liver injury. *In vivo* therapeutic effects of humanized anti-Fas mAb HFE7A against human rheumatoid arthritis and human adult T-cell leukemia in SCID mice will be also summarized.

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C5a controls the Fc γ R activation in inflammatory processes

R Schmidt

Department of Clinical Immunology, Hannover Medical School, Germany
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The role of Fc γ receptors and complement in various immune complex-mediated diseases has been debated for a long time. The advent of gene knockout technology as well as the characterization of different Fc and complement receptors as well as new cytokines have now allowed one to dissect the different pathogenetic elements of innate immunity in the autoimmune inflammatory response.

Using various murine knockout models, in particular Fc γ receptor-deficient, mast cell-defective animals as well as complement-deficient animals, during the past years we have demonstrated that Fc γ RIII and the C5a receptor are both critical for induction of immune complex-mediated vasculitis. In several studies it became clear that mast cells have a critical role in initiation in some of the inflammatory processes. This has also been demonstrated for rheumatoid arthritis meanwhile. On these mast cells, again, Fc γ RIII is the critical activating receptor used by immune complexes. When examining the effects of immune complexes in glomerular mesangial cells as well as glomerular basement membrane nephritis, we could show that IgG immune complexes had opposing regulatory effects on Fc γ RII and Fc γ RIII receptors in glomerular mesangial cells. Whereas activation by tumor necrosis factor alpha/IL-1 β induces substantial Fc γ RII expression, IFN- γ showed a complete downregulation of Fc γ RII. At the same time, IFN- γ induced the Fc receptor γ -chain as well as the low-affinity IgG receptor Fc γ RIII. Triggering of Fc γ RIII again induced chemoattractant protein 1, MCP-1, MCP-5 and RANTES. Examining the regulatory role in the cooperation of Fc γ receptors and complement in interstitial pneumonitis, we could demonstrate that C5a is critical in amplifying the inflammatory response to IgG. On one hand, C5a is important in downregulating the inhibitory Fc γ RII; on the other, inducing the activating Fc γ RIII. Altogether, the dissection of the different innate components of pathogenesis allows for new strategies to intervene in this inflammatory process.

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Cas-L associates with human T-lymphotropic virus type I Tax and regulates its transactivation of NF- κ B: possible role of Cas-L in pathophysiology of rheumatoid arthritis

S Iwata, A Souta-Kuribara, R Miyake-Nishijima, T Sasaki, H Kobayashi, H Tanaka, C Morimoto

Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Japan

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The Crk-associated substrate lymphocyte type (Cas-L) is a docking protein that is heavily tyrosine phosphorylated by FAK and Src family

kinases upon the engagement of $\beta 1$ integrins in T cells. Transfection of Cas-L into Jurkat cells markedly enhances cell motility and IL-2 production by engagement of $\beta 1$ integrins through its tyrosine phosphorylation. These results clearly indicate the involvement of Cas-L in $\beta 1$ -integrin-mediated costimulation of signal transduction and cell migration. We have recently reported that expression of Cas-L is markedly elevated in human T-lymphotropic virus type I (HTLV-I) tax transgenic mouse, a murine model for rheumatoid arthritis, as well as in rheumatoid arthritis patients.

In the present study, we show the interaction of Cas-L and Tax, and its biological significance in detail.

We initially found that tyrosine phosphorylation as well as the expression of Cas-L was markedly elevated through the induction of HTLV-I Tax protein in JPX-9 cells, in which Tax is induced under the control of metallothionein promoter. Biochemical study showed that autophosphorylation of Src family PTKs, fyn and lck, were significantly enhanced in correlation to tyrosine phosphorylation of Cas-L. Consistent with our previous result, it was revealed that those cells had remarkably increased motile behavior on a fibronectin-coated Transwell insert.

A two-hybrid screen for Cas-L binding proteins resulted in identification of Tax as one of the candidates for Cas-L binding partners. We subsequently confirmed protein-protein interaction of Cas-L and Tax using an overexpression experiment and HTLV-I transformed T-cell lines.

Finally, expression of Cas-L was found to inhibit Tax-mediated trans-activation of NF- κ B, whereas it has virtually no effects on Tax-mediated trans-activation of HTLV-I LTR.

To date, HTLV-I has been associated with a variety of diseases affecting organs other than malignancies arising from the lymphoid system. Those chronic inflammatory diseases involve the nervous system in HTLV-I-associated myelopathy, the eyes in HTLV-I-associated uveitis, the salivary glands in Sjögren's syndrome, and the articular joints in the case of HTLV-I-associated arthropathy.

These results may present the basis for a pathological role as well as for therapeutic application of Cas-L in HTLV-I-related inflammatory diseases.

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The impact of exogenous and endogenous nucleic acids on the development of joint inflammation

A Tarkowski, J Bjersing, M Bokarewa, LV Collins, GM Deng, S Hajizadeh, F Zare

Department of Rheumatology and Inflammation Research, Göteborg University, Sweden

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Introduction Chronic joint inflammation, as in the case of rheumatoid arthritis (RA), is characterized by an aseptic disease course. Nonetheless, free nucleic acids of bacterial origin are readily found in the inflamed synovial fluid of RA patients. The aim of the present study was to assess the potential of exogenous and endogenous nucleic acids to trigger joint inflammation in a healthy joint.

Materials and methods Isolated and highly purified bacterial DNA, viral dsRNA, and endogenous mitochondrial (mt) and nuclear DNA, as well as synthetically produced homologues of these molecules, were all assessed for their *in vitro* and *in vivo* inflammatory potential. In addition, the intracellular pathways involved in the resulting inflammatory cascades were investigated.

Results Bacterial DNA-triggered inflammatory joint responses were primarily mediated by macrophages and their products (tumour necrosis factor). This inflammation was due to the cytidine phosphate guanosine content of DNA, which, as we have previously shown, promotes both macrophage and polymorphonuclear leukocyte activation and production of proinflammatory cytokines. Endogenous mtDNA but not nuclear DNA also gave rise to joint inflammation. However, in this case we have shown that the additional driving force for the inflammation is the oxidation status of the DNA. Importantly, mtDNA was readily detected in synovial fluid of a great majority of patients with RA. Finally, dsRNA of both synthetic and viral origin gave rise to joint inflammation, characterized by influx of both macrophages and T lymphocytes. *In vitro*, dsRNA induced NF- κ B activation and production of chemokines and cytokines.

Conclusion The capacities of endogenous and exogenous nucleic acids to promote joint inflammation depend on the occurrence of cyti-

dine phosphate guanosine moieties, on the oxidation status of the DNA, and on the double stranded configuration of RNA. These findings indicate the regulation of inflammatory responses in the joint by specific interactions between certain exogenous as well as endogenous nucleic acids and host cells expressing Toll-like receptor 3 and Toll-like receptor 9.

Poster Discussion (6) RA Group (1)

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Serum activity of joint destruction proteinases in management of juvenile rheumatoid arthritis

E Yarygina¹, A Bogatyreva³, S Zhanaeva¹, M Soboleva², T Korolenko¹

¹Laboratory of Cell Biochemistry and Physiology, Institute of Physiology, Russian Academy of Medical Science, Novosibirsk, Russia; ²Pediatric Department, Novosibirsk Medical Academy, Novosibirsk, Russia; ³Cardiorheumatological Department, The First Novosibirsk Children Clinical Hospital, Novosibirsk, Russia
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Background At the onset of juvenile rheumatoid arthritis (JRA), its common features including joint damage and cartilage destruction are absent. Therefore the onset of JRA is often assessed as an event of arthritis associated with infections (AAI), which precludes an early initiation of aggressive treatment with disease-modifying antirheumatic drugs to have an impact on the formation of joint destruction mediated by cathepsins and matrix metalloproteinases (MMPs).

Objectives To determine the differences in serum activity of joint destruction enzymes between different types of inflammatory arthritis for differential diagnosis and management of JRA.

Methods The activity of some kinds of cathepsins (B and L) by the Barrett and Kirschke method, using L-Arg-Arg-MCA and L-Phe-Arg-MCA as substrates and CA-074 as a selective inhibitor of cathepsin B, and the general activity of MMPs by the method of Nagase, using MCA-Pro-Leu-Gly-DPA-Ala-Arg-NH₂ as substrate, was measured in serum of 45 established JRA cases including relapses of disease compared with 23 AAI cases, two cases of lupus-associated arthritis, two cases of arthritis associated with ankylosing spondylitis, and 10 healthy children.

Results On admittance to the hospital, all investigated enzyme serum activity was higher in the group of children with JRA compared with the healthy controls ($P < 0.001$) and the AAI group ($P > 0.05$) and had no differences from cases of lupus-associated arthritis and of arthritis associated with ankylosing spondylitis. But in advanced JRA cases with marked proliferative changes in the joint tissues, the initial enzyme activity was lower and increased under the treatment ($P < 0.001$ for cathepsin L, $P > 0.05$ for MMP).

Conclusion Determining the activity of joint destructive proteinases in serum could be useful for differential diagnosis between JRA and AAI cases. Unreliable differences between these groups in our study may be explained by finding debut JRA cases in the AAI group that could be solved under follow-up study. Low initial activity with following increasing activity under the treatment has to be suspected to the predisposition to the proliferative changes in the joint tissues required more intensive treatment.

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Comparison of the nodule and synovial lesions of rheumatoid arthritis

J Highton¹, P Hessian²

¹Medicine, Medical and Surgical Sciences, University of Otago, Dunedin, New Zealand; ²Physiology, Health Sciences, University of Otago, Dunedin, New Zealand

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We have undertaken a number of investigations of the rheumatoid nodule. The infiltrating cell populations are activated macrophages migrating centrally to the palisade, and a diffuse infiltrate of T cells that

tend to cluster outside the palisade. In this area we have also demonstrated the presence of putative dendritic cells, suggesting the potential for presentation of local antigen within the nodule. Macrophages within the nodule stain positively for tumour necrosis factor and IL-1. We have recently used RT-PCR to demonstrate the presence of mRNA for INF- γ but not IL-4 or IL-5. Monokines included IL-12, IL-15 and IL-18 as well as tumour necrosis factor alpha and IL-1 β . We interpreted this as best fitting a Th1 profile.

As well as similarities, there are significant differences between the nodule and the synovial lesions of rheumatoid arthritis. A notable difference is the lack of B cells and lymphoid follicles in the rheumatoid nodule. Thus, we were able to demonstrate expression of the follicular dendritic cell-specific gene CD 21L in synovial membranes but not in the nodule. However, the chemokine SLC was present in both lesions, as was BCA-1, despite the absence of B cells in the nodule. Similarly, lymphotoxin alpha and lymphotoxin beta, which are important in follicle formation, were present in both lesions. Expression of the SLC receptor CCR7 was rare, but an alternative receptor, CCBP2, was present in both lesions. CXCR5, the BCA-1 receptor expressed by B cells, was present in the synovium. Thus, expression of chemokines for B cells and key cytokines important to follicle formation are present in both the nodule and synovial membrane. Therefore, other differences must explain the lack of B cells and follicles in the nodule.

The data suggest that the destructive inflammatory processes in the nodule and the synovial membrane may be essentially similar T-cell-driven lesions. Discovering the basis of differences such as the lack of B cells and follicles in the nodule may help the definition of important aspects of the inflammatory process in rheumatoid arthritis.

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Collagenous colitis and rheumatoid arthritis: is there a connection?

F Wollheim¹, A Domargård², J Bohr³, C Tysk³, T Skogh⁴

¹Department of Rheumatology, Lund University Hospital, Lund, Sweden; ²Department of Rheumatology, Örebro University Hospital, Örebro, Sweden; ³Department of Medicine, Örebro University Hospital, Örebro, Sweden; ⁴Department of Rheumatology, Linköping University Hospital, Linköping, Sweden

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Background Collagenous colitis (CC), discovered by the Swedish pathologist Claes Lindström in 1976 [1], is characterised clinically by chronic watery diarrhoea, and a macroscopic normal colonic mucosa, where characteristic changes are found microscopically. CC is most prevalent in middle-aged women and has frequently been associated with connective tissue diseases, including rheumatoid arthritis (RA) [2]. **Objectives** To confirm the occurrence of RA in a large series of CC, and to search for distinguishing clinical features and possible clues to the connection.

Method Retrospective chart review and follow-up examination of patients a large referral center.

Results A registry of CC at the Örebro University Hospital contains 163 patients [3]. Sixteen of these patients had a previous diagnosis of RA. Fifty-three of the patients were residents of the immediate catchment area, and among them eight patients with RA have been identified, all women. The onset of RA preceded that of CC in seven out of eight patients by between 1 and 34 years. In the remaining case, CC started 14 years before RA. Radiographic erosions were present in six out of eight patients, rheumatoid factor in three out of eight patients, and seven out of eight patients fulfilled the ACR criteria for RA. Four out of eight patients had first-degree relatives with RA. Four out of eight patients had extra-articular manifestations, and three out of eight patients had monoclonal IgG components in the blood. Three out of eight patients had Sicca syndrome or full-blown Sjögren's syndrome.

Conclusions We confirm the occurrence of RA in at least 10% of a large series of CC, usually present before the onset of CC. This is further evidence for a real correlation rather than mere coincidence. We were not able to find any distinguishing features of RA, but it is suggested that familial RA and extra-articular manifestations are common. RA as such, or remedies used to treat RA, may contribute to the pathogenesis of CC

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Evidence for functional variation of the death receptor 3 gene predisposing rheumatoid arthritis

S Shiozawa¹, K Komai¹, K Osaka¹, Y Miura¹, A Hashiramoto¹, K Shiozawa²

¹Department of Rheumatology, Faculty of Health Science, Kobe University School of Medicine, Kobe, Japan; ²Department of Rheumatology, Konan Kakogawa Hospital, Japan

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Apoptosis of the cell is important for the pathogenesis of autoimmune diseases. We identified the death receptor 3 (DR3) gene, a family of apoptosis-inducing Fas gene6, containing four single nucleotide polymorphisms and one locus of a 14-nucleotide deletion within exon 5 and intron 5. This haplotype resulted in insertion of a portion of intron 5 into the coding sequence or deletion of exon 6, and generated premature stop codons. The thus generated mutant DR3 lacking death domain assembled with a wild-type DR3 molecule to inhibit apoptosis, and apoptosis induction by anti-DR3 antibody was impaired in the lymphocytes of individuals with mutation. We also found that experimental collagen-induced arthritis was ameliorated by anti-DR3 antibody treatment, and the haplotype was clinically found in increased frequency in familial patients with RA and the patients who underwent joint surgery as compared with controls ($P < 0.0006$). We surmise that this mutation is responsible for impairment of apoptosis induction in patients with rheumatoid arthritis, which may predispose to arthritic joint destruction and autoimmunity.

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Lymphoid neogenesis in chronic inflammation

C Pitzalis¹, A Manzo², S Paoletti³, F Barone¹, R Caporali², CM Montecucco², M Ugucioni³

¹Rheumatology Department, GKT School of Medicine, Guy's Hospital, London, UK; ²Cattedra di Reumatologia, IRCCS Policlinico San Matteo, Italy; ³Institute for Research in Biomedicine (IRB), Bellinzona, Switzerland

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Aims To investigate the relationship between 'lymphoid chemokines' CXCL13 and CCL21 and the developmental dynamics of organized lymphoid tissue in rheumatoid synovitis, by determining their local production sites in the context of progressively enlarging perivascular cell aggregates. **Materials and methods** Forty synovial samples from 20 rheumatoid arthritis patients were studied. The organizational features of cell aggregates were analysed by staining serial sections for T cell, B cell and follicular dendritic cell (FDC) markers and peripheral node addressin (a group of vascular adhesion molecules constitutively expressed by high endothelial venules in secondary lymphoid tissues). The dimensional grading analysis was performed according to the radial cell count from the central blood vessel to the point of widest infiltration: Grade 1 (two to five cells), grade 2 (six to 10 cells) and grade 3 (> 10 cells). CXCL13 and CCL21 expression was analysed by immunohistochemistry and *in situ* hybridization with radiolabelled CXCL13-specific and CCL21-specific RNA probes.

Results In 17 patients grade 2 and/or grade 3 aggregates were demonstrated. Fully formed follicular-like aggregates, with detectable centrally located CD21⁺ dendritic cell clusters and T/B segregation, were identified exclusively in 7/20 patients (35%) of grade 3 aggregates. CXCL13 was observed in 7.66% grade 1, 39.9% grade 2 and 82.6% grade 3 aggregates, while CCL21 expressing cells were observed in 3.06% grade 1, 15.93% grade 2 and 43.55% grade 3. CXCL13 and CCL21 were expressed within lymphocytic clusters in 18/20 and 15/20 patients, respectively, both at protein level and mRNA level, in mature CD21⁺ FDC containing T/B cell follicular-like structures as well as in not organized T cell/B cell aggregates devoid of CD21⁺ FDC networks.

Conclusions This study demonstrates for the first time, by *in situ* hybridization, the site of production of CXCL13 and CCL21 in the context of synovial ectopic lymphoid tissue. The morphological and grading analysis provides evidence of the independence, in the synovial inflammatory microenvironment, between the ectopic production of lymphoid chemokines and the presence of a mature CD21⁺ FDC-rich/germinal centre lymphoid-like structures. This supports the hypothesis, similar to that shown in animal models, of a potential role of these factors in prefollicular stages of synovial lymphoid neogenesis.

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T-cell regulation in the pathogenesis of autoimmune diseases

H Schulze-Koops¹, PE Lipsky², JR Kalden¹, A Skapenko¹

¹Nikolaus Fiebiger Center for Molecular Medicine, Clinical Research Group III, University of Erlangen–Nuremberg, Erlangen, Germany;

²NIAMS, National Institutes of Health, Bethesda, Maryland, USA
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Evidence suggests that chronic inflammation in several human autoimmune diseases, such as rheumatoid arthritis (RA), is mediated by activated Th1 cells. Delineation of the regulatory mechanisms controlling a Th1-biased human immune reaction and its pathologic potential is, therefore, a critical step in the understanding of chronic autoimmune inflammation. We analyzed T-cell subsets from patients with RA with regard to their regulatory capacity for Th1 inflammation. Flow cytometric analysis of freshly isolated peripheral blood (PB) and synovial fluid (SF) T cells revealed that rheumatoid inflammation is characterized by the absence of Th2 cells and their cytokines. Moreover, resting T cells from patients with RA expressed an impaired ability to differentiate into Th2 effectors with a potential to downmodulate Th1 inflammation. Thus, altered Th2 cell differentiation might contribute to the imbalance in favor of inflammatory Th1 cells in RA. Whereas Th2 cytokines, such as IL-4, play essential roles in regulating the development and perpetuation of Th1-mediated autoimmune responses, a novel subset of regulatory CD4 T cells that express CD25 on their surface and perform their suppressor function on the development of autoimmune inflammation by cytokine-independent mechanisms has recently been described in animals and humans. However, their *in vivo* role in human inflammation is largely elusive. We identified CD4CD25^{pos} T cells in the PB and the SF of patients with active RA. Importantly, CD4CD25^{pos} T cells from both RA PB and RA SF exerted potent suppressive activity as proliferation of autologous peripheral blood mononuclear cells was significantly inhibited by the presence of CD4CD25^{pos} T cells compared with proliferation in the presence of CD4CD25^{neg} T cells. CD4CD25 regulatory T-cell-mediated inhibition of the proliferation was abrogated by addition of exogenous IL-2, a characteristic of CD4CD25 regulatory T cells in animals. Moreover, RA CD4CD25 T cells had a markedly decreased proliferative capacity in response to anti-CD3 monoclonal antibody, suggesting an anergic phenotype typical for CD4CD25 regulatory T cells. Together, the data indicate that, in contrast to immunomodulatory Th2 effectors, CD4CD25^{pos} T cells with potent suppressive potential are present in rheumatoid inflammation. The data further suggest that CD4CD25^{pos} regulatory T cells are involved in the continuous regulation of the developing human Th1 inflammation *in vivo*. However, their activity appears to be insufficient to silence the chronic immune response in RA.

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Autoantibodies in very early rheumatoid arthritis: diagnostic tools or pathogenic players?

G Steiner¹, V Nell¹, G Eberl², S Hayer¹, K Machold¹, E Hoefler², J Smolen¹

¹Division of Rheumatology, Department of Internal Medicine III, University of Vienna, Austria; ²Department of Internal Medicine II, Lainz Hospital, Vienna

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Background Among the autoantibodies (autoAb) described to occur in patients with rheumatoid arthritis (RA), autoAb to citrullinated antigens (anti-CCP) have proven to be highly specific for RA. Other

autoAb such as anti-RA33 are less specific but may still have some diagnostic value.

Objectives To assess the diagnostic and prognostic value of RF, anti-CCP and anti-RA33 autoAb in patients with very early arthritis, and to investigate the role of autoAb in the pathogenesis of RA.

Methods Patients with very early arthritis of less than 3 months' duration were included in this prospective study. So far, a final diagnosis of RA could be made in 100 patients, while 80 patients developed other diseases. To investigate pathogenetic involvement of autoAb, tumour necrosis factor transgenic (TNFtg) mice were used as an animal model of RA.

Results At first visit, rheumatoid factor (RF) was present in 54% of RA patients, anti-CCP in 42% and anti-RA33 in 26%, respectively. Specificity was 89% for RF, 98% for anti-CCP and 90% for anti-RA33, respectively (Table 1). However, while 46% of RA patients showed RF > 50 U/ml, only two non-RA patients were above this value. Thus, RF > 50 U/ml and anti-CCP both showed a high positive predictive value > 95%. Although anti-RA33 was less specific, the combined occurrence of (low) RF and anti-RA33 was also highly predictive of RA since it was exclusively observed in 15% of RA patients. Concerning the prognostic value, we found a significant correlation between anti-CCP or RF positivity and radiological outcome. To investigate the pathogenetic involvement of anti-RA33 autoimmunity, TNFtg mice (which spontaneously develop anti-RA33 autoAb) were immunized with the antigen or two antigen-derived peptides. Treatment with a peptide harbouring a major epitope or the complete antigen enhanced arthritis significantly. On the contrary, treatment with osteoprotegerin not only inhibited bone erosion, but also led to a significant reduction in anti-RA33 autoAb; a similar observation was made in *c-fos*-deficient TNFtg mice that lack osteoclasts.

Table 1

Sensitivity, specificity and positive predictive value of RF, anti-CCP and anti-RA33 autoantibodies in patients with very early RA

	RA (n = 100)	Non-RA (n = 80)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)
RF (≥ 20 U/ml)	54	9	54	89	86
RF (≥ 50 U/ml)	46	2	46	97	96
Anti-CCP	42	1	42	98	97
Anti-RA33	26	8	26	90	76
RF + anti-CCP	32	0	32	100	100
RF + RA33	15	0	15	100	100

Conclusion Determination of anti-CCP and also anti-A2/RA33 in addition to RF may be very helpful in the early diagnosis of RA. The data obtained in TNFtg mice suggest a molecular link between inflammation, tissue destruction and the generation of a pathogenic autoimmune response.

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Expression of IgVH mRNAs in plasma cells derived from rheumatoid arthritis synovium detected by single-cell RT-PCR

J Vencovský¹, Z Cimburek¹, J Niederlová¹, O Horvath², O Kryštůfková¹, T Dörner³, Š Růzicková⁴

¹Institute of Rheumatology, Prague, Czech Republic; ²Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic;

³Department of Rheumatology/Immunology, Medical Faculty, Charité, Humboldt University, Berlin, Germany; ⁴Laboratory of Gene Expression, Czech Academy of Sciences, Prague, Czech Republic

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Introduction The synovial membrane in rheumatoid arthritis (RA) contains large lymphocytic infiltrates, sometimes organized in germinal-like centres. The presence of plasma cells within the synovium and the pro-

duction of clonally related immunoglobulin transcripts have been observed. The exact nature of clonal expansion and its role in generation of specific, mutated antibodies in RA is not known. Somatic mutations and isotype switching in synovial B lymphocytes might suggest the antigen-driven process. This would be supported by the expression of recombination-activating genes 1 and 2 (Rag1 and Rag2).

Objectives To calculate mutational frequencies of immunoglobulin heavy-chain transcripts in the single plasma cells generated from RA synovium. To analyze the frequency of isotype-switched plasma cells and Rag1 and Rag2 gene expression in inflamed RA synovial tissue.

Methods Individual CD19⁺CD38⁺ plasma cells were isolated from digested synovium of two Caucasian RA patients using single-cell deposition. The cDNA from each single plasma cell was generated and a nested PCR specific for VH genes and Rag1 and Rag2 genes was performed. After sequencing, the VBASE database was used to assign VH, DH and JH gene segments and somatic mutations.

Results Three different subsets of CD19⁺CD38⁺ plasma cells were detected. The first subset represents cells expressing only IgM transcripts (IgM⁺, 13.5%), cells in the second subset expressed only IgG transcripts (IgG⁺, 48.7%), and the cells in the third subset expressed both IgM and IgG mRNAs (IgM⁺IgG⁺, 37.8%). All of these detected IgVH mRNAs contained mutated sequences, indicating their memory cell origin. Significant differences in mutational frequencies were found between the subsets (IgM⁺ plasma cells, 3.8%; IgG⁺ plasma cells, 11.2%; and IgM⁺IgG⁺ cells, 6.3%). Interestingly, either Rag1 or Rag2 mRNA was observed in 83.3% of all analyzed CD19⁺CD38⁺ plasma cells, with the highest frequency in the IgM⁺IgG⁺ subset (71.4%).

Conclusions The population of CD19⁺CD38⁺ plasma cells differentially expressing mutated IgVH mRNAs and reinducing Rag1 and Rag2 genes was observed in RA synovium. The IgM⁺IgG⁺ cells might represent cells switching from the IgM to the IgG isotype, and IgG⁺ plasma cells might correspond to post-switched cells producing high-affinity (auto)antibodies. The high mutational rate and reinduction of Rag genes suggest an antigen-driven process.

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T cell contact-mediated induction of tumor necrosis factor alpha in peripheral blood monocytes is inhibited by autologous serum in rheumatoid arthritis

U Wagner¹, M Rossol¹, S Kaltenhäuser¹, S Hauschild², H Häntzschel¹

¹Department of Medicine IV, University of Leipzig, Germany;

²Department of Immunobiology, University of Leipzig, Germany

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Background and objective Cell contact of monocytes with preactivated T cells is one of the strongest known stimuli of monocyte cytokine production and has been implied in the pathogenesis of rheumatoid arthritis (RA). T cells from the synovial membrane of RA patients have been found to induce high levels of tumor necrosis factor alpha (TNF- α) production in synovial macrophages, and monocytes from healthy individuals react similarly to preactivated T cells. The response of peripheral monocytes from RA patients to T-cell contact, however, has not been analyzed previously, and was therefore investigated in the study presented here.

Methods Peripheral blood monocytes from 20 patients with RA and from age-matched healthy donors were isolated by immunomagnetic separation and used in a coculture system with T lymphocytes from unrelated donors. T cells were stimulated with immobilized CD3 and CD28 antibodies, subsequently fixed with glutaraldehyde, and then used in the coculture system at a T cell:monocyte ratio of 7:1. In control experiments, unstimulated fixed T cells or lipopolysaccharide were used.

Results Lipopolysaccharide-induced TNF- α production of monocytes from RA patients did not differ from the controls. Upon cocubation with preactivated T cells, however, monocytes from RA patients produced significantly lower amounts of TNF- α . This difference was independent of previous or current disease-modifying antirheumatic drug

therapy, since it was also found in patients with recent onset RA who had not received immunosuppressive therapy prior to study inclusion. Serum transfer experiments showed the presence of inhibitory factors in sera from RA patients to be one mechanism contributing to the diminished response of RA monocytes, since those sera were also able to inhibit T-cell-dependent TNF- α production in healthy monocytes.

Conclusion The suppressed response of peripheral blood monocytes from RA patients to preactivated T cells shows that they are not likely to contribute significantly to the excessive levels of TNF- α that are associated with this disease. The inhibitory activity of sera from RA patients in the cell contact-dependent system suggests a counter-regulatory mechanism in the systemic circulation that prevents excessive activation of circulating monocytes in this disease.

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The declining incidence of nonsteroidal anti-inflammatory drug gastropathy in rheumatoid arthritis patients: rates and reasons

J Fries, K Murtaugh, M Bennett, E Zatarain, B Lingala, B Bruce

Department of Medicine, Stanford University School of Medicine,

Stanford, California, USA

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Background Nonsteroidal anti-inflammatory drug (NSAID) gastropathy is recognized as a major cause of hospitalizations and deaths, with more than 100,000 annual hospitalizations and more than 10,000 annual deaths in the United States alone. Trends in this epidemic over time have not been examined.

Objective We studied the possibility that new preventive approaches to NSAID gastropathy may have reduced the magnitude of this epidemic.

Methods We studied 5598 patients with rheumatoid arthritis from longitudinal data banks previously employed to help establish the epidemiology of NSAID gastropathy. Consecutively enrolled patients were followed with bi-annual Health Assessment Questionnaire assessments and medical record audits between 1981 and 2000. Annual rates of hospitalization involving gastrointestinal (GI) bleeding, obstruction, or perforation were calculated and curves fitted using spline regression.

Results Annual GI hospitalization rates rose from 0.6% in 1981 to a peak of 1.5% in 1992, then declined again to 0.6% again in 2000. The period of rising incidence was associated with an increase in patient age and GI risk propensity score. The period of declining incidence was correlated with use of lower doses of aspirin and ibuprofen, a decline in use of 'more toxic' NSAIDs from 52% to 42%, a rise in use of 'safer' NSAIDs from 19% to 48%, and increasing use of proton pump inhibitors. The decline was not associated with changes in age, NSAID exposure, or GI risk.

Conclusions The risk of GI hospitalization for NSAID gastropathy has declined by 60% in these cohorts since 1992. We estimate a 16% decline attributable to dose reductions, 10% to use of proton pump inhibitors, and 10% to use of less toxic NSAIDs. Given continued trends in these three factors, these declines are likely to continue.

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Significance of fatigue in patients with rheumatoid arthritis

G Singh¹, M Bennett¹, B Lingala¹, A Singh²

¹Department of Medicine, Division of Immunology and Rheumatology,

Stanford University, Stanford, California, USA; ²Wyeth Research, USA

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Background Fatigue is a frequent and debilitating problem in people with rheumatoid arthritis (RA). However, there are few prospective studies evaluating the economic impact of fatigue in people with RA.

Objectives To study the economic correlates of fatigue in patients with RA, and to evaluate the impact of fatigue on costs of medical care and missed days of work.

Patients and methods A total of 6551 consecutively diagnosed RA patients with 35,371 person years of follow-up are currently enrolled in

the ARAMIS postmarketing surveillance program. Patients complete semi-annual Stanford Health Assessment Questionnaires on their disease symptoms, severity, medication use (including over-the-counter medications), adverse events and healthcare resource utilization including hospitalizations and emergency room visits. Patients also report on measures of indirect costs such as missed days of work and days when they were unable to do nonemployment-related activities. All self-reported hospitalizations are audited, and discharge summaries are reviewed by a physician. Complete ascertainment of all deaths is obtained from regular searches of the US National Death Index database. Since 1987, all patients were asked to report on the presence of tiredness or fatigue in their semi-annual assessment. Medical costs of care are estimated based on 2002 Medicare reimbursement rates and on 2002 average wholesale medication costs. Statistical comparisons were made between periods when patients reported fatigue and those when no fatigue was reported.

Results A total of 2699 patients (mean age, 58.3 years; 78% female) with 12,458 person-years of follow-up answered the fatigue question. The point prevalence of fatigue varied from 57.5% in 1987 to 67.2% in 2000 (average, 56.9%). For comparison, the point prevalence of fatigue in 1916 patients with osteoarthritis (mean age, 66 years; 76% female) was 49.6% ($P < 0.001$, after adjustment for confounding demographic variables). Patients with fatigue reported significantly higher annual direct medical costs of care compared with those who were not fatigued ($\$4621.70 \pm 241.66$ vs $\$2131.42 \pm 265.68$ respectively, $P < 0.001$). During the 6-month periods when they reported fatigue, patients missed an average of 1.93 days (standard error of the mean [SEM], 0.11) of work, and were unable to do nonemployment-related activities for 12.31 days (SEM, 0.34). In the 6-month time periods when no fatigue was reported, patients only missed an average of 0.98 days (SEM, 0.10) of work and were unable to do nonemployment-related activities for 5.44 days (SEM, 0.34) ($P < 0.001$ for both comparisons). Multivariate analysis showed that the costs of medical care were strongly correlated with the presence of fatigue ($P < 0.02$), after adjusting for age, gender, duration of RA and education level.

Conclusions Fatigue is a common symptom in patients with RA. Patients with fatigue have significantly higher costs of medical care and miss more days of work compared with those who do not have fatigue. It is important to measure fatigue in clinical trials of innovative therapies since this is an important reason for poor quality of life, and is strongly correlated with disease severity and direct and indirect medical costs.

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Skewed T-cell receptor BV14 and BV16 expression, and shared complementarity-determining region 3 sequence and common sequence motifs in synovial T cells of rheumatoid arthritis

W Sun^{1,2}, H Nie^{1,3}, N Li^{1,4}, Y Zang^{2,5}, D Zhang^{1,3}, G Feng^{1,6}, L Ni^{1,6}, R Xu^{1,6}, E Sercarz⁴, JZ Zhang^{1,2,3,5,6}

¹Shanghai Institute of Immunology and Immunology Division, E-Institutes of Shanghai Universities, People's Republic of China;

²Department of Immunology, Baylor College of Medicine, Houston,

Texas, USA; ³Health Science Center, Chinese Academy of Sciences, Shanghai Second Medical University, People's Republic of China;

⁴Torrey Pines Institute for Molecular Studies, San Diego, California, USA; ⁵Hong Kong Chinese University, People's Republic of China;

⁶Guanghua Rheumatology Hospital, Shanghai, People's Republic of China

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints, in which T cells are thought to play an important role in the rheumatoid synovium. We hypothesized that if the T-cell receptor (TCR) repertoire of infiltrating T cells is shaped by interaction with common self-antigens or microbial antigens in the context of susceptible human leukocyte antigen genes, these synovial T cells may share some TCR structural features in different patients. In this study, syn-

ovial lesion tissue, synovial fluid and blood specimens from 37 RA patients and seven control patients were analyzed for TCR repertoire. The results indicated highly skewed BV14 and BV16 in the synovial lesion tissue and BV16 in synovial fluid of RA, while control synovial material had diverse BV distribution. A trend for correlation between the skewed BV16 but not BV14 and the expression of DRB1*0405 was found in this cohort of Chinese RA patients. Immunoscope analysis of the V-D-J region showed oligoclonal expansion of BV14⁺ and BV16⁺ T cells in some cases, while polyclonal patterns were frequently seen in other specimens. Refinement of the V-D-J region profile by detailed immunoscope analysis using BV and BJ primers revealed common clonotypes that utilized the same BV14 or BV16 and the same BJ, and had similar complementarity-determining region 3 (CDR3) length. DNA cloning and sequence analysis of the clonotypes confirmed identical CDR3 sequences and common CDR3 sequence motifs among different RA patients. The findings are important in the understanding of BV gene skewing and the CDR3 structural characteristics in synovial infiltrating T cells of RA, and have implications in novel immunotherapy.

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Changes in the synovial cell proteome induced by hypoxia

H El-Gabalawy¹, J Wilkins¹, K Dasuri¹, D Duggan²

¹Arthritis Centre, University of Manitoba, Winnipeg, Manitoba, Canada;

²NIAMS, National Institutes of Health, Bethesda, Maryland, USA

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Purpose Fibroblast-like synovial cells (FLS) have provided considerable insight into the mechanisms underlying joint inflammation and destruction. There is currently an incomplete knowledge of the global FLS proteome and of how this compares with the proteome of other fibroblasts. The rheumatoid arthritis synovial microenvironment is known to be chronically hypoxic. We studied the FLS proteome using two-dimensional gels and mass spectrometry to evaluate the effects of hypoxia on the synovial proteome, comparing this with the expressed genome.

Methods FLS were isolated from rheumatoid arthritis and osteoarthritis synovia obtained at the time of joint arthroplasty. Cells from passages 2-4 were cultured under normoxic and hypoxic conditions for variable time points prior to analysis. Total proteins found in whole-cell FLS lysates were separated on immobilized pH-gradient two-dimensional (2D) PAGE and stained with Coomassie blue. To date, the in-gel digests of >300 protein spots have been analyzed using matrix-assisted laser desorption/ionization time of flight mass spectrometry. Spectra were analyzed using the Knexus automation client and the Profound search engine (Proteometrics Canada, Winnipeg, Manitoba, Canada). 2D gels from hypoxic and normoxic FLS were superimposed using image analysis software and were compared. Differences were identified, and were related to differences in the expressed genome demonstrated in a microarray experiment using the same cell lines and conditions.

Results Under normoxic conditions, more than 300 proteins were fully characterized in the FLS lines. Of particular note, FLS intensely expressed uridine diphosphoglucose dehydrogenase, which is an enzyme needed for the synthesis of hyaluronic acid. Other proteins identified fell into the following groups: enzymatic, transcription and cell-cycle regulation, protein binding, cytoskeletal, extracellular matrix, heat shock, protein synthesis and assembly, and membrane channel. In comparing the proteome expressed under hypoxic conditions, most of the apparent differences were quantitative, with shifts in position suggestive of phosphorylation and glycosylation. The microarray data demonstrated a spectrum of known, as well as novel, hypothetical proteins that were regulated by hypoxia. Identification of these proteins in the 2D gels is currently underway.

Conclusions We demonstrate techniques used in the detailed exploration of the synovial proteome. These techniques are applied to the examination of the effects of hypoxic stimulation on FLS. Comparisons between the expressed proteome and genome provide novel insights into the response of FLS to hypoxia

Poster Discussion (7) RA Group (2)

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CD44 regulates bone erosion and osteoclastogenesis in arthritis

G Schett¹, S Hayer¹, K Redlich¹, EF Wagner², G Kollias³, G Steiner¹, JS Smolen¹

¹Division of Rheumatology, Department of Internal Medicine III, University of Vienna, Austria; ²Research Institute for Molecular Pathology, Vienna, Austria; ³Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece
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Objective CD44 mediates cell-matrix interaction and is thought to play a role in cell adhesion, fusion and migration. Blocking of CD44 is considered a potential target in the therapy of rheumatoid arthritis.

Methods To elucidate the role of CD44 in arthritis, human tumour necrosis factor transgenic (hTNFtg) mice were crossed with CD44 knockout mice.

Results Clinical evaluation revealed a significantly increased severity of arthritis in CD44^{-/-} hTNFtg mice than in hTNFtg mice. Wild-type mice and CD44^{-/-} mice were normal. Histologically, bone destruction was dramatically increased in the arthritic paws of CD44^{-/-} hTNFtg mice. Changes were based on a significant increase of size and number of osteoclasts in the synovial inflammatory tissue. *Ex vivo* analysis of osteoclastogenesis revealed that osteoclasts differentiated more rapidly and were increased in size and number in CD44^{-/-} hTNFtg mice compared with in hTNFtg controls. In addition, a bone resorption assay showed increased 'pit' formation by osteoclasts of CD44^{-/-} hTNFtg mice.

Conclusion CD44 deficiency does not block, but rather increases the severity of TNF-mediated arthritis. This was due to increased bone damage caused by deregulation of osteoclastogenesis. We conclude that CD44 is of benefit for TNF-mediated arthritis due to its regulatory role on osteoclasts.

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Repair of local bone erosions by combined treatment with parathyroid hormone, osteoprotegerin and anti-tumor necrosis factor in tumor necrosis factor-transgenic mice

K Redlich¹, B Goertz¹, N Doerr², G Kollias³, G Steiner⁴, J Smolen¹, G Schett¹

¹Division of Rheumatology, Department of Internal Medicine III, University of Vienna, Austria; ²Department of Pathology, Amgen, Inc., Thousand Oaks, California, USA; ³Molecular Genetics Laboratory, Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece; ⁴Center of Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria
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Local bone erosions and systemic bone loss are hallmarks of rheumatoid arthritis and cause progressive disability. Tumor necrosis factor (TNF) is a key mediator of arthritis and acts catabolically on bone by stimulating bone resorption and inhibiting bone formation. We hypothesized that the concerted action of parathyroid hormone (PTH), which stimulates bone formation, osteoprotegerin (OPG), which blocks bone resorption, and anti-TNF, which reduces inflammation, could lead to repair of local bone erosions and to inhibition of systemic bone loss. To test this, human TNF-transgenic mice with erosive arthritis and established systemic bone loss were treated with PTH, OPG and anti-TNF. Local bone erosions almost fully regressed, suggesting repair of inflammatory skeletal lesions. In contrast, OPG or anti-TNF alone led to arrest of bone erosions but did not achieve repair. Treatment with PTH alone had no influence on the progression of bone erosions. Local bone erosions showed all signs of new bone formation such as the presence of osteoblasts, osteoid formation and mineralization. Systemic bone loss was completely reversed upon combined treatment, and this effect was mediated by osteoblast stimulation and osteoclast blockade. In contrast to local and systemic bone loss, joint inflammation was only affected by anti-TNF. In summary, we conclude that local and systemic

inflammatory bone loss due to TNF can regress, and that repair requires a combined approach by reducing inflammation, blocking bone resorption and stimulating bone formation.

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Essential role for migration inhibitory factor in IL-1 activation of mitogen-activated protein kinases

E Morand, D Lacey, Y Yang, M Leech, M Toh

Centre for Inflammatory Diseases, Monash University, Melbourne, Australia

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Macrophage migration inhibitory factor (MIF) is a pluripotential proinflammatory cytokine with a possible role in the pathogenesis of rheumatoid arthritis (RA). MIF is expressed in RA synovium, and directly activates RA synoviocyte gene expression and proliferation, as well as exerting anti-apoptotic effects via inhibition of p53. MIF activates ERK and p38 mitogen-activated protein (MAP) kinase, but evidence for direct activation of NF-κB by MIF is lacking.

Anti-MIF monoclonal antibodies prevent IL-1 activation of fibroblast-like synoviocytes *in vitro*, implicating MIF in the activation of cells by IL-1. The mechanism of this action of MIF has not been adduced. We studied the activation of signal transduction pathways by IL-1 in cells deficient in MIF. Dermal fibroblasts were cultured from MIF^{-/-} and WT mice, and were exposed to IL-1. MAP kinase and NF-κB activation were studied by Western blotting, electrophoretic mobility shift assay (EMSA), and reporter gene assays.

IL-1 rapidly induced phosphorylation of p38, JNK, and ERK MAP kinases in WT cells. In contrast, MIF^{-/-} cell p38, JNK, and ERK activation in response to IL-1 was reduced. Consistent with this observation, MIF^{-/-} cells were hyporesponsive to IL-1-induced AP-1 DNA binding as measured by EMSA ($P = 0.03$). IL-1-induced activation of an AP-1 luciferase reporter gene system was also reduced in MIF^{-/-} cells ($P = 0.037$). Confirming the functional significance of these results, MIF^{-/-} cells were hyporesponsive to IL-1-induced proliferation. In contrast, MIF^{-/-} cells were normally responsive to IL-1-induced MKK3 and MKK7 phosphorylation. No significant difference in NF-κB activation was detected between MIF^{-/-} and WT cells, as measured by cellular IκB content, NF-κB EMSA, or NF-κB luciferase reporter gene assay.

These data demonstrate that MIF is required for cellular MAP kinase responses to IL-1. This represents a novel mechanism of action of MIF in the support of the inflammatory response. The absence of an effect on MKK3/MKK7 or NF-κB suggests the effects may be mediated distally in the signal transduction cascade. Therapeutic MIF antagonism may limit the cellular effects of IL-1.

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Regulatory T cells in experimental arthritis

R Bräuer¹, O Frey¹, PK Petrow¹, A Scheffold², A Radbruch²

¹Institute of Pathology, Friedrich Schiller University, Jena, Germany;

²German Rheumatism Research Centre, Berlin, Germany

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Background It is now generally accepted that central and peripheral immune tolerance is in part mediated by the action of suppressor cells. In particular, CD4⁺ T cells coexpressing CD25 (CD4⁺CD25⁺ regulatory T cells [T_{reg}]) have been shown crucial for the prevention of autoimmunity in several animal models. We investigated the role of this cell population in murine antigen-induced arthritis, a well-characterized experimental model of human rheumatoid arthritis.

Methods For this purpose, we used two different approaches. First, the depletion of CD25-expressing cells *in vivo* using a monoclonal antibody against this molecule, and second, the transfer of purified CD4⁺CD25⁺ T_{reg} from naive donors into arthritic mice.

Results Depletion of CD25-expressing cells resulted in a clinical and histological aggravation of arthritis. The increased severity of arthritis was due to a lack of T_{reg} cells, since transfer of purified CD4⁺CD25⁺ cells from naive donors into depleted animals ameliorated the clinical signs of arthritis. The aggravated arthritis in depleted mice was accompanied by exaggerated humoral (serum IgG) and cellular (ELISPOT) immune responses against the inducing antigen (methylated bovine

serum albumin) and to cartilage matrix constituents (collagen type I, collagen type II, proteoglycans). Thus, CD4⁺CD25⁺ T_{reg} cells may regulate the severity of arthritis by adjusting the systemic immune responsiveness to arthritis-related antigens. Furthermore, adoptive transfer of purified CD4⁺CD25⁺ T_{reg} cells, isolated from naive mice, into arthritic animals resulted invariably in a marked decrease of the severity of arthritis. Especially with use of *in vitro* preactivated T_{reg} cells, this effect could be achieved with very low cell numbers (1 × 10⁵ cells per animal). Analysis of cellular and humoral immune responses in the recipients did not show signs of systemic immunosuppression. Local immunoregulatory effects of these cells are possibly involved, but their exact mechanism of action is hitherto unknown.

Conclusions Taken together, these data implicate a pivotal role of CD4⁺CD25⁺ immunoregulatory T cells in experimental arthritis, and it can be hypothesized that they are a new diagnostic or prognostic tool for rheumatoid arthritis patients. Hopefully, enhancement of their function may be beneficial for these patients and represents a new therapeutic strategy in autoimmune diseases.

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Antirheumatic effects of humanized anti-Fas monoclonal antibody in human rheumatoid arthritis/SCID mouse chimera

H Matsuno¹, K Yudoh², K Nishioka²

¹Bioengineering Research Center, Toin-Yokohama University, Yokohama, Japan; ²Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan

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Objective Anti-Fas monoclonal antibodies (mAbs) are considered a potential therapeutic agent for rheumatoid arthritis (RA). However, Fas-mediated liver and chondrocyte damage is a serious problem in its clinical application. m-HFE7A, a novel anti-Fas mAb, selectively induces apoptosis in inflammatory cells. We succeeded in humanizing m-HFE7A to obtain h-HFE7A. We investigated the therapeutic effects of h-HFE7A mAb in RA.

Methods We investigated the apoptosis-inducing activities of h-HFE7A on human Fas ligand transfected cells and cultured human activated lymphocytes (human peripheral blood mononuclear cells and isolated human RA synovial lymphocytes), synoviocytes, and chondrocytes. We then examined the effects of h-HFE7A mAb *in vivo* using SCID-HuRag mice implanted with human RA tissue.

Results Administration of h-HFE7A mAb alone did not induce apoptosis in cultured human Fas ligand transfected cells and activated lymphocytes. However, apoptosis-inducing activities were noted by this mAb crosslinking with a secondary antibody or Fcγ receptor-positive cells. In contrast, no apoptosis induction by h-HFE7A was observed on cultured synoviocytes and chondrocytes with or without crosslinking. Thus, the crosslinking with Fcγ receptor-positive cells is essential for the efficacy of this mAb *in vivo*. In the implanted tissue of the SCID-HuRag mice, the number of inflammatory cells was significantly decreased in the h-HFE7A mAb-treated group compared with the IgG-treated control group. Moreover, there were only negligible effects in synoviocytes and chondrocytes with the h-HFE7A mAb.

Conclusion Administration of this novel humanized anti-Fas mAb may provide a new treatment for RA by inducing Fas-mediated apoptosis in inflammatory cells.

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TNF-alpha blockade by nonviral gene therapy in collagen-induced arthritis

MC Boissier¹, C Bloquel¹, P Bigey², V Deleuze², D Scherman², N Bessis¹

¹UPRES EA-3408 and Department of Rheumatology, University of Paris 13 and Avicenne Hospital, Bobigny, France; ²Laboratoire de Pharmacologie clinique et génétique, U266 INSERM, FRE2463 CNRS, University of Paris 5, France

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Objectives Gene therapy using electrotransfer (ET) is a safe method for transferring therapeutic transgenes *in vivo*. We have used this

method to administer transgenes encoding three human tumour necrosis factor alpha soluble receptor I (hTNFRIs) variants engineered in Scherman's research unit. The ET parameters and therapeutic effects in collagen-induced arthritis (CIA) in mice were studied.

Methods The following three plasmids were used: pCOR(hTNFRIs)₁, pCORhTNFRIs/mlgG₁, and pCOR(hTNFRIs)₂. These encode, respectively, the monomeric (hTNFRIs)₁ form, the chimeric hTNFRIs/mlgG₁ form or the dimeric (hTNFRIs)₂ form of hTNFRIs. They were electrotransferred in the tibial cranial muscle. The hTNFRIs concentrations were determined by ELISA. Biological activity (tumour necrosis factor alpha neutralization) of the fusion protein encoded by each of the plasmids was determined using L929 cells. Detection of the plasmid genome was determined by PCR. CIA was induced in DBA/1 mice with bovine type II collagen in complete Freund adjuvant.

Results ET of the three plasmids allowed dose-dependent hTNFRIs production in sera and muscle after 10 days. Local expression in the muscle lasted for at least 6 months. Systemic expression in the serum lasted for at least 6 months for the hTNFRIs/mlgG₁ form, while it was shorter for the two other forms (about 3 weeks). After intramuscular ET of any of the three plasmids, muscle lysates were able to inhibit tumour necrosis factor alpha cytotoxicity on L929 cells *ex vivo*. No plasmid DNA was found in the organs distant from the injected muscle (liver, spleen, kidney, gonads, heart, lung, brain and distant muscle). ET of 50 μg pCOR/sTNFR1/IgG₁ plasmid at the onset of clinical disease induced a clear-cut decrease of clinical and histological signs of arthritis. The dimeric (hTNFRIs)₂ form was also efficient, although the effect was weaker than with the fusion protein. The monomeric form had no effect on arthritides.

Conclusion Intramuscular ET of plasmids encoding the three forms of hTNFRIs (monomeric, dimeric and the IgG chimera) leads to a production of biologically active protein and, most importantly, is followed by a long-term secretion of hTNFRIs in the serum. CIA is efficiently inhibited when ET of plasmids encoding either the chimera or the dimeric form of the hTNFRIs was performed at the onset of the clinical signs.

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Therapeutic inhibition of tumour necrosis factor alpha reduces oligoclonality in the selected T-cell receptor BV families in rheumatoid arthritis

M Pierer, U Wagner, S Kaltenhäuser, S Arnold, H Häntzschel

Department of Medicine IV, University of Leipzig, Germany

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Background Treatment of rheumatoid arthritis (RA) with the recombinant p75 tumour necrosis factor (TNF) receptor fusion protein etanercept is clinically highly effective. Clonally expanded, autoreactive CD4⁺CD28 null T cells can be found in the peripheral blood of patients with RA, and have been shown to use distinct BV-BJ combinations in their T-cell receptor (TCR).

Objective This study was undertaken to investigate the influence of anti-TNF-α treatment on expanded clonotypes in the peripheral CD4⁺ T-cell compartment.

Methods We investigated the size of the CD4⁺CD28 null compartment and the TCR β-chain repertoire of expanded CD4⁺ clonotypes in 19 patients with RA, before the initiation of treatment with etanercept and after 2, 4 and 18 months of TNF-α blocking therapy by complementarity-determining region 3 (CDR3) length analysis (spectrotyping) in the BV6 and BV14 TCR families. This was done with primers specific for three arbitrarily chosen β-chain joining elements (BJ1S2, BJ2S3 and BJ2S7).

Results TNF-α inhibition significantly reduced the total number of expanded clonotypes found in the TCR BV6 family after 4 months of treatment, and was even more pronounced after 18 months. In contrast, however, the number of detected BV14 clones was not affected. The percentage of CD4⁺CD28 null cells, which has been shown to correlate with expanded clonotypes using BV14-BJ1S2 and BV14-BJ2S3 combinations, but not with the number of BV6 clones, remained unchanged under therapy.

Conclusion Therapeutic blockade of the proinflammatory cytokine TNF-α, which leads to a profound decrease of disease activity in treated patients, was also found to influence the size of expanded CD4⁺ clonotypes in the peripheral circulation in RA patients. This effect was dependent on the TCR BV-BJ combinations used in the

clonotypes, however, and was restricted to the BV6 family in our study, while BV14 clonotypes were not affected.

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Pathogenic roles of IL-6 and IL-6 blocking therapy on rheumatoid arthritis

K Yoshizaki¹, N Nishimoto¹, N Miyasaka², K Yamamoto³, S Kawai⁴, T Takeuchi⁵, T Kishimoto⁶

¹Department of Medical Science I, School of Health and Sport Sciences, Osaka University, Suita, Japan; ²School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan; ³Graduate School of Medicine, University of Tokyo, Japan; ⁴St Marianna University of Medicine, Kawasaki, Japan; ⁵Saitama Medical Center/School, Kawagoe, Japan; ⁶Osaka University, Suita, Japan

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Background In rheumatoid arthritis (RA), chronic inflammation followed by the destruction of joints and systemic symptoms is constructed and progressed with a continuous inflammatory and immunological hyperactivity. IL-6 is considered a proinflammatory cytokine produced in inflammatory synovium in RA, and to participate in the lymphocyte activation, synovial cell growth, angiogenesis, cartilage and bone destruction, and generation of AA amyloidosis. However, precise functions of IL-6 in RA are still unknown.

Objective To know the actual pathogenic roles of IL-6 in RA and to develop IL-6 blocking therapy on RA, a humanized anti-IL-6 receptor antibody (MRA) has been used for the treatment of RA as a specific blockade of IL-6 function.

Method To evaluate the therapeutic effects of MRA to patients with RA, three clinical studies have so far been performed in Japan: a pilot study of 11 severe patients, an open-labeled, dose-escalating, multi-dose clinical study of 15 patients, and a randomized, placebo-controlled trial of a multidose study on 163 patients. In most clinical trials in Japan, 2–8 mg/kg MRA was administered intravenously every 4 weeks.

Results The treatment was well tolerated at all doses. The clinical efficacy of MRA evaluated by ACR criteria seems to be same as or more than those of tumor necrosis factor alpha blockades. MRA treatment also improved the abnormal laboratory findings observed in active RA patients. In most MRA-treated patients, elevated levels of acute phase proteins, such as C-reactive protein, fibrinogen and SAA, were rapidly normalized after first administration, and thrombocytosis, anemia and hypoalbuminemia were improved within 2 months. Rheumatoid factor levels were gradually decreased.

Conclusion These therapeutic effects and improvements of abnormal findings by MRA treatment confirm that IL-6 is one of the key cytokines in the pathogenesis of RA arthritis, and IL-6 blocking therapy is a cytokine-specific, safe and effective therapy for patients with RA.

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Repeated B-cell depletion with rituximab in rheumatoid arthritis

J Edwards, M Leandro

Centre for Rheumatology, University College, London, UK
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Thirty-seven patients have been recruited to a programme of repeated B-cell depletion therapy for rheumatoid arthritis. Protocols have been based on rituximab, combined with cyclophosphamide initially. Sixty-two treatments have been given over 4.5 years. Patients were withdrawn for inadequate response (<ACR20), for successful reintroduction of disease-modifying antirheumatic drug (DMARD) or for toxicity.

Twenty-five patients continue on the programme with evidence of response, and a further three patients have regained control on reintroduction of DMARD (methotrexate, sulphasalazine). All four IgM rheumatoid factor-negative patients failed to respond. One seropositive patient achieved no benefit, and two achieved benefit of 5 months or less. Two patients have been lost to follow-up. The mean period of benefit (>ACR20) was 15 months, with a maximum of 35 months. One patient showed inadequate depletion. Secondary failure of clinical response or depletion was not observed in up to four repeat treatments. Coexistent psoriasis on three patients showed no change.

B-cell depletion protocols were well tolerated. Eight febrile episodes with pulmonary symptoms occurred in 85 patient-years of follow-up. All settled rapidly on antibiotic. Although most were assumed infective, at least four may have been late reactions to therapy or been disease associated. One suspected joint prosthesis infection was sterile on culture. Three patients who received rituximab in combination with cyclophosphamide have developed breast carcinoma (two patients) or carcinoma *in situ* (one patient), although in one case there was evidence that this predated therapy. (No increase in the incidence of carcinoma has been reported in surveillance of rituximab usage elsewhere.) In three cases who have received three courses of therapy in rapid succession, serum IgM levels have fallen to undetectable during the period of depletion. Falls in IgG levels have been modest and antibacterial antibody levels well maintained.

In summary, experience with repeated B-cell depletion therapy in rheumatoid arthritis suggests that approximately 80% of seropositive patients may become susceptible to continuing control of disease, but seronegative disease appears unresponsive. Secondary resistance appears not to be a problem over 2–4 years. Susceptibility to chest infection may be increased. Cumulative effects on immunoglobulin levels may occur with frequently repeated usage.

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Gene transfer of p21^{Cip1} exerts multiple molecular effects in the treatment of arthritis

H Kohsaka, Y Nonomura, K Nagasaka, N Miyasaka

Department of Bioregulatory Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Japan
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Forced expression of the cyclin-dependent kinase inhibitor gene p21^{Cip1} in the synovial tissues was effective in treating animal models of rheumatoid arthritis (RA). Synovial hyperplasia in the treated joints was suppressed, reflecting the inhibitory effect of p21^{Cip1} on cell-cycle progression. Additionally, lymphocyte infiltration, expression of inflammatory cytokines, and destruction of the bone and cartilage were inhibited. To determine why the cell-cycle regulator gene exerted such anti-inflammatory effects, we investigated gene expression by rheumatoid synovial fibroblasts with or without the p21^{Cip1} gene transferred. We have found that p21^{Cip1} gene transfer downregulates the expression of various inflammatory mediators and tissue-degrading proteinases that are critically involved in the pathology of RA. These molecules included IL-6, IL-8, type I IL-1 receptor (IL-1R1), monocyte chemoattractant protein-1, macrophage inflammatory protein-3a, cathepsins B and K, and matrix metalloproteinase-1 and matrix metalloproteinase-3. Downregulation of IL-1R1 by p21^{Cip1} resulted in attenuated responsiveness to IL-1. Inhibition of the inflammatory gene expression by p21^{Cip1} was seen even when IL-1 is absent. This IL-1-independent suppression was accompanied by reduced activity of c-Jun N-terminal kinase, which was associated with p21^{Cip1}, and by inactivation of NF-κB and AP-1. These multiple regulatory effects should work in concert with the primary effect of inhibiting the cell cycle in ameliorating the arthritis, and suggest a heretofore unexplored relationship between cyclin-dependent kinase inhibitors and inflammatory molecules.

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The innate immune system as a therapeutic target in arthritis

M Corr, JY Choe, K Pekny

Department of Medicine and The Sam and Rose Stein Institute for Research on Aging, University of California San Diego, USA
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Rheumatoid arthritis is a chronic inflammatory disease that may be characterized by several phases. In the initial induction phase of the disease there may be a breach in the normal mechanisms that protect the host from destruction. In the later phase, pathways that are independent of the adaptive immune system may perpetuate inflammation. These pathways may be active in patients who are refractory to biologic therapies that target key cytokines, IL-1 and tumor necrosis factor alpha. The Toll-like receptors (TLRs) are an integral part of the innate immune system, and are present on synovial lining cells, as well as the cells of the

immune system, which may amplify their contribution to the inflammatory cascade. We have found that TLR-4 and its coreceptor CD14 can modulate arthritis and can modulate inflammatory joint disease in murine models of arthritis. In particular, exposure to a TLR-4 ligand stimulates release of IL-6 from synovial fibroblast-like cells in culture, and circumvents the requirement for IL-1 receptor signaling in a serum transfer model of arthritis. These data suggest that triggering the innate immune response through the TLRs with either microbial or autologous ligands plays a role in the initiation or perpetuation of rheumatoid arthritis.

Poster Discussion (8) RA Group (3)

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Soluble 4A11 antigen: a novel mediator of leukocyte-endothelial adhesion and monocyte recruitment in rheumatoid arthritis

A Koch, K Zhu, M Amin, Y Zha, M Kim, C Park, H Ruth, H Castro-Rueda, C Haas

Northwestern University Medical School and V.A. Chicago Healthcare, Lakeside Division, Chicago, Illinois, USA

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The rheumatoid arthritis (RA) synovial pannus is characterized by a number of processes, including monocyte recruitment, leukocyte-endothelial adhesion, and angiogenesis. We have generated a novel monoclonal antibody (mAb) (mAb 4A11), which detects the blood group antigens Lewis y and H-5-2 (collectively termed the 4A11 antigen). We have shown that this antigen, in soluble form, as well as a glucose analog of H-5-2 (H-2g), are potent mediators of angiogenesis. The 4A11 antigen is selectively expressed in skin, lymphoid organs, the thymus and synovium, and is upregulated on RA synovium compared with normal synovium, suggesting a role in leukocyte recruitment and homing. We have also shown that H-2g significantly upregulates cell surface expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) on human dermal microvascular endothelial cells (HMVECs), but not vascular cellular adhesion molecule-1 or E-selectin. In accord with this, H-2g induces ICAM-1-mediated leukocyte-endothelial adhesion. Moreover, H-2g or the 4A11 antigen activate the endothelial janus kinase 2 (JAK2)/STAT3 pathway, as demonstrated by inhibition by chemical inhibitors of these pathways as well as antisense oligodeoxynucleotides directed against these signaling intermediates. H-2g may also signal via the Erk1/2 and PI3kinase-Akt pathways. Moreover, H-2g induced endothelial NF- κ B activation, and blockade of PI3kinase or JAK2 inhibited H-2g-induced endothelial NF- κ B activation. These results suggest that the H-2g and the 4A11 antigen are mediators of leukocyte-endothelial adhesion via ICAM-1.

We next sought to determine whether H-2g could induce monocyte recruitment. We performed monocyte chemotaxis *in vitro* in modified Boyden chambers. H-2g induced monocyte chemotaxis in the picomolar or greater range. *In vivo*, injection of H-2g intraperitoneally into the peritoneum of mice resulted in monocyte recruitment. The migration of monocytes *in vitro*, as with induction of HMVEC ICAM-1 expression, appeared to occur via the mitogen-activated kinase and PI3kinase pathways, but also via the Src pathway. These results suggest that H-2g and the 4A11 antigen are potent mediators of leukocyte-endothelial adhesion and monocyte recruitment, and that efforts aimed at targeting this antigen or its signaling pathways may be useful in the treatment of RA.

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Galectin-3 and galectin-3 binding protein in rheumatoid arthritis

S Ohshima^{1,2}, S Kuchen², C Seemayer², FT Liu³, M Neidhart², RE Gay², S Gay²

¹Department of Molecular Medicine, Osaka University Graduate School of Medicine, Japan; ²Center of Experimental Rheumatology, University Hospital, Zurich, Switzerland; ³Department of Dermatology, University of California, Davis, School of Medicine, USA
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Galectin-3 (Gal-3) is one of the soluble lectins that has key functions in inflammation, chemotaxis, cell adhesion and apoptosis. We examined

the role of Gal-3 and Gal-3 binding protein (Gal-3BP) in rheumatoid arthritis (RA). Localization of Gal-3 and Gal-3BP in rheumatoid synovium was examined by immunohistochemistry and *in situ* hybridisation. Gal-3 and Gal-3BP concentrations in plasma and synovial fluid were measured by ELISA. Gal-3 protein and mRNA stained throughout the synovial membrane, and Gal-3BP expressed at the site of erosion in RA. Lower and scattered expression of both Gal-3 and Gal-3BP were detected in osteoarthritis synovium and normal synovium. ELISA revealed a marked increase of Gal-3 and Gal-3BP in synovial fluid of patients with RA compared with osteoarthritis patients and normal controls. Furthermore, the level of Gal-3 in serum correlated with the level of C-reactive protein in RA. *In vitro*, Gal-3 can induce the production of matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 in synovial fibroblasts in a dose-dependent manner. Our data indicate that Gal-3 and Gal-3BP represent novel markers of disease activity in RA. Moreover, Gal-3 might contribute to the destruction of bone/cartilage in RA by the induction of MMPs.

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Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis

D McGonagle, E Jones, A English, P Emery

University of Leeds, Academic Unit of Musculoskeletal Disease, Department of Rheumatology, Leeds, UK

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Objectives Synovial fluid (SF) contains rare clonogenic fibroblastic cells, but it is unknown whether these are true mesenchymal progenitor cells (MPCs) capable of trilineage differentiation or whether they participate in the pathogenesis of rheumatoid arthritis (RA). The present work evaluated SF for the presence of MPCs, to characterize these cells in relationship to bone marrow (BM) MPCs and to enumerate them in early and established RA, other inflammatory arthropathies and osteoarthritis (OA).

Methods One hundred SF samples were evaluated (53 RA samples, 20 OA samples, 27 other samples). To investigate multipotentiality, cultures of SF-derived fibroblasts were subjected to chondrogenic, osteogenic and adipogenic differentiation assays, with BM-derived and skin-derived cultures used as positive and negative controls of differentiation. Trilineage differentiation of the individual clonogenic fibroblasts present was determined by direct measurements of produced Ca²⁺, sulphated glycosaminoglycan and lipid. Having established the MPC nature of individual SF clonogenic fibroblasts, fibroblast-colony forming unit assays were then used for enumeration of SF MPCs in early and late RA, OA and other arthropathies. The immunophenotype of SF MPCs before and after expansion was determined by multiparameter flow cytometry.

Results Regardless of the nature of arthritis, polyclonal cultures of SF-derived fibroblastic cells possessed trilineage mesenchymal differentiation potentials. Individual clonogenic SF fibroblasts were also tripotential, confirming the MPC nature of SF cells. The number of MPCs in 1 ml SF was significantly higher in OA (median, 37) compared with RA (median, 2; $P < 0.00001$), corresponding to an average frequency of 2000 and 2 cells per 10⁶ of total cells, respectively. These differences were independent of SF volumes and inflammatory cell influx. No significant differences in MPC numbers were found between early and late RA (median, 3 and 2 cells/ml, respectively). Culture-expanded SF and BM MPCs had a similar phenotype (CD45^{neg}D7-FIB^{pos}CD13^{pos}CD105^{pos}CD55^{pos}CD110^{pos}) but rare SF MPCs expressed low-affinity nerve growth factor receptor prior to expansion, a distinct marker of *in vivo* BM MPCs.

Conclusion This work shows the presence of rare clonogenic tripotential MPCs in the SF, and is the first study to demonstrate that MPCs from two different anatomic sites have the same distinctive phenotype *in vivo*. The relative absence of SF MPCs in early and late RA does not support the concept of significant involvement in disease pathogenesis, but the relative abundance of these in OA provides the first direct evidence for MPC involvement in attempted joint repair in adults.

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IL-15 and its role in rheumatoid arthritis

M Kurowska-Stolarska¹, O Distler¹, W Rudnicka², J Distler¹, RE Gay¹, W Maslinski², S Gay¹

¹Center of Experimental Rheumatology, University Hospital, Zurich, Switzerland; ²Department of Pathophysiology and Immunology, Institute of Rheumatology, Warsaw, Poland
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Background IL-15 is involved in all phases of rheumatoid arthritis. Recently we have shown that rheumatoid arthritis synovial fibroblasts (RASf) express both IL-15 and functional IL-15 receptor [1].

Objective The aim of present study was to identify pathways that are regulated by autocrine IL-15 (IL-15R) in RASf.

Methods RASf were transfected with plasmid encoding IL-15R antagonist (CRB-15, Cardion AG) or control constructs. RNA from transient transfectants were used for Microarray analysis. The differential expression of genes obtained by microarray analysis was verified by SYBR Green real-time PCR. The expression of IL-15R α , cell proliferation and the expression of p16 and p21 were evaluated in stably transfected cells.

Results The IL-15R antagonist produced by transfected RASf blocked the endogenous IL-15/IL-15R α interaction, which resulted in an inhibition of cell proliferation (45 \pm 10%) via an increase of the expression of p16. In addition, we found that inhibition of IL-15R α induced the expression of mRNA for FGFR-3. Since two isoforms of FGFR-3 have been identified (FGFR-3b and FGFR-3c) [2], we tested the effect of IL-15R α inhibition on their expression. In contrast to FGFR-3b, the level of mRNA for FGFR-3c was strongly increased in cells transfected with the IL-15R antagonist (4.71 \pm 2.5 in transient transfectants and 6.1 \pm 1 fold in stable transfectants). FGFR-3c isoform binds specifically FGF-9, but also FGF-2 [2]. Besides FGFR-3, FGF-2 that is abundant in RA joints binds to FGFR-1. *In vitro* studies revealed that FGFR-1 transmits a potent mitogenic signal, whereas FGFR-3 usually has no stimulatory effect or inhibits cell proliferation. In contrast to FGFR-3c, blocking of IL-15R α did not change the mRNA expression for FGFR-1 in RASf. Moreover, we checked whether FGF-2 affects the expression of IL-15R α . Indeed, FGF-2 strongly decreased the spontaneous and tumor necrosis factor alpha-triggered expression of IL-15R α at the mRNA and protein levels.

Conclusion Our findings raise the possibility of a negative loop between FGF-2/FGFR-3c and IL-15/IL-15R signaling in RASf. Moreover, the activation of RASf by FGFs could depend on the ratio of FGFR-1/FGFR-3 expression, which is controlled by the endogenous IL-15/IL-15R system.

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Floating anchorage-independent fibroblast-like cells mediate cartilage destruction independently from the hyperplastic synovial tissue

M Neidhart, CA Seemayer, BA Michel, RE Gay, S Gay

Center of Experimental Rheumatology, Zurich, Switzerland
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We characterized the morphological and immunological features of adherent synovial fluid cells derived from patients with rheumatoid arthritis (RA), and explored their potential function *in vitro* and *in vivo* by focusing on cartilage destruction. Synovial fluid adherent cells were obtained from patients with RA and from controls, and were characterized by immunohistochemistry and flow cytometry. *In vitro*, these cells were cultured in the presence of cartilage particles. Cartilage destruction was monitored by the release of sulphated glycosaminoglycans (sGAG) into the medium, and the level of MMP-1 in the cell culture supernatant was measured by ELISA. To inhibit cartilage destruction *in vitro*, the matrix metalloproteinase (MMP) inhibitor Marimastat was tested in this system. *In vivo*, in the SCID mouse coimplantation model, RA synovial fluid adherent cells and RA synovial fibroblasts (as positive controls) were coimplanted with human cartilage under the kidney capsule, and were maintained there for 60 days. Synovial fluid adherent cells *in vitro* consist of two subpopulations, large round-shaped macrophage-like cells

(CD68⁺) and spindle-shaped fibroblast-like cells (Thy-1⁺). When passaged, the latter cells proliferate and organize themselves in three-dimensional formations. The majority (>90%) of passaged RA synovial fluid adherent cells expressed the Thy-1⁺, CD68⁻, CD86⁻ phenotype. Compared with synovial tissue fibroblasts, synovial fluid adherent cells expressed lower levels of adhesion molecules, including CD54 and galectin-3. Using RA synovial fluid adherent cells, the *in vitro* release of sGAG associated with cell activity was 2.5-fold higher compared with negative controls. The release of sGAG correlated with the concentration of MMP-1 and was inhibited by the broad range MMP inhibitor Marimastat in a dose-dependent manner. RA synovial fluid adherent cells coimplanted with cartilage in SCID mice showed the same invasive behaviour as tissue-derived RA synovial fibroblasts. Taken together, our data showed that RA synovial fluid adherent contains 'floating' anchorage-independent fibroblast-like cells similar to tissue-derived RA synovial fibroblasts, contributing to cartilage destruction independently of the process mediated by the hyperplastic synovial tissue.

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Pathogenetic roles of Synoviolin in synovial hyperplasia of rheumatoid arthritis

S Yamasaki¹, N Yagishita¹, T Amano¹, K Tsuchimochi¹, I Maruyama², K Nishioka³, T Nakajima¹

¹Department of Genome Science, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Department of Dermatology and Laboratory of Molecular Medicine, Kagoshima University, Faculty of Medicine, Kagoshima, Japan; ³Rheumatology, Immunology and Genetics Program, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan
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Background Synovial hyperplasia is a pathological hallmark of rheumatoid arthritis (RA). However, the mechanisms that regulate synovial cell outgrowth are not fully understood. We recently isolated a novel membrane-type molecule named 'Synoviolin'. This molecule was cloned from the cDNA library of RA synovial cells by immunoscreening using antirheumatoid synovial cell antibody. It is thus expected that the molecule plays important roles in synovial hyperplasia of RA. We examine the pathogenetic role of Synoviolin in RA *in vivo* and *in vitro*.

Methods We examined the protein expression of Synoviolin in RA synovia by immunohistochemistry. We also examined the protein expression of the molecule in cultured synovial cells by Western blotting. To examine the effects of this molecule on synovial cell proliferation, the expression of Synoviolin in cultured synovial cells was knocked down using the RNAi technique.

Results Synoviolin is highly expressed in synovia of RA compared with synovia of osteoarthritis. Correspondingly, the protein expressions of Synoviolin in cultured RA synovial cells are higher than that in osteoarthritis synovial cells. The proliferation of synovial cells is significantly inhibited by downregulation of Synoviolin mediated by RNAi.

Conclusion Our results implicate the important roles of Synoviolin in synovial hyperplasia of RA. These findings will provide a novel pathogenetic mechanism in RA, and suggest that further research on Synoviolin will provide new therapeutic strategies for RA.

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Transcriptional regulation of Synoviolin is important for the proliferation of rheumatoid synovial cells

K Tsuchimochi¹, S Yamasaki¹, N Yagishita¹, T Amano¹, S Komiya², I Maruyama², K Nishioka¹, T Nakajima¹

¹Department of Genome Science and Rheumatology, Immunology and Genetics Program, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Department of Orthopedic Surgery and Department of Dermatology and Laboratory of Molecular Medicine, Kagoshima University, Faculty of Medicine, Kagoshima, Japan
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We recently cloned Synoviolin, a transmembrane protein, by immunoscreening from the human cDNA library of rheumatoid synovial cells (RSCs) using anti-RSC antibodies.

Synoviolin was highly expressed in rheumatoid synovial fibroblast cells. The mice overexpressing Synoviolin developed spontaneous arthropathy. Conversely, synoviolin^{+/-} mice were resistant to collagen-induced arthritis.

These results indicate that it is important for the prevention of arthritis to reduce the amount of Synoviolin.

We thus analyzed the transcriptional regulation of Synoviolin promoter and identified that an Ets binding site (EBS) was crucial for the transcription of Synoviolin *in vitro* and *in vivo*.

Furthermore, to investigate the effect of downregulation of Synoviolin in RSCs, we transfected the EBS decoy oligodeoxynucleotide (ODN) or Synoviolin antisense ODN into RSCs and carried out a proliferation assay using Alamar blue reagent, which resulted in the repression of proliferation activity through the suppression of Synoviolin expression. Our results suggest that the EBS decoy ODN provides a new therapeutic approach for the treatment of arthritis.

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The importance of Synoviolin in embryogenesis

N Yagishita¹, T Amano¹, S Yamasaki¹, T Tsuchimochi¹, K Nishioka², I Maruyama³, A Fukamizu⁴, T Nakajima¹

¹Department of Genome Science, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan;

²Rheumatology, Immunology and Genetics Program, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ³Department of Laboratory of Molecular Medicine, Kagoshima University, Faculty of Medicine, Kagoshima, Japan;

⁴Institute of Applied Biochemistry and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Japan

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Rheumatoid arthritis (RA) is one of the most critical articular diseases with synovial hyperplasia followed by impairment of quality of life. However, the mechanism(s) that regulates synovial cell outgrowth is not fully understood.

To clarify its mechanism(s), we carried out immunoscreening using antirheumatoid synovial cell antibody, and cloned 'Synoviolin'. Synoviolin was highly expressed in the rheumatoid synovium, and mice overexpressing this molecule developed spontaneous arthropathy. Thus, it was demonstrated that Synoviolin is a novel pathogenic factor for arthropathy.

In this study, to understand the physiological role of Synoviolin *in vivo*, we generated *synoviolin*-deficient (*syno*^{-/-}) mice by gene-targeted disruption. Surprisingly, all fetuses lacking *synoviolin* died *in utero* by embryonic day 12.5–13.5. Histologically, *syno*^{-/-} embryos showed generalized low cellular density and aberrant apoptosis. In the hematopoietic system, apoptosis, nuclear fragmentation of erythroblasts, Howell–Jolly body formation and hemophagocytosis were observed. These results indicate that lack of Synoviolin is associated with abnormal erythroid differentiation caused by augmentation of apoptosis, and it was probable that *syno*^{-/-} embryos died of anemia as a result of a reduced number of circulating erythroblasts.

In conclusion, Synoviolin has an anti-apoptotic effect, and is essential for embryogenesis. For further study, clearing the molecular mechanism of Synoviolin in apoptosis could lead to cure of RA.

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Synoviolin as a novel pathogenic factor for arthropathy

T Amano¹, S Yamasaki¹, N Yagishita¹, K Tsuchimochi¹, A Fukamizu², I Maruyama³, K Nishioka¹, T Nakajima¹

¹Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Institute of Applied Biochemistry and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Ibaraki, Japan;

³Department of Dermatology and Laboratory of Molecular Medicine, Kagoshima University, Faculty of Medicine, Kagoshima, Japan

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Rheumatoid arthritis (RA) is one of the most critical articular diseases with synovial hyperplasia followed by impairment of quality of life.

However, the mechanism(s) that regulate synovial cell outgrowth is not fully understood. To clarify its mechanism(s), we carried out immunoscreening using antirheumatoid synovial cell antibody, and identified and cloned Synoviolin. Synoviolin was highly expressed in the rheumatoid synovium, and mice overexpressing this protein developed spontaneous arthropathy. Conversely, *synoviolin*^{+/-} mice were resistant to collagen-induced arthritis by enhanced apoptosis of synovial cells. We conclude that Synoviolin is a novel causative factor for arthropathy by triggering synovial cell outgrowth through its anti-apoptotic effects. Our findings provide a new pathogenetic model of RA, and suggest that Synoviolin could be targeted as a therapeutic strategy for RA.

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Laminin, but not lipopolysaccharide, IL-1 or tumor necrosis factor alpha, induces IL-16 gene activity in synovial fibroblasts

W Aicher¹, D Alexander¹, T Pap², S Gay³

¹Basic Science Research Laboratory, Department of Orthopaedic Surgery, UKT University Tuebingen, Germany; ²Medical School, University of Magdeburg, Germany; ³WHO Rheumatology Centre, University Hospital Zurich, Switzerland

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In rheumatoid arthritis (RA), synovial fibroblast-like cells (SF) contribute significantly to articular inflammation. They express elevated levels of cytokines and chemoattractant factors, including IL-16. We analyzed the induction pathways for IL-16 mRNA expression in synovial fibroblasts from eight RA patients in comparison with SF from six osteoarthritis (OA) patients and dermal fibroblasts (DF) (*n*=6) by real-time quantitative RT-PCR. Stimulation of cAMP-dependent signal transduction by forskolin induced a twofold and 2.5-fold enhancement of IL-16 RT-PCR signals in DF and OA-SF, respectively, whereas the kinase inhibitor staurosporine induced IL-16 transcripts to a lesser extent in DF and OA-SF. In contrast, in RA-SF, staurosporine significantly augmented IL-16 RT-PCR signals (2.7-fold, *P*<0.022), but forskolin failed to do so (1.3-fold, *P*<0.15). Phorbol ester induced IL-16 mRNA only in RA-SF (1.5-fold) but not in OA-SF, and reduced IL-16 signals in DF (0.6-fold). Most interestingly, growth of cells on laminin significantly induced the expression of IL-16 mRNA (1.88-fold, *P*<0.001). Collagen and other matrix proteins had no such effects. Induction of IL-16 mRNA by laminin was more pronounced in RA-SF than in OA-SF. Addition of lipopolysaccharide (LPS) to SF induced different cytokines including IL-1a, IL-1b, IL-6 and IL-8, but not IL-16, indicating that induction of IL-16 mRNA by laminin was not a LPS artifact. Addition of IL-1b or tumor necrosis factor alpha did not upregulate IL-16 mRNA, indicating that the NF-κB and p38 pathways are not the main activators of the IL-16 gene. We conclude that OA-SF and RA-SF differ in their IL-16 mRNA responses, in that OA-SF respond primarily to adenylate cyclase-dependent pathways while RA-SF respond prominently to protein kinase pathways. Laminin activates the IL-16 gene in synovial fibroblasts but this activation is not dependent on NF-κB or p38 signal transduction.

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Triptolide, an active compound identified in a traditional Chinese herb, induces apoptosis of rheumatoid synovial fibroblasts

N Kusunoki¹, R Yamazaki², S Kawai¹

¹Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Yakult Central Institute for Microbiological Research, Tokyo, Japan

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Introduction Extracts of *Tripterygium wilfordii* Hook F (TWHF), a traditional Chinese herb, have been reported to show efficacy in patients with rheumatoid arthritis (RA). It was also reported that TWHF extracts suppress inflammation in animal models of RA, and inhibit the production of proinflammatory cytokines by several kinds of cells *in vitro*.

Objectives Since RA is not only characterized by inflammation, but also by synovial proliferation in the joints, we examined whether triptolide (a constituent of TWHF) could influence the proliferation of rheumatoid synovial fibroblasts (RSF) by induction of apoptosis.

Methods RSF were obtained from RA patients during surgery and were treated with triptolide or other disease-modifying antirheumatic drugs under various conditions. The viability and proliferation of RSF were measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate assay and by 5-bromo-2'-deoxyuridine incorporation, respectively. Apoptosis was identified by detection of DNA fragmentation using an ELISA and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. The role of caspases in apoptosis of RSF was analyzed by measuring caspase-3 activity, and we also studied DNA fragmentation with or without caspase inhibitors. Activation of the peroxisome proliferator-activated receptor gamma (PPAR γ) was assessed by a luciferase reporter gene assay using RSF transfected with a plasmid containing the peroxisome proliferator response element.

Results Triptolide decreased viability, inhibited proliferation, and induced apoptosis of RSF in a concentration-dependent manner at very low (nanomolar) concentrations, whereas bucillamine, D-penicillamine and methotrexate did not affect cell proliferation at all. Although PPAR α activation was induced by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, triptolide did not induce it under the same experimental conditions. Caspase-3 activity was increased by treatment with triptolide and was suppressed by a pan-caspase inhibitor. Triptolide-induced DNA fragmentation was inhibited by inhibitors of caspase-3, caspase-8 and caspase-9.

Conclusions Although the mechanism of action remains to be studied, triptolide may possibly have a disease-modifying effect in patients with RA.

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The fibrinolytic pattern of rheumatoid synoviocytes is invasive like neoplastic cells

M Mattedi-Cerinic, S Guiducci, A Del Rosso, M Cinelli, G Fibbi, M Del Rosso

Department of Medicine, Section of Rheumatology, University of Florence, Italy

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Background In rheumatoid arthritis (RA), the synovial membrane proliferates and invades the underlying tissues. The fibrinolytic cascade is involved both in the genesis and maintenance of synovial inflammation, and is pivotal in cell invasion and proliferation.

Aim To evaluate the fibrinolytic pattern and the proliferative potential of urokinase-plasminogen activator (u-PA) on RA synoviocytes (SY), in order to establish the role of each component of the fibrinolytic cascade in the genesis and progression of the RA synovitis.

Materials and methods The levels of plasminogen activators and inhibitors, and the u-PA-dependent proliferation were studied *in vitro* on SY from four controls and four RA patients undergoing joint surgery. SY monolayers were used within the seventh passage in culture: u-PA and plasminogen activator inhibitor-1 (PAI-1) were assayed on supernatants, and urokinase-plasminogen activator receptor (u-PA-R) was determined on cell lysates. Levels of u-PA, u-PA-R and PAI-1 were assayed by specific ELISA and by RT-PCR of mRNAs. To evaluate the SY proliferative potential, cells were seeded on to multiwell plates with RPMI 1640 supplemented with 10% FCS and were incubated for 48 hours. The FCS concentration was then reduced to 0.1% for an additional 48 hours. SY were then treated with 500 ng/ml u-PA and/or antibodies to anti u-PA (5B4) and anti-u-PA-R (R3) for 48 hours, and were counted. Cell invasion was measured with the Boyden chamber.

Results RA SY showed significantly higher levels of PAI-1 (6.3 μ g/million cells \pm 1.1 standard deviation [SD] versus 2.9 μ g/million cells \pm 0.7 SD for controls, $P=0.01$), lower levels of u-PA (2.6 ng/million cells \pm 1.4 SD versus 10.9 ng/million cells \pm 2.1 SD for controls, $P=0.01$), and higher u-PA-R on their surface (28.5 ng/million cells \pm 4.8 SD versus 13 ng/million cells \pm 3.0 SD for controls, $P<0.05$). Treatment

of RA SY with u-PA provided a proliferative effect similar to 10% FCS ($P<0.05$), blocked by 5B4 and R3, and different in RA patients with respect to controls ($P<0.05$). RA SY are more prone than controls to spontaneous and u-PA-challenged invasion and proliferation, which are counteracted by antagonists of the fibrinolytic system.

Conclusions RA SY present the typical fibrinolytic pattern of the invasive tumor-like cells. RA SY proliferation is stimulated by u-PA. The fibrinolytic system thus provides the extracellular proteolysis required for the synovial invasion of articular tissues and for the first steps of synovitis. Antagonists of the fibrinolytic system may revert growth and invasion of RA SY. The components of this system may be a future target for new RA therapies.

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The synovial cadherin (cadherin-11) mediates tissue formation and promotes invasion of fibroblast-like synoviocytes

H Kiener, J Higgins, X Valencia, M Brenner

Department of Medicine, Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

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Cadherins are integral membrane homophilic adhesion proteins that mediate adhesion between cells of the same type within a tissue. Cadherins mediate tissue morphogenesis during development and maintenance of tissue architecture in adults. We recently found that cadherin-11 is expressed in human fibroblast-like synoviocytes. We demonstrate that the expression of the synovial cadherin promotes cell invasion and confers on cells the ability to become organized into synovial lining-like architecture *in vitro*.

We generated cadherin-11-expressing fibroblast L-cell clones and empty vector transfected L-cell controls. Phase-contrast microscopy analysis demonstrated that cadherin-11-transfected L-cells form extensive and intimate contacts along their surfaces, and condense together at higher cell density to form a continuous sheet of cells *in vitro*. In contrast, L-cells transfected with control empty vector are loosely organized and do not form a tissue-like structure. We have established an *in vitro* model for synovial lining formation. Interestingly, at the edge of fibronectin-coated culture areas, the cadherin-11-expressing L-cells piled up and formed a continuous lining layer-like architecture after 2 days in culture, whereas the control L-cells failed to form this lining morphology.

Matrigel invasion assays revealed a twofold increased invasive capacity of the cadherin-11-transfected L-cells when compared with L-cells transfected with control vector. To determine the molecular mechanisms of cadherin-11-dependent cell invasion, we generated cadherin-11 constructs of the cytoplasmic tail that delete domains implicated in binding critical intracellular molecules that control cadherin function. Strikingly, deletion of the juxtamembrane domain, known to bind p120-catenin, profoundly altered the invasive capacity of these cells.

These *in vitro* studies suggest a role for cadherin-11 in tissue and lining layer formation in the synovium and the invasive nature of fibroblast-like synoviocytes in chronic synovitis and in rheumatoid arthritis.

Poster Discussion (9) SLE, Vasculitis Group

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Antiphospholipid antibodies harness innate effectors, neutrophils and an alternative pathway of complement, to induce fetal damage

J Salmon¹, G Girardi¹, J Berman¹, J Thurman², VM Holers²

¹Department of Medicine, Hospital for Special Surgery-Weill Medical College of Cornell University, New York, USA; ²Departments of Medicine and Immunology, University of Colorado Health Sciences Center, Denver, Colorado, USA

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In a murine model of antiphospholipid antibody (aPL)-induced pregnancy loss, we have demonstrated that activation of complement, specifically C5a-C5a receptor interactions, leads to fetal loss and growth restriction. Yet, the effectors of tissue injury, the role of individ-

ual complement activation pathways and the precise targets for treatment remain unknown. Because C5a recruits and activates polymorphonuclear leukocytes (PMN) and because PMN infiltration is evident at sites of fetal resorption, we considered the possibility that PMN are critical cellular effectors of fetal damage. To assess the effects of aPL antibodies in mice depleted of granulocytes, we treated pregnant mice with anti-mouse Ly-6G (α -Gr) or control murine IgG2K prior to administration of human IgG containing aPL (aPL-IgG). In the absence of granulocytes, mice did not develop aPL-induced pregnancy complications. The frequency of fetal resorptions was similar to that of mice treated with IgG from healthy controls (NH-IgG), while treatment with control IgG2bK was not protective (% fetal resorption, aPL-IgG + α -Gr, $8.5 \pm 1.5\%$; NH-IgG, 10 ± 2 ; aPL + IgG2bK, $39 \pm 5\%*$; fetal weight (mg), aPL + α -Gr, 352 ± 48 ; NH-IgG, 370 ± 56 ; aPL + IgG2bK, $187 \pm 28*$; * aPL vs NH-IgG, $P < 0.01$).

Immunohistochemical analysis of decidual tissues showed that, in the absence of neutrophil infiltration, either as a consequence of neutrophil depletion or due to blockade of the C5a–C5aR interaction, there was limited C3 deposition coincident with improved pregnancy outcomes. It has been suggested that neutrophils promote complement activation by secreting C3 and/or properdin at sites of inflammation, and thereby amplify complement activation via the alternative pathway. To examine the contribution of the alternative pathway to aPL-induced pregnancy loss, we performed studies in mice deficient in factor B (fB). In the absence of fB, mice were protected from aPL-induced fetal resorption and growth restriction (% fetal resorption fB^{-/-} mice, aPL-IgG vs NH-IgG, 9.5 ± 2.4 vs 10.9 ± 2.1), indicating a role for the alternative pathway in fetal damage. This report, taken together with our previous work, shows that fB, C5 and C5aR are required for pregnancy complications triggered by aPL antibodies. Specifically, we identified the proinflammatory sequelae of C5a–C5aR interactions and the recruitment of neutrophils as the critical intermediates linking pathogenic aPL antibodies to fetal damage. Our findings identify the key innate immune effectors engaged by aPL antibodies that mediate poor pregnancy outcomes and provide novel and important targets for the prevention of pregnancy loss in antiphospholipid syndrome.

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The systemic lupus erythematosus V λ J λ repertoire harbors characteristics of the natural antibody repertoire

J Lee¹, PE Lipsky²

¹Department of Internal Medicine, Ewha Womans University College of Medicine, Seoul, Korea; ²NIAMS, National Institutes of Health, Bethesda, Maryland, USA

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The molecular mechanisms that lead to autoantibody production in systemic lupus erythematosus (SLE) are poorly understood. There is evidence that pathogenic IgG autoantibodies characteristic of human SLE may arise from the physiologically autoreactive natural antibody repertoire of fetal or CD5⁺ B cells. To address this hypothesis, the SLE V λ repertoire obtained from B cells of three SLE patients was analyzed and compared in detail with the V λ repertoire obtained from IgM⁺ B cells of three human fetal spleens and IgM⁺CD5⁺/CD5⁻ B cells of two normal adults. V λ J λ rearrangements were amplified from genomic DNA of individual B cells by PCR. The expressed V λ repertoire of SLE patients contained several similarities with the expressed repertoire of the fetal and adult CD5⁺ B cells. The V λ gene, 1G, was a major component of the SLE, fetal and adult CD5⁺ B-cell repertoire, but not in the adult CD5⁻ B-cell population. The restriction of junctional diversity by utilization of homology-directed joining (H-joining) together with the absence of N-regions was a prominent feature of the fetal and adult CD5⁺ B-cell repertoires. This restriction was also observed in the SLE repertoire, but was less significant in the adult CD5⁻ B-cell population. Furthermore, profound expansion of V λ clones employing identical CDR3s were observed in the adult CD5⁺ B cells, the fetal cells, and the SLE repertoire, whereas the frequency of the V λ clones were much lower in the adult CD5⁻ B-cell population. Notably, significant numbers of expanded adult CD5⁺ B cells, and fetus and SLE V λ clones utilized

H-joining at the junctions. These data demonstrate that the SLE V λ J λ repertoire harbors characteristics of the natural antibody repertoire. These observations imply that the fetal antibody repertoire with restricted antigen specificities can be conserved through development via CD5⁺ B cells, and during abnormal immune responses can potentially give rise to SLE-associated pathogenic autoantibodies.

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Enhanced proliferative response of CD4⁺ T cells from patients with systemic lupus erythematosus by T-cell receptor stimulation

S Park¹, I Kang², H Kim¹, J Craft²

¹Division of Rheumatology, Department of Internal Medicine, The Catholic University of Korea, Seoul, Korea; ²Section of Rheumatology, Department of Internal Medicine, Yale University Medical School, New Haven, Connecticut, USA

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Objective To investigate the expression of the chemokine receptor CCR7, which defines distinct subsets of naive and memory T lymphocytes with different homing and effector capacities, and the proliferative capacity of CD4⁺ T cells by T-cell receptor (TCR) stimulation in patients with systemic lupus erythematosus (SLE)

Methods Heparinized venous blood from SLE patients (23–53 years old; mean, 36.7 years) and age-matched and sex-matched healthy controls (HC) was collected. Peripheral blood mononuclear cells (PBMC) were freshly isolated by Ficoll-Hypaque. We stained 106 PBMC with anti-human CCR7 antibody, anti-CD45RA, anti-CD4 and anti-CD8 to characterize the phenotype of T-cell subsets. PBMC were stimulated with anti-CD3+anti-CD28 monoclonal antibody, and cell division was analyzed by carboxyfluorescein diacetate succinimidyl ester labeling and flow cytometry in different subsets of CD4⁺ T cells. Data was acquired on a FACS caliber system, and analyzed using Flow-Jo software (Tree Star Inc., San Carlos, CA, USA).

Results SLE patients ($n=27$) had fewer CD45RA⁺CCR7⁺CD4⁺ naive T cells (32.2 ± 13.2 vs 43.5 ± 12.3 , $P < 0.05$) and higher CD45RA⁺CCR7⁻CD4⁺ effector memory T cells (20.1 ± 12.2 vs 12.9 ± 5.6 , $P < 0.05$) as compared with the HC ($n=27$). No significant differences were found in the CD45RA⁻CCR7⁺ central memory CD4⁺ T cells and the CD8⁺ T-cell compartment of SLE patients as compared with HC. The appearance of cell division was more rapid in the population of CD45RA⁻CCR7⁻CD4⁺ T cells, and the frequency of CD45RA⁻CCR7⁻CD4⁺ effector memory T cells did not correlate with disease activity, disease duration, age, or treatment within the SLE group. Enhanced cell division was observed in SLE CD4⁺ T cells by TCR stimulation as compared with HC, but there are no significant correlations between frequency of effector CD4⁺ T cells and proliferative capacity within the SLE group. Furthermore, naive CD4⁺ T cells from SLE patients showed increased proliferative capacity compared with that of HC, but the difference was not so significant.

Conclusion These results suggest that enhanced proliferative response of CD4⁺ T cells of SLE by TCR stimulation may be caused by increased distribution of the effector memory population and intrinsic defects of SLE CD4⁺ T cells.

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Evidence for an IFN-inducible gene, *Iff202*, in the susceptibility to systemic lupus

S Rozzo¹, D Choubey², T Vyse³, S Izui⁴, J Allard⁵, G Peltz⁵, B Kotzin¹

¹Departments of Medicine and Immunology, The University of Colorado Health Sciences Center, Denver, Colorado, USA;

²Department of Radiation Oncology, Stritch School of Medicine, Loyola University Medical Center, Maywood, Illinois, USA;

³Rheumatology Section, Imperial College School of Medicine, London, UK;

⁴Department of Pathology, Centre Medical Universitaire, University of Geneva, Switzerland; ⁵Roche Bioscience, Palo Alto, California, USA

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The *Nba2* locus is a major genetic contribution to disease susceptibility in the (NZB \times NZW)F1 mouse model of systemic lupus. We generated

C57BL/6 mice congenic for this NZB locus, and these mice produced antinuclear autoantibodies characteristic of lupus. F1 offspring of congenic and NZW mice developed high autoantibody levels and severe lupus nephritis similar to (NZB × NZW)F1 mice. Expression profiling with oligonucleotide microarrays revealed only two differentially expressed genes, IFN-inducible genes *Iff202* and *Iff203*, in congenic mice versus control mice, and both were within the Nba2 interval. Quantitative PCR localized increased *Iff202* expression to splenic B cells and non-T cells/non-B cells. Moreover, recent results show *Iff202* shows inducible expression in NZB mice in response to both type I and type II IFNs, both *in vivo* and *in vitro*. Other experiments using mice with a targeted mutation of the type I IFN receptor show that this pathway is required for type I interferon-mediated responsiveness. Studies using splenic cells demonstrate that multiple subpopulations show enhanced *Iff202* expression in response to type I IFN, and indicate that dendritic cells show the greatest increased expression of *Iff202*. These results together with analyses of promoter region polymorphisms, strain distribution of expression, effects on cell proliferation and apoptosis, in addition to recent results characterizing responsiveness to type I and type II IFNs, implicate *Iff202* as a candidate gene for lupus.

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IgM and C1q function in the same pathway to promote the clearance of apoptotic cells *in vivo*

K Elkon¹, R Kowaleski¹, V Montenegro¹, SJ Kim²

¹Departments of Medicine and Immunology, University of Washington, Seattle, Washington, USA; ²Department of Immunology, Weill Medical College of Cornell University, New York, USA

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Background Dying cells may be the source of the autoantigens that initiate and/or perpetuate systemic autoimmunity. C1q-deficient mice develop a lupus-like disease and accumulate apoptotic cells in their kidneys, and our studies [1] suggested that classical complement components facilitate the clearance of apoptotic cells. Mice deficient in serum IgM (slgM) also develop a lupus-like disease.

Objective Since IgM binds to apoptotic cells and activates the classical complement on the apoptotic cells *in vitro* [2], we asked whether slgM was required for complement activation and rapid removal of dying cells by phagocytic cells *in vivo*.

Methods Apoptotic thymocytes were injected into the peritoneum of mice that had received thioglycollate 3 days previously. Thirty minutes after intraperitoneal injection, apoptotic cell uptake by elicited peritoneal macrophages was determined by light microscopy.

Results The percentages of peritoneal macrophages that ingested apoptotic cells were (mean ± standard error): wild type (WT) (*n*=12), 31.2 ± 4.7%; C1q^{-/-} (*n*=4), 9.4 ± 1.8%; slgM^{-/-} (*n*=16), 8.5 ± 0.9%. The differences between C1q-deficient and WT mice as well as between slgM deficient and WT mice were highly significant (*P*=0.0036 and *P*=0.0003, respectively). Mice heterozygous for slgM showed an intermediate result (17.9 ± 1.8%). To determine whether the clearance defect in the slgM-deficient mice could solely be attributed to IgM deficiency, apoptotic thymocytes were preincubated with purified murine IgM prior to intraperitoneal injection into slgM-deficient mice. IgM completely restored the ability of slgM-deficient mice to ingest apoptotic cells. Finally, to prove that IgM and C1q function in the same pathway to facilitate clearance of apoptotic cells, we created C1q/IgM double knockout mice. In contrast to the IgM single knockout mice, addition of IgM to the double knockout mice failed to restore phagocytosis of apoptotic cells.

Conclusions These findings indicate that IgM plays a pivotal role in clearance of apoptotic cells, and that this occurs through activation of the classical pathway of complement. IgM upstream of complement-mediated opsonization of dying cells provides a unifying mechanism explaining why mice with either early complement component or slgM deficiency develop lupus-like diseases. These findings have important implications for understanding the pathogenesis of lupus in humans.

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New aspects of the etiopathogenesis of systemic lupus erythematosus

US Gaipf, TD Beyer, RE Voll, S Franz, JR Kalden, M Herrmann

Institute for Clinical Immunology and Rheumatology, Friedrich-Alexander-University of Erlangen-Nuremberg, Erlangen, Germany
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Introduction Impaired clearance of apoptotic cells has been supposed to be involved in the etiopathogenesis of systemic lupus erythematosus (SLE). Furthermore, antibodies against retroviral proteins can frequently be detected in sera of SLE patients without overt retroviral infections. Decreased levels of serum DNase I activities as well as deficiencies in components of the classical complement pathway are well established to predispose to SLE.

Objectives We analysed the role of serum factors that could be responsible for the degradation and clearance of human chromatin, and followed up the processing of the HERV-K-10gag polyprotein during apoptosis of Tera-1 cells. Furthermore, we investigated the clearance of apoptotic cells in the germinal centers of patients with SLE and controls.

Methods The chromatin degradation and uptake was monitored by measuring the residual DNA content by flow cytometry. The HERV-K-10gag polyprotein was used to investigate the processing of viral proteins during apoptosis. Lymph node biopsies obtained from SLE patients and non-SLE patients with benign follicular hyperplasia were stained with monoclonal antibodies against macrophages (CD68) and follicular dendritic cells (CR2/CD21). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed to detect apoptotic nuclei.

Results Whereas an excess of recombinant DNase I degraded human necrotic cell-derived chromatin in the absence of C1q, an efficient uptake of the predigested material by monocyte-derived phagocytes required the presence of C1q. During apoptotic cell death, Tera-1 cells showed an altered HERV-K-10gag processing compared with viable cells. In addition, granzyme B was able to cleave HERV-K-10gag isolated from viable Tera-1 cells. Regarding the fate of dying cells in germinal centers of SLE patients, we found that the numbers of tingible body macrophages were significantly reduced in a subgroup of patients with SLE. TUNEL-positive apoptotic material was observed to be associated with the surfaces of follicular dendritic cells.

Conclusions We conclude that C1q or DNase I deficiencies may precipitate human autoimmunity. Furthermore, the immunogenicity of retroviral antigens in SLE patients may result from a similar mechanism as described for nuclear autoantigens. In general, nuclear autoantigens bound to follicular dendritic cells may provide a survival signal for autoreactive B cells, thereby over-riding an important initial control mechanism of B-cell tolerance.

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C-reactive protein is an important immunoregulatory molecule that decreases systemic inflammatory response and ameliorates autoimmune disease

T Du Clos

Department of Internal Medicine, The University of New Mexico, Albuquerque, New Mexico, USA

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C-reactive protein (CRP) is an ancient member of the innate immune system. CRP is also a member of the pentraxin family of proteins and is a strong acute phase reactant in man. CRP has been shown to bind to phagocytes through interaction with Fcγ receptors, to affect the production of cytokines, and to enhance phagocytosis. CRP has been shown

to protect mice from infection, to bind to nuclear antigens and to delay the onset of systemic lupus erythematosus (SLE) in autoimmune mice. The mechanism for these diverse effects has remained obscure. Both CRP and serum amyloid P (SAP) have been shown to bind to nuclear antigens that are targets of autoantibodies in patients with SLE. Additional studies have shown that CRP and SAP can mediate the uptake and clearance of apoptotic cells. These studies suggested that the pentraxins might prevent exposure and reactivity of the immune system to self-antigens. Our studies have shown that CRP and SAP can delay the onset of autoimmune disease accelerated by the injection of chromatin into autoimmune mice. More recently, it has been demonstrated that expression of human CRP as a transgene on autoimmune disease can delay the onset of autoimmune disease. We report now that a single injection of CRP can delay the onset of proteinuria in NZB×NZW female mice. In all three cases, CRP failed to substantially decrease anti-DNA autoantibodies yet protected mice from glomerular injury and death. We suggest that CRP delays the onset of autoimmune disease by modulating the effect of immune complexes on inflammatory cells. As we have shown recently, the anti-inflammatory effect of CRP in the lipopolysaccharide challenge model requires Fcγ receptor expression. It is proposed that CRP mediates protection from nephritis in SLE by the induction of anti-inflammatory cytokines and altering the reactivity of macrophages to inflammatory stimuli.

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Infliximab for severe ocular inflammation in patients with Behcet's disease

P Sfikakis¹, N Markomichelakis²

¹First Department of Propedeutic and Internal Medicine, Athens University Medical School, Athens, Greece; ²Ocular Inflammation/Immunology Service, General Hospital, Athens, Greece
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A single infusion of the anti-tumour necrosis factor monoclonal antibody Infliximab, given in five patients with Behcet's disease (BD) and sight-threatening panuveitis relapse, resulted in rapid and effective suppression of ocular inflammation [1]. Until May 2003, a single Infliximab infusion (5 mg/kg) has been given in a total of 24 such patients (bilateral relapse or unilateral relapse in 18 and six patients, respectively). At day 1 post-treatment, a significant improvement of visual acuity, a striking decrease of anterior chamber cells, and a 50% decrease of vitreous haze was evident in all patients but one. Within 2 weeks, acute ocular inflammation, including retinal lesions and vasculitis, resolved completely in 26 eyes, and by 80–90% in six eyes. Visual acuity returned to the prerelapse levels (at least) in all.

We are currently looking at the safety and efficacy of continuous Infliximab infusions (at week 0, weeks 3–4, and every 6–8 weeks thereafter, at 5 mg/kg) in patients who do not respond, or in patients with an inadequate response to prednisolone (nine patients), combined either with cyclosporin A (six patients) and/or azathioprin (five patients), or cyclophosphamide (one patient). In the 17 affected eyes, visual acuity has improved by a mean of three lines in the Shellen chart at 6 months compared with at baseline. This effect has been sustained during further follow-up (6 months–2 years). Concomitant immunosuppressive therapy has been significantly tapered in all. No patient has experienced an ocular relapse requiring other than topical treatment, or a major extra-ocular relapse of the disease. No possible side effects, including opportunistic infections or an exacerbation of previous neurological disease (present in three of nine patients), have been noted.

Similar results have been obtained in a parallel study looking at safety and efficacy of continuous Infliximab infusions as monotherapy in previously untreated patients, which is also underway.

Since conventional immunosuppressive treatment BD-associated acute ocular relapse is often unable to rapidly control ocular inflammation, which is critical to avoid development of chronic lesions, we suggest that a single infusion of Infliximab with observation for objective improvement is the treatment of choice in these patients, and that continuous treatment of Infliximab is a safe, effective and immunosuppressive therapy-sparing approach for patients with refractory, relapsing ocular disease.

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Identification of kinectin as a novel Behcet's disease-related autoantigen

Y Lu¹, P Ye¹, X Feng¹, S Chen¹, EM Tan², EKL Chan²

¹Department of Rheumatology, Shanghai Ren Ji Hospital, Shanghai, China; ²Keck Autoimmune Disease Center, the Scripps Research Institute, La Jolla, California, USA

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Background Lines of evidence support that an autoantibody reaction contributes to the immunological abnormalities underlying Behcet's disease (BD), yet no specific antibody has been reported in the disease. In our previous study, common immune reactivities against unknown cellular proteins were uncovered in 23.1% patient sera from that of 39 Chinese BD patients as determined by immunoblotting.

Objectives This project set out to identify target antigen(s) in BD in view of the fact that it may provide new insights into the immunopathogenesis of the disease, and may have serodiagnostic usefulness.

Methods Immunoscreening of a ZAP cDNA library followed by transcription/translation *in vitro* of candidate clones in a reticulocyte lysate system were carried out. The antigenicity of clone product was analyzed by immunoprecipitation using sera from all 39 BD patients in parallel with normal controls and control sera from Lupus and Sjögren syndrome patients. Three truncated gene products spanning the full length of the target protein were expressed in *Escherichia coli*, and their respective antigenicities in BD were preliminarily analyzed using an ELISA approach. A full-length gene cloned in the pEGFP vector was transfected into HEp-2 cells as an overexpression antigen substrate for immune fluorescent study.

Results Six independent candidate clones were isolated from a gene library, being identified as overlapping human kinectin cDNA clones. The antigenic identity of the partial gene product from the largest clone of the six was preliminarily verified since nine out of 39 (23.1%) BD patients' sera could immunoprecipitate the translation product, whereas sera from controls showed no reactivity. The antigenicity of kinectin was found to mainly reside in the middle and carboxyl portion of the intact protein, and the overexpression GFP-kinectin showed distinct perinuclear cytoplasmic staining and can be recognized by BD patient sera but not normal sera under a confocal fluorescent microscope.

Conclusions BD patient sera contain autoantibodies to cellular proteins, and one of the related autoantigens was kinectin, as unraveled by gene library screening, the antigenicity of which mainly resides in the middle and carboxyl portion of the intact protein. Further in-depth work is needed to clarify the significance of kinectin in the disease entity.

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Expanded circulating transitional B cells in a patient with systemic lupus erythematosus

R Ettinger¹, GP Sims¹, E Tackey², C Yarboro², G Illei², PE Lipsky^{1,2}

¹National Institutes of Health, Autoimmunity Branch, Bethesda, Maryland, USA; ²National Institutes of Health, Office of Clinical Director, Bethesda, Maryland, USA

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B-cell development begins in the bone marrow and results in the generation of immature transitional B cells that leave the bone marrow and enter the spleen, where they complete their maturation into mature B cells. Transitional B cells undergo intense selection, resulting in the survival and entry of only a small fraction into the mature B-cell pool. The possibility that abnormalities in transitional B-cell biology might contribute to the development of autoimmunity in systemic lupus erythematosus (SLE) stimulated an examination of the phenotype and immunoglobulin repertoire of transitional B cells in patients with SLE. Initially, we sought to identify human transitional B cells phenotypically, as this had not previously been done in humans. This was facilitated by the identification of an unusual patient with SLE and transient hypogammaglobulinemia. This patient had a very large population of IgD⁺CD27⁺

naive B cells, which included an expanded population of what we identified as transitional B cells. These were comparable in phenotype with transitional B cells that we found in normal human bone marrow. These were small, CD19⁺ CD20^{hi} IgD^{lo} IgM^{hi} CD21⁻ CD23⁻ CD10⁺ CD24^{hi} CD38^{hi} CD44^{lo} cells. This population was expanded in this SLE patient and also in children compared with normal adults. Immunoglobulin heavy chain genes were completely unmutated in this population and the repertoire differed significantly from that found in normal adult mature B cells. These results suggest that abnormalities in transitional B-cell biology may contribute to the development of autoantibodies in SLE.

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Immunological abnormality and regulation in patients with Sjögren's syndrome

T Sumida

Department of Internal Medicine, University of Tsukuba, Tsukuba City, Japan

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The majority of infiltrating cells into labial salivary glands and into lacrimal glands in patients with Sjögren's syndrome (SS) is CD4⁺TCRab T. Analyses of the T-cell receptor on T cells in both glands support the notion that infiltrating T cells are induced by antigen-driven stimulation. Thus, the identification of autoantigens recognized by T cells in inflamed lesions is important to clarify the pathogenesis of SS. T-cell epitopes on autoantigens in labial salivary glands have been examined using several strategies such as T-cell lines, PCR-single-strand conformational polymorphism, and West-Western methods. Our results showed the following four autoantigens; Ro/SS-A, heat shock protein, α -amylase, and muscarine receptor 3. Especially, the common T-cell epitope on α -amylase in HLA-DR B1*0405-positive SS patients was NPFRPWW-ERYQWPV (amino acids 68–80). The analog peptide of α -amylase might regulate autoimmunity in an antigen-specific fashion. Furthermore, TCRAV24⁺BV11⁺ double-negative natural killer T (NKT) cells are thought to be regulatory T cells. In patients with SS, these NKT cells are significantly decreased in peripheral blood, inducing the autoimmune response. *In vitro* stimulation by α -galactosylceramide, which is one of the antigens for NKT cells, was able to enrich NKT cells more than 10–100 times. These findings suggest that the upregulation of NKT cells by α -galactosylceramide might be a new therapeutic strategy in patients with SS.

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Humoral, hormonal and genetic determinants of cholinergic dysfunction in Sjögren's syndrome

M Rischmueller

Department of Rheumatology, The Queen Elizabeth Hospital, Adelaide, South Australia, Australia

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Functional anti-muscarinic receptor autoantibodies have been demonstrated in Sjögren's syndrome (SS) in a mouse bladder contraction assay. Most patients with these antibodies complained of severe lower urinary tract disturbances, not usually recognised as a feature of SS. In a cross-sectional study, we evaluated urological, sleep and fatigue symptoms in female SS patients ($n=76$) compared with osteoarthritis (OA) controls ($n=43$), utilising the American Urological Symptom Index (AUA-7), the Epworth Sleepiness Scale (ESS) and the Facit-F fatigue instruments. Patients were comparable with respect to hormone-replacement therapy use, bladder operations, urinary tract infections, parity and diuretic therapy; OA patients were slightly older. Sixty-one percent of SS patients reported severe urological symptoms compared with 40% of OA patients ($P=0.04$). This difference was attributable to urge incontinence and not nocturia. Daytime somnolence was more severe in SS ($P=0.02$), independent of nocturia. A trend towards increased fatigue was observed in SS that did not reach statistical significance ($P=0.15$). These results suggest that urological symptoms may be an under-recognised feature of SS, and that fatigue in SS may in some cases be secondary to an underlying sleep disorder such as obstructive sleep apnea due to dry airways, or circadian rhythm disorder. These symptoms are consistent with functional distur-

bances of muscarinic receptors, possibly mediated by muscarinic receptor autoantibodies.

Many features of SS overlap with menopausal symptoms. The T allele of the IVS1-0401 (T/C) polymorphism in the estrogen receptor alpha gene (ER- α) is thought to be associated with decreased responsiveness to estrogen. IVS1-0401 (T/C) genotyping was performed by PCR-restriction fragment length polymorphism using the restriction enzyme PvuII. No difference was seen in genotype frequencies between SS patients ($n=154$) and controls ($n=163$, $P=0.26$). Within female SS patients who completed the AUA-7 and ESS ($n=73$), the ER- α IVS1-0401 T allele was associated, in a dose-dependent manner, with increasing daytime somnolence ($P=0.003$) and urological ($P=0.08$) symptom severity. This polymorphism appears to influence the severity of commonly reported symptoms in SS that are also linked with estrogen deficiency. We predict that it will be a risk factor for severity of menopausal symptoms and sleep disturbances in non-SS patients.

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Apoptosis, loss of B-cell tolerance and lymphoid neogenesis in Sjögren's syndrome

R Jonsson

Broegelmann Research Laboratory, The Gade Institute, University of Bergen, Norway

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Background Sjögren's syndrome is a chronic autoimmune and rheumatic disorder of the mucous membranes caused by lack of proper exocrine secretions, with prominent sicca complaints. The molecular mechanisms of the pathogenesis are virtually unknown.

Objectives To investigate functional properties of lymphoid aggregates and its surroundings observed in salivary glands of patients with Sjögren's syndrome and in *in vitro* systems combined with serological analysis of blood and saliva.

Methods Biopsy material from 178 inflamed minor salivary gland biopsies formed the basis for studies of lymphoid neogenesis. Cell surface markers, proliferation markers, adhesion molecules, chemokines, B-cell activating factor (BAFF) and local production of autoantibodies were investigated by immunohistochemistry, ELISPOT, ELISA and apoptosis determination.

Results About every sixth biopsy contained lymphoid aggregations with germinal center-like morphology. Elevation of autoantibody production was observed related to inflamed salivary glands. Germinal center-positive patients had high expression of the lymphocyte-homing and retention chemokines and adhesion molecules. Induced apoptosis disclosed subcellular redistribution and cell surface exposure of autoantigens of high relevance for Sjögren's syndrome. Attenuated apoptosis was detected among BAFF⁺ B cells.

Conclusions Ectopic secondary lymphoid follicles in Sjögren's syndrome contain all elements of relevance for driving an autoimmune response. BAFF seems to direct the lifespan of infiltrating B cells by enhancing their proliferation and maturation.

Altogether, the studies have shed light on factors involved in directing lymphocytes into inflamed tissue and maintaining inflammation in Sjögren's syndrome.

Poster Discussion (10) OA & Other Rheumatic Diseases Group

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Hepatocyte growth factor in osteoarthritis: when bone and cartilage decide to have a chat

P Reboul¹, M Guévremont¹, J Martel-Pelletier¹, F Massicotte¹, P Ranger², JP Pelletier¹, D Lajeunesse¹

¹Department of Medicine, University of Montreal, Quebec, Canada;

²Hôpital Sacré-Coeur, Montreal, Quebec, Canada

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Recently, hepatocyte growth factor (HGF) has been identified by immunohistochemistry in cartilage and, more particularly, in the deep

zone of human osteoarthritic (OA) cartilage. By investigating HGF expression in cartilage, we found that chondrocytes did not express HGF; however, they express the two truncated isoforms, namely HGF/NK1 and HGF/NK2. As the only other cells localized near the deep zone are osteoblasts from the subchondral bone plate, we hypothesized that they were expressing HGF. Indeed, we found that HGF was synthesized by osteoblasts from the subchondral bone plate. Moreover, OA osteoblasts produced five times more HGF than normal osteoblasts. Because prostaglandin E_2 and proinflammatory cytokines such as IL-1 and IL-6 were involved in OA progression, we investigated whether these factors impact HGF produced by normal osteoblasts. Prostaglandin E_2 was the only factor able to stimulate HGF synthesis. However, the addition of NS398, a selective inhibitor of cyclooxygenase-2, had no effect on HGF produced by OA osteoblasts. When investigating signaling routes that might be implicated in OA osteoblast-produced HGF, we found that protein kinase A and protein kinase C were involved. In summary, this study raises the hypothesis that the HGF found in articular cartilage is produced by osteoblasts, diffuses into the cartilage and may be implicated in the progression of OA. Furthermore, we investigated joints in HGF transgenic mice. We found that the subchondral bone was remodeled and that cartilage matrix was qualitatively different from the control mice. These results reinforced the idea of a role played by HGF in the joint.

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Potentiality of mesenchymal stem cells with *ex vivo* gene therapy for osteochondral defect in arthritis

H Inoue¹, N Abe¹, J Lieberman²

¹Science of Functional Recovery and Reconstruction, Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; ²Department of Orthopaedic Surgery, UCLA School of Medicine, Los Angeles, California, USA
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Ex vivo gene therapy includes culture-expansion of pluripotent stem cells, genetic manipulations *in vitro* and reimplantation into a recipient. This cell-mediated gene transfer with adenoviral vectors may be safer than *in vivo* techniques since it avoids the inoculation of viral particles into the body. Previous studies have shown that the transduced cells can contribute to bone and cartilage regeneration when osteoprogenitor cells or mesenchymal stem cells were used as the carrier to transfer the BMP-2 protein. However, these studies did not reveal that these infected cells continue to secrete the transduced protein. Also, the fate of these cells remained unknown, whether they could become incorporated into host tissue and differentiated into cartilage and bone. To investigate these parameters, we constructed an adenoviral vector encoding the *myc* epitope-tagged BMP-2 gene (AdBMP-2^{Myc}). Rat bone marrow cells (rBMCs) transduced with AdBMP-2^{Myc} produced biologically active BMP-2 protein, which was confirmed by Western blot analysis and alkaline phosphatase assay. *Ex vivo* studies using rBMCs infected with AdBMP-2^{Myc} and implanted into the hindlimbs of SCID mice demonstrated orthotopic bone formation by 1 week and greater consolidation at the later time periods. Immunohistological analysis revealed that the *Myc*-positive cells differentiated into chondrocytes and became introduced into the bone during the endochondral ossification process. The different distribution of BMP-2 and *Myc*-positive cells suggested that exogenous BMP-2 protein, delivered by infected rBMCs, can induce endogenous BMP-2 protein expression and bone formation from host osteoprogenitor cells. Our study indicated that osteoprogenitor cells can be utilized as the delivery vehicle for therapeutic osteochondral inductive genes, and their transduced cells themselves can develop into terminal differentiated mesenchymal tissues, like cartilage and bone. Therefore, the cell selection is critical when using an *ex vivo* gene therapy strategy, and this adenoviral construct is useful for the evaluation of cell-tracing experiments to determine the distribution of the secreted protein.

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Oxidative stress induces chondrocyte telomere instability and chondrocyte dysfunctions in osteoarthritis

K Yudoh¹, N van Trieu¹, H Matsuno², K Nishioka¹

¹Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan; ²Bioengineering Research Center, Toin-Yokohama University, Yokohama, Japan
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Objective To clarify the implication of oxidative stress in the progression to osteoarthritis (OA) from the point of view of oxygen free radical-induced genomic instability, including telomere instability and resultant replicative senescence and dysfunction in human chondrocytes.

Methods Human chondrocytes and articular cartilage explants were isolated from knee joints in patients undergoing arthroplastic knee surgery for OA. The oxidative damage/antioxidative capacity in OA cartilage was investigated in the donor-matched pairs of the intact and degenerative region that were isolated from same OA cartilage explants. The results were histologically confirmed by immunohistochemistry for nitrotyrosine, which has been considered a marker of oxidative damage. Under treatment with reactive oxygen species or antioxidative agent (ascorbic acid), cellular replicative potential, telomere instability and production of proteoglycan aggrecan glycosaminoglycan (GAG) were assessed in cultured chondrocytes.

Results Lower capacity of antioxidant and stronger staining of nitrotyrosine were observed in the degenerative regions of OA cartilages as compared with those of intact regions from the same cartilage explants. Immunopositivity for nitrotyrosine was associated with the grade of histologic change of OA cartilage, suggesting the correlation of oxidative damage with articular cartilage degeneration. During continuous culture of chondrocytes, the telomere length, replicative capacity and GAG production were decreased by treatment with reactive oxygen species. In contrast, treatment with an antioxidative agent showed a tendency to elongate the telomere length and replicative lifespan in cultured chondrocytes.

Conclusion Our findings clearly showed the presence of oxidative stress that induces telomere genomic instability, replicative senescence and dysfunction of chondrocytes in OA cartilage, suggesting the implication of oxidative stress in the chondrocyte senescence and cartilage aging responsible for the development of OA. New efforts to prevent the development and progression of OA may include the strategies and interventions aimed at reducing oxidative damage in articular cartilage.

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Expression of focal adhesion kinase, Akt/PKB, Elk-1 and p90RSK in rheumatoid arthritis tissues but not at sites of cartilage invasion

CA Seemayer^{1,2}, S Kuchen¹, P Kuenzler¹, V Rihosková¹, J Schedel³, M Neidhart¹, BA Michel¹, RE Gay¹, S Gay¹

¹Centre for Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich, Switzerland; ²Institute of Pathology, University Hospital Basle, Switzerland; ³Department of Internal Medicine I, University of Regensburg, Germany
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Objective To investigate expression of the focal adhesion kinase (FAK), Akt/PKB and the transcription factors Elk-1 and p90RSK in rheumatoid arthritis (RA).

Materials and methods Synovial tissues from eight RA synovia (FAK, Akt/PKB) or six RA synovia (Elk-1, p90RSK), one osteoarthritis (OA) synovia and two normal synovia were investigated by immunohistochemistry with antibodies recognizing the phosphorylated of the respective molecules. In addition, cultured rheumatoid arthritis synovial fibroblasts (RASf) and normal (N) synovial fibroblasts were coimplanted with human cartilage for 60 days. The invasion score was determined on H & E stained sections, and sections with strong cartilage invasion were also stained for the aforementioned molecules by immunohistochemistry utilizing the avidin-biotin detection system.

Results In RA tissues, FAK was expressed in the lining, sublining layer and perivascular layer. Limited perivascular FAK expression was detected in two normal synovia and no staining occurred in one OA tissue. In the lining and sublining, Akt/PKB was present in all investigated RA tissue, but not in the normal synovia and only in a few cells of the OA sample. Cartilage invading RASF neither stained for FAK or for Akt/PKB. The transcription factor Elk-1 was expressed in RA tissues in both the lining and sublining layer. No staining was detected in normal synovium, and only limited Elk-1 expression in the lining layer of one OA tissue. In addition, p90RSK was also expressed in RA tissues in the lining and sublining layer; however, no staining occurred in the normal synovium. In OA tissue, staining was restricted to the lining layer. SCID mice sections with strong cartilage invasion of RASF did not stain positive for Elk-1 or for p90RSK.

Conclusions We suggest that expression of the phosphokinases FAK, Akt/PKB and the transcription factors Elk-1 and p90RSK are associated with inflammation, but not with cartilage invasion in RA. Therefore, we assume that these molecules might not be therapeutic targets to inhibit cartilage destruction in RA.

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T cell–chondrocyte interaction in the pathogenesis of osteoarthritis

H Nakamura, T Kato, K Masuko-Hongo, M Tanaka, A Shibakawa, K Nishioka

Department of Bioregulation, St Marianna University, Kawasaki, Japan
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Objectives Several lines of evidence have recently suggested that some immune responses are involved in the pathogenesis of osteoarthritis (OA). Aside from humoral immunity, T cells seem relevant to the development of OA. Previously, we showed that T cells responded to autologous chondrocytes only in OA, suggesting involvement in some role in the pathogenesis of OA. In this study, we investigated the response of chondrocytes contacting with autologous T cells.

Methods Human chondrocytes are obtained during arthroplasty for OA and fracture. The latter samples served as normals. Enzymatically isolated chondrocytes were cultivated and used within the second passage for the following experiments. Both cells were cultured together with or without separate wells and the production of matrix metalloproteinases (MMPs) and RANTES were measured using ELISA kits. The contact responses were blocked by antibodies for adhesion molecules (LFA-4 and VLA-4).

Results MMP-1, MMP-3, and MMP-13 were expressed only in chondrocytes. The production of all MMPs was enhanced by contact coculture of chondrocytes with autologous T cells, whereas the production was not enhanced by separate coculture. Blockade of adhesion molecules had no influence on these responses. RANTES was expressed both in T cells and chondrocytes without stimulation. RANTES production was enhanced both in contact and in separate conditions only in OA samples, not in normal samples.

Conclusions The augmentation of MMP production required T cell–chondrocyte contact mediated by mechanisms different from the adhesion molecules tested. As chondrocytes are surrounded by plenty of extracellular matrix, its contact with other types of cells is extremely rare; however, the chance of contact will appear with the degradation of cartilage. Thus, T-cell-mediated development of OA is supposed possible.

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Pentosidine in serum and synovial fluid in patients with knee osteoarthritis and its potential role of prediction of osteoarthritis progression

K Pavelka, L Senolt, V Vilim, P Spacek, M Braun, S Forejtova

Institute of Rheumatology, Prague 2, Czech Republic
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Background The role of molecular markers for prediction of osteoarthritis (OA) progression is not yet defined. Pentosidine, one of the well-characterized advanced glycation endproducts, may be a candidate.

Objectives To study the role of pentosidine as a marker of knee OA, as a marker of progression of knee OA, and for correlation of pentosidine in serum and synovial fluid and correlation with other markers.

Methods Pentosidine was estimated by our own HPLC method [1], and cartilage digomeric matrix protein (COMP) by an original sandwich ELISA with monoclonal antibodies 16-F 12 and 17 C 10 [2]. MMP-9, TIMP and YKL-40 were estimated by commercial ELISA kits. Joint space width was measured in the narrowest point of the tibiofemoral compartment by 0.1 caliper and magnifying glass.

Results Eighty-nine patients with knee OA were included in the study and were followed for 2 years. The paired samples of serum and synovial fluid were obtained from 48 patients in second study. The joint space narrowing in 2 years was 0.4 ± 0.79 mm. The patients with knee OA had higher initial serum values of pentosidine than controls ($P=0.04$) and also of TIMP ($P=0.04$), MMP-9 ($P=0.02$) and COMP ($P=0.05$). The patients with initially higher serum levels of pentosidine had more rapid radiological progression than controls ($r=0.30$), and the same was true for hyaluronic acid ($r=0.56$). Serum pentosidine levels correlated with synovial fluid pentosidine levels ($r^2=0.78$). Mild correlation occurred between synovial fluid pentosidine and COMP levels ($r^2=0.11$, $P<0.05$).

Conclusions Serum pentosidine levels may be a new molecular marker for prediction of OA progression.

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Exercise: medicine for knee cartilage?

L Dahlberg¹, E Roos², J Svensson³, P Leander⁴, CJ Tiderius⁵

¹Department of Orthopedics, Malmö University Hospital, and Lund University, Sweden; ²Department of Orthopedics, University Hospital, Lund, and Lund University, Sweden; ³Department of Radiation Physics, Malmö University Hospital, Malmö, Sweden; ⁴Department of Radiology, Malmö University Hospital, Malmö, Sweden; ⁵Department of Orthopedics, Malmö University Hospital, Malmö, Sweden
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Many of the 10–60% of the working-age population with knee pain will develop osteoarthritis (OA), a progressive joint disease with cartilage deterioration and increased disability. In knee OA, exercise decreases joint pain and improves function. Lack of human *in vivo* monitoring methods has made studies of influence of exercise on cartilage composition impossible. Delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC) can estimate joint cartilage glycosaminoglycan (GAG) content. It is based on the principle that the negatively charged Gd-DTPA²⁻ ions distribute in the cartilage in an inverse relationship to the GAG content. A high GAG content results in a low contrast medium distribution and a long T₁ relaxation time that can be measured by magnetic resonance imaging. We used dGEMRIC in a cross-sectional study in healthy subjects with different exercising levels, and in a longitudinal exercise study in patients at risk for OA.

Methods Study 1 included healthy, nonexercising individuals ($n=12$), moderately exercising individuals ($n=16$), and elite male track and field athletes ($n=9$). Study 2 included medially meniscectomized patients that were randomized to exercise three times weekly for 4 months ($n=22$) or to a control group ($n=23$).

T₁ measurements were made in sagittal slices in a 1.5T magnetic resonance imaging system, using sets of turbo inversion recovery images with different inversion times in a region of interest in weight-bearing cartilage 2 hours after intravenous Gd-DTPA²⁻ injection at 0.3 mmol/kg body weight. T₁ (s) was calculated using the mean signal intensity from each region of interest as input to a three-parameter fit. In study 2, subjects were examined before and after the exercise period.

Results In study 1, there was a significant relationship between T₁ relaxation time and the level of physical exercise, with longer T₁ values in physically active subjects. In study 2, the exercise group showed a significant higher mean change in T₁ than the control group.

Conclusions *In vivo* cartilage monitoring by dGEMRIC indicates, for the first time, that human articular cartilage in mature individuals seems to adapt to physical activity levels by increasing its matrix GAG content. This may improve the resistance to mechanical compression, and thus protect the collagen network and prevent knee OA development.

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Structural and functional changes in the brains of fibromyalgia subjects evaluated with single photon emission computed spectrometry and magnetic resonance imaging

K Pile¹, L Barnden², S Behin-Ain², L Yelland², D Danda³, R Casse², R Kwiatek³

¹University of Adelaide Department of Medicine, Queen Elizabeth Hospital, Adelaide, Australia; ²Department of Nuclear Medicine, Queen Elizabeth Hospital, Adelaide, Australia ³Department of Rheumatology, Queen Elizabeth Hospital, Adelaide, Australia
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Fibromyalgia (FM) is the most common chronic pain syndrome in the community. The absence of reproducible peripheral pathology has supported the hypothesis of abnormal central pain sensitisation/processing, with both psychological and biological theories invoked. Seminal work by Mountz *et al.* identified reduced regional cerebral blood flow (rCBF) in the thalamus and caudate nuclei. Our aim was to verify variation in rCBF of subjects with FM, and to refine its anatomical localisation and clinical correlation. Twenty-seven females with ACR criteria FM were matched with 22 age-matched, gender-matched and education-matched controls. Detailed physical and psychological inventories, 1.5T T₁/T₂ magnetic resonance imaging (MRI), and triple-headed ⁹⁹Tc single photon emission computed spectrometry (SPECT) scanning were performed. We have developed a colocalisation of SPECT-measured rCBF with MRI imaging, accurate to 2 mm. This allowed highly definable quantitative rCBF analysis using statistical parametric mapping (SPM). Initial studies identified a highly significant 16% reduction in rCBF of the pontine tegmentum/upper medulla, and less robust reduction in the thalamus. Clinical correlates favoured physical rather than psychological variables. In a subcohort of 11 FM subjects and 11 controls, rCBF has been measured twice over a 5-year period. None of the subjects were cured. rCBF remained significantly reduced in the pontine tegmentum and midbrain of FM subjects with normalisation of caudate and thalamic rCBF. In a novel analysis, SPM has been uniquely used for the detection of subvisual changes on T₁ and T₂ MRI scans. The MRI scans of 27 FM subjects were compared with those of 19 controls. Analysis of T₁ images identified a 6% reduction in signal among FM subjects within the cerebellar tonsils and midbrain, the former in keeping with reports of Arnold–Chiari type 1 malformations. T₂ analysis identified widespread diffuse reduction in the white matter of FM subjects. Our studies have confirmed and localised objective abnormalities of rCBF among FM subjects. These changes probably reflect altered metabolic demands in pain processing centres, and for the first time have been shown to persist over time. Canonical and discriminant statistical analysis was not able to utilise these findings for diagnostic purposes. Additionally, subvisual abnormalities on MRI suggested structural and functional abnormalities within the brain.

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Clinical feature of fibromyalgia syndrome in Japan and novel strategy in the treatment by bioproduct (Neurotropin)

H Nakamura¹, K Hoshi¹, T Kato¹, S Ozaki², M Nishioka³, K Nishioka¹

¹Department of Bioregulation, Institute of Medical Science, St Marianna University, Kawasaki, Japan; ²Department of Rheumatology, St Marianna University, Kawasaki, Japan; ³Jikei University School of Medicine, Tokyo, Japan
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Purpose Fibromyalgia syndrome (FMS) is a controversial disorder of unknown etiology. Because of lack of laboratory and objective findings,

many patients have been ignored, misdiagnosed or referred to a psychiatrist. Recently, the number of FMS patients has been increasing in Japan. The purpose of this study is to elucidate the clinical feature of FMS and to evaluate the clinical efficacy of Neurotropin (extracted from inflammatory cutaneous tissue of rabbits inoculated with vaccinia virus) for FMS associated with neck or back pain.

Methods Subjective complaints, tender points and clinical findings were assessed and routine laboratory examination was carried out in 70 FMS patients. Among them, 23 patients were treated with Neurotropin, administered orally and intravenously. Patients' assessment and the number of tender points were evaluated after the treatment.

Results Subjective complaints were composed of fatigue, sleep disturbance, depression, anxiety and headache, as well as widespread pain. The mode of pain ranged from cutting pain to touch pain. In serious cases, patients could not even move the joints nor stand by their selves. Neurotropin treatment was effective in 16 out of 23 patients. Further investigation will be needed to comprehend the present status of FMS in Japan and to elucidate its etiology. The effect of Neurotropin on FMS also needs to be evaluated.

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From clinical pharmacology to clinical rheumatology: bench to bedside

P Pispati

Rheumatology Division, Jaslok and Bhatia Hospitals, Bombay, India
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The mainstay of management of rheumatic diseases is based on drug therapy. A number of drugs, from gold salts of yesteryear to methotrexate, leflunomide and the biologicals of today, are in vogue. These are used both singly and in permutations and combinations. Patient response varies enormously, from impressive results to abysmal failure. Why this great variability of response? The following factors would determine patient response: the choice of drug (factors in the drug and factors in the patient), the dosage forms, the dose, the pharmacokinetics, the pharmacodynamics, the drug interactions (synergism, additive effects, and antagonism), the adverse reactions, the pharmacoeconomics, and the patient education.

This presentation highlights nuances in drug therapy that will minimise failure rate, enhancing the success rate in the realm of rheumatology.

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Celecoxib: a selective cyclooxygenase-2 inhibitor

M Gandelman

Pfizer, Inc., New York, USA
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Selective cyclooxygenase-2 (COX-2) inhibitors were developed to fill an important medical need. The rationale for the development of selective COX-2 inhibitors will be discussed using celecoxib as an example. A brief description of the pharmacology and selective binding to the COX-2 enzyme will be presented. Both preclinical and clinical data will be shown to demonstrate important clinical aspects of celecoxib. The efficacy data will include studies of osteoarthritis, rheumatoid arthritis, and acute pain conditions. Both gastroenterology and cardiovascular safety of celecoxib will be discussed. Finally, potential new therapeutic areas for celecoxib will be presented.

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Bioethics and research: scientific autonomy versus social responsibility. Should we lead or be led?

A Rubinow

Rheumatology Unit, Division of Medicine, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
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From the dawn of the time, discoveries have been made to improve the human condition. Today, science and biotechnology have transcended to the very essence of life itself. The inexorable urge to extend the frontiers of genetics and biology threatens to impinge on the balance

between the well-being of mankind and its detriment. UNESCO, through the Universal Declaration on the Human Genome and Human Rights (1998) and via the International Bioethics Committee, is drafting an international instrument of bioethics based on the principles of human dignity, freedom, autonomy, justice and equity across cultures, values and religions. It emphasizes the noncommercialization of the human body, cautions against the pursuit of indiscriminate research in embryonic stem cells and cloning, gives directives in the collection, storage and treatment of genetic material, and outlaws the patenting of human genes. The voice of scientists/physicians in this arena is muffled

and distant. Before binding legislation intended to protect the liberty of human beings is thrust upon us, scientists need to lead the public debate. Society, still recovering from the era of eugenics where science was heralded as being able to provide solutions for social dilemmas, wants to be fully informed of its choices. The education of the public requires strategic planning so that efforts may not be counterproductive and generate mistrust. This presentation to scientists/clinicians who are at the cutting edge of innovative research in our field will highlight bioethical issues of mutual interest, emphasizing the applications and implications of scientific advances.
