Introduction

α-Actinin is an actin-binding cytoskeletal protein present in a variety of cells [1] and in focal adhesion sites where cells adhere to the substrate [2]. There is biochemical [3] and histologic [4] evidence that focal adhesion complexes, containing α-actinin and other footpad material, are left behind as a result of normal movement of cells [2], perhaps at increased rates when neutrophils and monocytes move into inflammatory tissue. We have shown that α-actinin is abundant in the bone marrow stroma matrix, presumably at focal adhesion sites [5]. We have also reported that a 31 kDa amino-terminal α-actinin fragment, which we have named mactinin, is generated by the degradation of extracellular α-actinin by monocyte-secreted urokinase [6]. Furthermore, we have demonstrated that mactinin is present in inflammation caused by Pneumocystis carinii pneumonia, by examining bronchoalveolar lavage fluid from mice with infection [6]. It was not present in mice not challenged with P. carinii, suggesting that inflammation is necessary for mactinin formation. We have also reported that mactinin promotes monocyte/macrophage maturation [7]. For example, α-actinin fragments significantly increase lysozyme secretion and tartate-resistant acid phosphatase staining in peripheral blood monocytes. In contrast, intact α-actinin has no maturation-promoting activity. We proposed that mactinin is present in the microenvironment at sites of various types of inflammation, perhaps owing to migrating cell populations, and there it might contribute to the recruitment and maturation of monocytes.
Monocyte/macrophage infiltration has a key role in the pathogenesis of chronic arthritis [8]. The release of pro-inflammatory cytokines, chemokines, growth factors, and enzymes by the synovial lining macrophages is important for the onset, propagation, and flare of arthritic inflammation [9]. The finding that the number of synovial tissue macrophages is correlated with joint destruction in rheumatoid arthritis is evidence of their importance [9,10]. Monocytes and macrophages are believed to have a similar role in other chronic inflammatory joint diseases, such as gout [11] and psoriatic arthropathy [12]. Therefore in this study we assessed the effects of mactinin on monocyte chemotaxis in vitro. We have also tested synovial fluid from patients with various types of arthritis, including rheumatoid arthritis, psoriatic arthritis, reactive arthritis, gout, and ankylosing spondylitis, for the presence of the monocyte/macrophage maturation-promoting fragment, mactinin. We have also investigated whether mactinin is present in the antigen-induced arthritis model in rabbits [13,14]. Macrophages are believed to be important in this model of rheumatoid arthritis [15,16], and both the arthritic and control joint fluid can be tested for mactinin.

Materials and methods

Source of mactinin

As described previously [6], a pGEX2 vector, encoding the actin-binding domain, residues 2–269 of chicken smooth muscle α-actinin, fused with the carboxy terminus of glutathione S-transferase (GST) with an engineered thrombin cleavage site, was kindly provided by Dr DR Critchley of the University of Leicester, UK. Fusion protein was expressed in Escherichia coli, and the cleavage products of the fusion protein were purified by affinity chromatography of cell extracts on immobilized glutathione. The fusion protein was then cleaved with thrombin (Calbiochem, San Diego, CA) to yield the actin-binding domain of α-actinin and the GST carrier. The cleavage products were then separated by reverse-phase high-performance liquid chromatography on a C-4 column [6]. SDS–PAGE demonstrated that the α-actinin fragment was more than 90% of the total protein of pooled fractions, with the remaining 10% being carrier GST. The calculated molecular mass of this α-actinin fragment was 30,700 Da. In this report, both the active product of urokinase degradation of α-actinin formed in vivo [6] and the active recombinant actin-binding domain, which are of similar molecular masses, will be referred to as mactinin. Mactinin and GST routinely assay negative for protein endotoxin with a Pyrotell chromogenic assay kit, which can detect more than 0.25 endotoxin units/ml (Associates of Cape Cod, Woods Hole, MA).

Isolation of peripheral blood monocytes

Mononuclear cells were isolated from buffy coat preparations of healthy blood donors by density gradient centrifugation with Histopaque 1077 (Sigma). Contaminating red cells were then lysed in distilled water, and the sample was applied to an LS separation column with a magnetic monocyte isolation kit in accordance with the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). This negative selection method resulted in a cell population containing more than 90% monocytes as determined by CD14 expression.

Chemotaxis assay

Cell migration was assessed by a 48-well micro-chemotaxis chamber (NeuroProbe, Gaithersburg, MD). An aliquot of peptide was placed in the lower compartment, and a suspension of monocytes (30,000–35,000) was placed in the upper compartment of the well. The two compartments were separated by a polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μm. The chamber was incubated at 37°C for 90 minutes. At the end of the incubation period the filter was removed, fixed, and stained with a Hema 3 stain set (Fisher, Pittsburgh, PA). The cells that migrated through the membrane pore in three high-power fields (×400) were counted by light microscopy. Three chamber membranes were counted for each concentration.

Assessment of mactinin concentrations necessary for HL-60 cell maturation

HL-60 myeloid leukemia cells were seeded at a density of 10⁵/ml and grown for 3 days in RPMI medium with 50 μg/ml gentamicin and 15% fetal calf serum at 37°C and 5% CO₂, in the presence of various concentrations of recombinant mactinin. We have previously reported that mactinin promotes monocyte maturation as measured by morphology, non-specific esterase activity, and Fc rosette formation in this leukemia cell line [7]. Here we report the concentrations necessary to induce maturation as measured by non-specific esterase staining used as a maturation marker.

Antisera generation

To generate antisera with sensitivity for detecting mactinin, purified recombinant chicken α-actinin peptide was modified by coupling with dinitrophenol [17] and injected into two New Zealand white rabbits along with complete Freund’s adjuvant, as described previously [6]. Boosts were done with peptide and incomplete Freund’s adjuvant. These animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Minneapolis Veterans Affairs Medical Center. Antisera were screened for their ability to detect the immunizing peptide and were immunoaffinity-purified with columns of the recombinant fragment covalently bound to a Affi-Gel 15 matrix (Bio-Rad, Hercules, CA). We expected cross-reactivity of the purified antisera with fragments from rats, mice, or humans because of the highly conserved amino acid sequence.
Synovial fluid samples
Fluid from patients with arthritis undergoing therapeutic arthrocentesis was collected and tested for the presence of the fragment by Western blot analysis. The use of these fluid samples in this study was approved by the subcommittee on human studies of the Minneapolis VAMC. In brief, fresh samples were centrifuged at 895g for 10 minutes and frozen at ~80°C until the time of analysis. Because mactinin is a very small fraction of the total protein content (more than 50 μg/10 μl) of the fluid, immunoaffinity purification of mactinin was performed before Western blotting. Samples were thawed, dialyzed against phosphate-buffered saline, and centrifuged again; the total sample volume was then applied to a column of immunoaffinity-purified antibody covalently bound to an Affi-Gel 15 matrix, and eluted with 0.1 M sodium citrate in 0.3 M NaCl, pH 3.0. Fractions (2 ml) were collected and neutralized with 2 M Tris-HCl. Protein-containing fractions were pooled, dialyzed against phosphate-buffered saline, concentrated, and subjected to electrophoresis on a 12% SDS–PAGE gel under reducing conditions. Each lane contained 100 μl, representing about 1% of the total sample.

Immunoblot analysis
The proteins separated by SDS–PAGE were transferred electrophoretically to poly(vinylidene difluoride) membranes (Bio-Rad). The membrane was blocked with 5% nonfat milk in 50 M Tris-HCl/150 M NaCl, pH 7.5, and sequentially treated with affinity-purified rabbit antisera raised against recombinant mactinin, followed by second antibody conjugated with alkaline phosphatase (ICN, Costa Mesa, CA). Immunoreactive proteins were detected by alkaline phosphatase reaction with 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium. Control analyses were performed with rabbit IgG (Santa Cruz Biochemistry, Santa Cruz, CA).

Dissociation of immune complexes in rheumatoid arthritis fluid
To examine whether mactinin was present in immune complexes in rheumatoid arthritis fluid, some samples were acidified by dialysis against 0.1 M sodium acetate, pH 4.1, to dissociate complexes [18]. The acidified samples were first fractionated on an 800 ml G-75 Sephadex (Pharmacia, Piscataway, NJ) size-exclusion column. Fractions identified by Western blotting as containing mactinin were pooled, neutralized, and further purified by fractionation on a C-4 column. The C-4 column was equilibrated with 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient (0–100%) run at 1%/ml/min. Aliquots of protein-containing fractions were used for Western blots.

Measurement of mactinin in the antigen-induced arthritis model
To produce antigen-induced arthritis, New Zealand white rabbits were immunized by subdermal injection of ovalbumin emulsified in complete Freund’s adjuvant in accordance with modifications [14] of the method of Dumonde and Glynn [13] under IACUC approval. Three weeks later, animals with positive skin tests to ovalbumin received intra-articular injections of 1 mg of sterile ovalbumin into one knee and an equal volume of sterile saline in the contralateral control knee weekly for 3 weeks. Arthritic and control knee joints were then lavaged with saline and aspirated at the time of killing, when animals had developed chronic synovitis 10 weeks after the intraarticular injections were complete. Samples were frozen at ~80°C until the time of analysis. Samples were then concentrated and used for Western blots.

Results
Mactinin is a chemoattractant for peripheral blood monocytes
For analysis of the effect of mactinin on monocyte chemotaxis, peripheral blood monocytes were placed in the upper chambers of a 48-well micro-chamber plate with various concentrations of mactinin. The lower compartment of the wells also contained various concentrations of mactinin and was separated from the upper chamber by a polycarbonate filter. As shown in Table 1, 1–10 nM mactinin had significant chemotactic activity for monocytes. Intact α-actinin at 10 nM had no activity. Because our mactinin preparations were contaminated with up to 10% GST (thus containing 0.1 nM GST in 1 nM mactinin), we tested 0.1 nM GST alone and also found no activity. The number of concentrations tested per assay was limited by the 48 wells (16 combinations for triplicate wells), but in separate assays we have seen significant chemotactic activity at mactinin concentrations as low as 0.5 nM. The concentration of mactinin necessary for activity is similar to that of FMLP (0.1–10 nM) in our assay system, and mactinin and FMLP attract similar numbers of monocytes.

Measurement of mactinin’s maturation-promoting activity
Recombinant mactinin has maturation-promoting activity in vitro in HL-60 leukemia cells at the concentrations shown in Figure 1. That is, it induces non-specific esterase staining at 2.5 pM and activity reaches a plateau at 25 pM. For analysis of the effect of mactinin on monocyte chemotaxis, peripheral blood monocytes were placed in the upper chambers of a 48-well micro-chamber plate with various concentrations of mactinin. The lower compartment of the wells also contained various concentrations of mactinin and was separated from the upper chamber by a polycarbonate filter. As shown in Table 1, 1–10 nM mactinin had significant chemotactic activity for monocytes. Intact α-actinin at 10 nM had no activity. Because our mactinin preparations were contaminated with up to 10% GST (thus containing 0.1 nM GST in 1 nM mactinin), we tested 0.1 nM GST alone and also found no activity. The number of concentrations tested per assay was limited by the 48 wells (16 combinations for triplicate wells), but in separate assays we have seen significant chemotactic activity at mactinin concentrations as low as 0.5 nM. The concentration of mactinin necessary for activity is similar to that of FMLP (0.1–10 nM) in our assay system, and mactinin and FMLP attract similar numbers of monocytes.

Detection of mactinin in arthritis fluid
Affinity-purified rabbit antiserum raised against a recombinant chicken α-actinin fragment detected picogram amounts of the immunizing amino-terminal protein fragment (Fig. 2). As also shown in Figure 2, this antiserum reacted with a protein of the expected molecular mass in representative samples of immunoaffinity-purified synovial fluid from patients with psoriatic arthritis, reactive arthritis, gout, and ankylosing spondylitis. In all, six of seven samples from patients with these arthritides contained...
mactinin. In contrast, mactinin was detected in none of five rheumatoid arthritis samples ($P<0.05$; Fisher’s exact test). When the detected levels of mactinin in the upper and lower compartments of chemotactic chambers define the ‘checkerboard’ analysis of mactinin as a chemotactic or chemokinetic factor. Results (means ± SEM) are the average number of migrated cells per oil field (counting three fields) from three filters. Significant results, compared with controls with no mactinin below the membrane and either an equivalent amount of mactinin or no mactinin above the membrane by Student’s t-test, are indicated by asterisks ($P<0.05$). This represents one of two experiments with similar results. Neither 10 nM intact α-actinin nor 0.1 nM GST had significant activity. NT, not tested.

Figure 1

Percentage of HL-60 cells staining positive for nonspecific esterase after treatment with various concentrations of recombinant mactinin. Cells were incubated for 3 days with mactinin, then harvested and stained. The percentage of untreated HL-60 cells positive for staining was subtracted. Each value is the mean ± SD for a minimum of two assays of 100 cells each. The result of treatment with 100 nM 12-O-tetradecanoylphorbol-13-acetate is also shown (circle).

Figure 2

Western blot analysis with affinity-purified rabbit antisera. Each of the first seven lanes contains the immunizing peptide in the amount shown (in nanograms). The second band seems to be due to an alternative cleavage site in the fusion protein at amino acid 262. Lanes A–E contain synovial fluid from patients with various types of arthritis; it was immunoaffinity-purified to decrease the protein load before Western blotting. Lane A, psoriatic arthritis; lane B, reactive arthritis; lane C, gout; lane D, ankylosing spondylitis; lane E, rheumatoid arthritis. The samples in lanes A–D contained mactinin. Controls with rabbit IgG were negative for all samples.

Table 1

‘Checkerboard’ analysis of mactinin as a chemotactic factor

<table>
<thead>
<tr>
<th>Mactinin concentration above membrane (nM)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21 ± 7</td>
<td>25 ± 8</td>
<td>17 ± 6</td>
<td>32 ± 10</td>
</tr>
<tr>
<td>0.1</td>
<td>25 ± 8</td>
<td>39 ± 12</td>
<td>21 ± 9</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>70 ± 7*</td>
<td>77 ± 15*</td>
<td>48 ± 9*</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>10</td>
<td>78 ± 7*</td>
<td>82 ± 19*</td>
<td>NT</td>
<td>26 ± 7</td>
</tr>
</tbody>
</table>

Different concentrations of mactinin in the upper and lower compartments of chemotactic chambers define the ‘checkerboard’ analysis of mactinin as a chemotactic or chemokinetic factor. Results (means ± SEM) are the average number of migrated cells per oil field (counting three fields) from three filters. Significant results, compared with controls with no mactinin below the membrane and either an equivalent amount of mactinin or no mactinin above the membrane by Student’s t-test, are indicated by asterisks ($P<0.05$). This represents one of two experiments with similar results. Neither 10 nM intact α-actinin nor 0.1 nM GST had significant activity. NT, not tested.

Owing to the autoimmune nature of rheumatoid arthritis, we examined whether mactinin was present in immune complexes. That is, antibody-bound mactinin might not bind to the antibody-matrix column used in the isolation protocol to decrease the total protein load, resulting in mactinin being undetectable but potentially active. To dissociate immune complexes, an aliquot of a rheumatoid arthritis fluid sample was acidified and the proteins were fractionated on a C-4 column. As shown in Figure 3, mactinin is detectable by Western blot analysis after dissociation from immune complexes.

To confirm the presence of mactinin in rheumatoid arthritis fluids, some frozen aliquots were thawed and immediately subjected to electrophoresis on a 12% SDS–PAGE gel under reducing conditions. As shown in Figure 4, affinity-purified mactinin antisera reacted with several 30–40 kDa proteins, including 31 kDa mactinin in each of the samples tested. We have previously demonstrated that only the 31 kDa α-actinin degradation product has maturation-promoting activity [5]. These proteins were not seen in nonreduced samples, which did have immunoreactive material at the top of the gel (data not shown). Hence, the
detected proteins might represent mactinin bound to various immune complex fragments.

**Mactinin is present in inflammatory fluid from antigen-induced arthritis**

Arthritic and control knee joints of rabbits with chronic antigen-induced arthritis were lavaged with saline and aspirated at the time of killing. Samples were concentrated and used for Western blots run under reducing conditions. As seen in Figure 5, mactinin is present in arthritic joint fluid but absent from control fluid, suggesting that mactinin formation is dependent on the inflammatory response. As also seen in Figure 5, another α-actinin fragment of slightly higher molecular mass is present in both samples.

**Discussion**

During inflammatory processes, various mediators, such as cytokines and chemokines, regulate the recruitment of monocytes. Once in the tissue, monocytes undergo the poorly understood process of transformation to macrophages with altered morphology and function [19]. In arthritis, synovial macrophages might cause joint destruction by differentiating to bone-resorbing osteoclasts [20] or by releasing cartilage-degrading enzymes and cytokines, such as interleukin-1 and tumor necrosis factor-α [8]. It has therefore been suggested that therapies for chronic arthritis should be aimed at depleting joint mononuclear cells or controlling the activation of synovial macrophages [21]. Indeed, elimination of macrophages by clodronate-laden liposomes in rat models of adjuvant [22] and antigen-induced arthritis [23] induces amelioration of the arthritis.

Of the many mediators of inflammation, mactinin is the first example of a fragment of a cytoskeletal component that might be released during leukocyte influx into inflammatory tissue. Further, mactinin might have a role in promoting the response of mononuclear phagocytes to inflammation. The monocyte functional studies in vitro demonstrate that 0.5–10 nM levels of the fragment have significant chemotactic activity. We have previously reported that mactinin promotes monocyte maturation, as measured by lysozyme secretion and tartrate-resistant acid phosphatase staining [7]. Here we show that 25 pM levels of mactinin promote monocytic maturation of the HL-60 leukemia cell line.

Mactinin is present at sites of various types of arthritic inflammation at levels that are active in vitro, including synovial fluid samples from patients with psoriatic arthritis, reactive arthritis, gout, and ankylosing spondylitis. Although it was not initially detected in five immunoaffinity-purified rheumatoid arthritis samples, it was detected after the acid dissociation of immune complexes. Girard and Senecal [24] have reported that sera from patients with autoimmune diseases such as rheumatoid arthritis contain antibodies against microfilament-associated proteins, including α-actinin. In addition, auto-antibodies against actin, vinculin, integrins, or fibronectin could also form complexes with mactinin [24,25]. Our results suggest that mactinin is bound to immune complexes in rheumatoid arthritis joint fluid, which prevents its binding to the antibody-matrix during the isolation procedure. The finding that mactinin is detected by Western blotting of samples run under reducing conditions without immunoaffinity purification seems to confirm this. It is noteworthy that, unless the antibody is neutralizing, even antibody-bound mactinin might still be active.
The antigen-induced arthritis model of rheumatoid arthritis in rabbits demonstrates persistent active inflammation for several months after the intra-articular injection of antigen, including hypertrophy and hyperplasia of the synovial lining cells, pannus formation with articular cartilage erosion, and chronic infiltration of synovium by lymphocytes and plasma cells [13]. In addition, Dijkstra and colleagues [15] found macrophages in the superficial layer of the synovium, where they might secrete enzymes and oxygen radicals into the joint space, which can lead to cartilage erosion [9]. The protein levels in lavage fluid from arthritic joints in this model are low enough to allow direct testing by Western blot analysis without immunofinity purification, as was needed with the human aspirates. Mactinin was found in the lavage fluid from arthritic knee joints of rabbits with this immune arthritis and might contribute to macrophage function in this arthritis model.

Because this study was done using waste fluid from therapeutic synovial fluid aspirates, we did not have samples from noninflamed joints. The low mactinin recovery rate during the purification process and the low concentration of mactinin needed for activity make it necessary to assay at least 1 ml of fluid, and this amount is not available from any tissue and fluid bank. However, mactinin was not present in the control joint fluid in the rabbit antigen-induced arthritis model, suggesting that mactinin is specific for the inflammation process. Similarly, we have reported that bronchoalveolar lavage fluid from uninfected mice contains no mactinin, in contrast to fluid from mice infected with P. carinii [6].

The plasminogen activators, tissue type and urokinase type, have been reported to be both deleterious in inflammation, owing to the proteolysis of tissue proteins, and beneficial because of fibrinolytic activity [26]. The presence of both increased urokinase and plasmin inhibitors in rheumatoid arthritis synovial tissue suggests a complex role for urokinase in this disease [27–29] that seems pertinent to our finding of urokinase-generated mactinin in the arthritis fluid samples. The overall effect of urokinase in the antigen-induced arthritis model seems to be beneficial, because chronic joint inflammation and bone erosion are significantly worse in urokinase-deficient mice [30]. However, the ability of urokinase-generated mactinin to enhance proteolysis might be deleterious. Hence, future testing of specific mactinin inhibitors in animal models of arthritis seems warranted.

Conclusion

We conclude that mactinin is present in arthritic synovial fluid in levels that can promote mononuclear phagocyte chemotaxis and maturation. Therefore, increased numbers of mature monocytes might increase cartilage and bone destruction. These results lead us to speculate that inhibitors of mactinin might be of benefit in the treatment of some forms of chronic arthritis and form the basis for our plans to test the efficacy of mactinin antiserum to ameliorate antigen-induced arthritis.

Competing interests

None declared.

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References


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