

Review

Molecular action of methotrexate in inflammatory diseases

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Abstract

Despite the recent introduction of biological response modifiers and potent new small-molecule antirheumatic drugs, the efficacy of methotrexate is nearly unsurpassed in the treatment of inflammatory arthritis. Although methotrexate was first introduced as an antiproliferative agent that inhibits the synthesis of purines and pyrimidines for the therapy of malignancies, it is now clear that many of the anti-inflammatory effects of methotrexate are mediated by adenosine. This nucleoside, acting at one or more of its receptors, is a potent endogenous anti-inflammatory mediator. In confirmation of this mechanism of action, recent studies in both animals and patients suggest that adenosine-receptor antagonists, among which is caffeine, reverse or prevent the anti-inflammatory effects of methotrexate.

Keywords: adenosine receptor, inflammation, methotrexate, rheumatoid arthritis

Introduction

The demonstration in 1985 that low-dose, intermittent methotrexate is a potent and effective therapy for rheumatoid arthritis (RA) [1] led to a dramatic change in the way that patients with RA are treated. Indeed, methotrexate is no less efficacious than specific anti-tumor-necrosis-factor (anti-TNF) therapy for the relief of symptomatic joint inflammation in early RA, and the difference between methotrexate and etanercept with respect to protection from structural injury in RA is probably not biologically significant [2]. Thus, methotrexate remains the cornerstone of therapy for RA, and understanding the mechanism(s) responsible for the therapeutic efficacy of this agent may lead to the development of new therapies.

History and clinical pharmacology

Methotrexate was first developed in the 1940s as a specific antagonist of folic acid. This drug inhibits the proliferation of malignant cells, primarily by inhibiting the *de novo* synthesis of purines and pyrimidines. Because administration of high doses of reduced folic acid (folinic acid) or even folic acid itself can reverse the antiproliferative effects of methotrexate, it is clear that methotrexate does act as an antifolate agent. Interestingly, although not originally designed as such, methotrexate appears to be a 'pro-drug',

i.e. a compound that is converted to the active agent after uptake. Methotrexate is taken up by cells via the reduced folate carrier and then is converted within the cells to polyglutamates [3]. Methotrexate polyglutamates are long-lived metabolites that retain some of the antifolate activities of the parent compound, although the potency for inhibition of various folate-dependent enzymes is shifted [3–6].

Proposed mechanisms of action of methotrexate

Low-dose methotrexate was introduced for the treatment of RA because of its presumed antiproliferative properties, although it was unclear how inhibiting proliferation of the lymphocytes thought to be responsible for synovial inflammation in RA for one day a week might lead to effective suppression of disease activity. However, it soon became clear that inhibition of folic acid metabolism could not be completely responsible for the anti-inflammatory effect of methotrexate. During the past 15 years, it has become clear that administration of folic acid in doses of 1–5 mg per day helps to prevent much of the toxicity of methotrexate without interfering with the anti-inflammatory efficacy of the drug, whereas very high doses of folinic acid also prevent methotrexate toxicity but may interfere with its efficacy [7–20]. There are two potential explanations for the

AICAR = aminoimidazolecarboxamidribonucleotide; Fc = crystallizable fragment (of antibody); IFN = interferon; IL = interleukin; RA = rheumatoid arthritis; Th = T helper (cells); TNF = tumor necrosis factor.

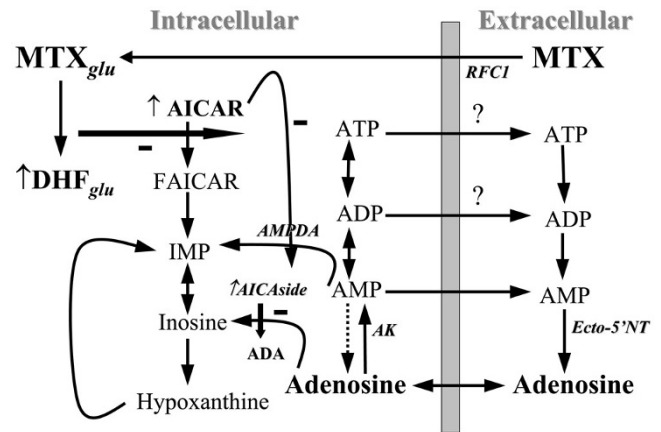
capacity of high doses of folic acid to reverse the therapeutic effects: first, folic acid may bypass the effects of methotrexate on reduction of folic acid and thereby bypass the therapeutic effects of the drug; alternatively, folic acid but not folic acid may compete with methotrexate for a single transport site into the cell (Fig. 1) and may thus interfere with cellular uptake of methotrexate [21]. Moreover, the expected inhibition of cellular proliferation is manifested as bone marrow suppression, and oral and gastrointestinal ulcers, and may require lowering the dose of the drug and, usually, the efficacy of the therapy, suggesting that inhibition of cellular proliferation alone is not responsible for the anti-inflammatory effects of methotrexate. Thus, folate antagonism appears to play, at most, a minimal role in the anti-inflammatory mechanism of methotrexate.

Another potential mechanism by which methotrexate may diminish inflammation in the joint is by diminishing cytokine production. Numerous studies have demonstrated diminished levels of inflammatory cytokines in the serum of patients. The adenosine A_{2A} receptor agonist CGS-21680 is a potent inhibitor of neutrophil leukotriene synthesis *in vitro*, and, similarly, methotrexate therapy leads to diminished production of leukotriene B_4 by neutrophils stimulated *ex vivo* [22,23]. The mechanism by which methotrexate diminishes these cytokine levels remains unexplained and it is difficult to determine from these studies whether the effects of methotrexate therapy on production of inflammatory mediators results in diminished inflammation or is secondary to other anti-inflammatory events.

Similarly, methotrexate-mediated effects on T-cell function, either *in vivo* or *in vitro*, have been demonstrated. Indeed, Genestier and colleagues have reported that methotrexate diminishes antigen-stimulated T-cell proliferation both *in vitro* and in T cells taken from patients taking methotrexate [24]. That the effects of methotrexate on T-cell function are completely reversed by folic acid and that the effects of therapy on T cells studied *ex vivo* are present for only 48 hours a week would strongly suggest that this cannot be responsible for the bulk of the anti-inflammatory effects of the drug.

A third proposed mechanism of action is based upon the observation that polyamines accumulate in the synovium of patients with RA and that metabolism of these polyamines by macrophages leads to the production of toxic oxygen products that diminish stimulated T-cell function [25–27]. Indeed, methotrexate therapy does diminish polyamine levels in the joints of patients with RA [28–30], but this effect, like that of methotrexate on T-cell proliferation, is reversed by folic acid. Moreover, there are more than enough toxic oxygen metabolites being generated in the rheumatoid synovium to mediate the tissue damage present in this disease; another source of toxic agents would add relatively little.

Figure 1



Methotrexate-induced metabolic changes lead to increased extracellular adenosine. ADA = adenosine deaminase; AICAR = aminoimidazolecarboxamidoribonucleotide; AICAside = aminoimidazolecarboxamidoribonucleoside; AK = adenosine kinase; AMPDA = AMP deaminase; DHF = dihydrofolate; DHF_{glu} = dihydrofolate polyglutamate; ecto-5'NT = ecto-5' nucleotidase; FAICAR = formyl-AICAR; IMP = inosine monophosphate; MTX = methotrexate; MTX_{glu} = methotrexate polyglutamate; RFC1 = reduced folate carrier 1.

Methotrexate induces adenosine release

Our laboratory originally proposed the hypothesis that the beneficial effects of methotrexate result from the intracellular accumulation of intermediates in purine biosynthesis that, by a mechanism that has not been completely worked out, leads to increased concentrations of adenosine in the extracellular space [31]. This hypothesis sprang from the prior demonstration that intracellular accumulation of specific intermediates in the *de novo* synthesis of purines leads to adenosine release [32] and from our interest in the anti-inflammatory effects of adenosine, which are mediated by specific receptors on inflammatory cells. Prior work had demonstrated that methotrexate polyglutamates inhibit the enzyme aminoimidazolecarboxamidoadenosineribonucleotide (AICAR) transformylase more potently than the other enzymes involved in purine biosynthesis [4,5,33]. This inhibition occurred at pharmacologically relevant concentrations of methotrexate and might be expected to occur more readily with infrequent loading with methotrexate, since methotrexate polyglutamates are long-lived metabolites (persisting for weeks). The presence of increased concentrations of AICAR metabolites in the urine of RA patients treated with methotrexate supports these findings [34,35]. The accumulation of AICAR and its metabolites has a direct inhibitory effect on at least two key enzymes, adenosine deaminase and AMP deaminase, with the end result of increased concentrations of adenosine and adenine nucleotides intracellularly [4]. Methotrexate in doses similar to that used in the treatment of RA has been known

to cause the accumulation of AICAR in animal models of RA, and this accumulation is associated with an elevation in adenosine concentration in the extracellular space [32,36]. The exact mechanisms by which the elevation of extracellular adenosine arises are not fully understood, but dephosphorylation of adenine nucleotides is likely to be a major contributor, partly because of the ubiquitous nature of ATP in tissues and partly because of the widespread existence of ecto-5'-nucleotidase, an enzyme that catalyzes the dephosphorylation of AMP to adenosine [37].

All this evidence points to adenosine as a key mediator in the anti-inflammatory actions of methotrexate. *In vivo* experiments support this contention. The nonselective adenosine receptor antagonist 8-phenyl theophylline potentiated inflammatory responses in a hamster-cheek-pouch model [38]. Infusion of adenosine directly into the knee in rats inhibited the development of adjuvant-induced arthritis, and an adenosine receptor antagonist effectively reduced the severity of joint inflammation in a collagen-induced arthritis model in mice [39,40]. We have previously shown that the anti-inflammatory effects of methotrexate in carrageenan-induced mouse air pouch inflammation is reversed by an antagonist to the adenosine A_{2A} receptor, or by the addition of adenosine deaminase, an adenosine-metabolizing enzyme, suggesting that adenosine is indeed responsible for the anti-inflammatory effects of methotrexate *in vivo* [36]. An interesting study by Silke *et al.* showed that ingestion of caffeine, a nonselective antagonist of adenosine receptors, in coffee correlates with poor clinical response to methotrexate, and patients with a high caffeine intake are more likely to discontinue methotrexate than those with a low caffeine intake [41].

To better appreciate how adenosine influences biological responses in the network of events taking place in an inflammatory milieu, something must be said about this autocoid and the cellular receptors with which it interacts to produce these physiological responses. Adenosine receptors, or P1 receptors, fall into four known subclasses: A₁, A_{2A}, A_{2B}, and A₃. These are members of the large, seven-transmembrane-receptor family of receptors that influence cell signaling mechanisms by coupling to G proteins. The receptor sequences have been characterized and, with the exception of the A₃ receptor, they are highly conserved during evolution. Adenosine receptors modulate a vast array of physiological functions, from heart rate to the state of wakefulness. Adenosine, acting on P1 receptors, exerts a number of actions on a variety of cell types relevant to the anti-inflammatory effect of methotrexate.

Cellular effects

Neutrophils

Neutrophils, a hallmark of acute inflammation, are among the first cells recruited into the inflammatory site. The limitation of neutrophil-mediated damage relies in part on

the modification of the adhesive capacity and ability to generate chemical damage, properties under purinergic influence. The resting neutrophil has a number of mechanisms that, once activated, can damage tissues. One of these is latent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multimolecular complex that is assembled at the plasma membrane upon activation of the neutrophil and that generates oxygen radicals [42]. The first in the chain of these oxygen radicals is superoxide anion, and it was the discovery in 1983 that superoxide generation, as stimulated by a variety of agents including the chemoattractant *N*-formyl-leucyl-phenylalanine (*f*MPLP), the complement component C5a, and the calcium ionophore A23187, was inhibited by adenosine that sparked an interest in the anti-inflammatory properties of adenosine [43,44]. This physiological action of adenosine has subsequently been ascribed to its action on the adenosine A_{2A} receptor, which is present on the neutrophilic surface membrane [45]. An important second messenger to adenosine-A_{2A}-receptor signaling in this respect appears to be 3',5'-cyclic adenosine monophosphate (cAMP), the intracellular concentration of which increases with neutrophilic adenosine A_{2A} receptor stimulation. cAMP further activates protein kinase A downstream and inhibition of protein kinase A reverses the effects of cAMP analogues but not of adenosine receptor agonists on stimulated neutrophilic superoxide anion generation [46]. The cAMP-protein-kinase-A-dependent adenosine inhibition of neutrophil oxidative activity is mediated via the adenosine A_{2A} receptor [47]. One direct consequence of the interruption of superoxide anion formation and respiratory burst reactions is the protection of vascular endothelial cells from neutrophil-mediated injury [48].

The adenosine-A_{2A}-receptor-mediated effects on neutrophil function are dose-related. At concentrations similar to those required to inhibit the release of superoxide anions, adenosine, acting through A_{2A} receptors, inhibits adherence to endothelial cells by stimulated neutrophils [49]. This may be related in part to dose-related preferential recruitment of receptor subtype, since the adenosine A₁ receptor exhibits many opposing physiological functions to those mediated by the A_{2A} receptor, including stimulation of neutrophil adherence to endothelial cells. Adenosine also inhibits the release of vascular endothelial growth factor from neutrophils, thereby enhancing vascular permeability [50]. The dose-dependent response in adenosine action is also seen with Fc-gamma-receptor-mediated neutrophil phagocytosis, which is enhanced by A₁ receptor stimulation but inhibited via A₂ receptors [51]. In addition, adenosine also inhibits the TNF-induced generation of elastase by neutrophils [52].

Expression of adhesive molecules is an important event that guides neutrophil recruitment into an inflammatory site through adhesion to the vascular endothelium.

Adenosine has been known to be a modulator of the expression or function of adhesive molecules including β_2 -integrin, L-selectin, and CD11b/CD18 [49,53,54]. The activity of adenosine in the modulation of neutrophil adhesion again demonstrates the opposing roles of A_1 and A_2 receptors [49].

Macrophages

Cells of the monocyte-macrophage series are abundant in the rheumatoid synovium and pannus and contribute significantly to the tissue damage seen in both acute and chronic disease, as recently reviewed by Kinne and colleagues [55]. Macrophages, the differentiated tissue form, are also critical producers of cytokines that play a prominent role in promoting proinflammatory responses that culminate in tissue damage. Like neutrophils, their capacity to phagocytose opsonized particles and to generate superoxide anions plays a major role in eliciting tissue damage. Inhibition of Fc-gamma-receptor phagocytic activity in cultured monocytes is exhibited by adenosine at high concentrations such as that seen with tissue damage and is a function mediated via adenosine A_2 receptors, while low concentrations of adenosine have the opposite effect on Fc-gamma-receptor phagocytic activity mediated via adenosine A_1 receptors [56]. Similarly, adenosine inhibits the generation of superoxide anions by monocytes stimulated with *N*-formyl-leucyl phenylalanine [57].

One of the well known though uncommon side effects of methotrexate treatment is the formation of subcutaneous nodules, often similar in histological appearance though not in distribution to those found in rheumatoid disease. A hallmark of these subcutaneous nodules is the existence of the multinucleated giant cell, formed by fusion of macrophages. The fusion of macrophages into multinucleated giant cells is enhanced by stimulation of the adenosine A_1 receptor and is inhibited by activation of the A_2 receptor [58,59].

The recent success of anti-TNF therapy highlights the role of cytokines as important mediators of inflammatory activity. Not surprisingly, methotrexate, still one of the most effective disease-modifying antirheumatic drugs for the treatment of RA, acting through the release of adenosine, also inhibits the production of TNF- α , although the adenosine receptor involved in this action remains controversial [60–63]. Modulation of cytokine production by adenosine extends far beyond TNF- α and includes observable effects on IL-6, IL-8, IL-10, IL-12, and macrophage inflammatory protein-1 α (MIP-1 α) [40,64,65]. Cytokines themselves can regulate the expression of adenosine receptors on monocytic cells and thereby modulate adenosine-mediated responses, as we and others have recently shown [66,67]. Macrophage production of nitric oxide and nitric oxide synthase is also inhibited by adenosine, probably via A_{2B} receptors [65,67].

Endothelial cells

Endothelial cells are effective transit barriers between vessels and tissue and as such are notable in inflammation not only because of their expression of adhesive molecules, which allow leukocytes their access to inflammatory sites. The effectiveness of this barrier function relies in part on the preservation of impermeability to circulating cells homing in to take part in inflammatory reactions in the tissues. Adenosine enhances this barrier function by decreasing endothelial permeability via A_{2B} receptor and helps limit potential tissue damage [68,69]. Production of inflammatory cytokines such as IL-6 and IL-8 and expression of adhesive molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin by endothelial cells are also suppressed by adenosine [70]. Another important aspect of inflammation lies in the proliferation and migration of endothelial cells in the process of angiogenesis, which is enhanced by the presence of adenosine, probably acting through A_2 receptors [71–73]. Adenosine may also induce apoptosis of endothelial cells, thus potentially enhancing the extravasation of inflammatory fluids [74].

Humoral and cellular immune responses

Rheumatoid factor, or autoantibodies directed against the Fc portion of IgG, is a hallmark of RA, although its exact role in the pathogenesis of the disease has been debated. The effect of methotrexate on the levels of circulating IgM rheumatoid factors has also been controversial. While some workers have reported no suppression of serum rheumatoid factor levels with methotrexate treatment, Alarcon *et al.* observed significant drops in the levels of both IgM and IgA rheumatoid factors in methotrexate-treated patients, and particularly of the concentration of IgM rheumatoid factor in those who showed clinical improvement [75]. These findings were confirmed by other groups in studies done both *in vivo* and *ex vivo* [76–80], although it is unclear whether this is a primary or secondary effect of adenosine.

T lymphocytes have received much attention in relation to the pathogenesis of RA and opinions differ in their contribution to the causation of the disease. The presence of these cells in the affected synovium and the strong ethnicity-dependent HLA-DR associations implicate T lymphocytes as key players in the disease process. One possible explanation of the beneficial actions of methotrexate in RA is the diminution of both the size and reactivity of the T-lymphocyte population. There are suggestions that this may be accomplished by the induction of apoptosis in activated T cells [24]. This suggestion is consistent with the observations of reductions in peripheral blood T and B lymphocyte populations after short-term methotrexate treatment [81], and methotrexate induction of apoptosis in inflammatory cells may be relevant to its antirheumatic actions *in vivo* [82]. In contrast, significant increases in the CD3- and CD4-positive peripheral blood cells and

enhancement of stimulated lymphocyte proliferation have been observed after long-term treatment with methotrexate [83], and adenosine, acting through A_{2A} and A_{2B} receptors, may play a role in T-cell deactivation [84,85]. Nonetheless, the role of these shifts in T-cell function and trafficking in the pathogenesis of RA is unclear.

Phlogistic responses

Cytokines are messengers with major roles in inflammatory and immune responses and have been targets of interest in recent therapeutic developments in chronic arthritis, with TNF- α and IL-1 as the focus of interest [86]. In animal models of chronic arthritis, methotrexate was thought to be useful in reducing the production of IL-1 [87,88]. In support of these findings, clinical studies of RA patients receiving methotrexate treatment have documented reductions in monocytic IL-1 production but not serum concentrations of IL-1 [89]. Others have disputed this view and suggested that alterations in IL-1 responses were related to diminutions in the ability of cells to respond to IL-1 rather than to direct inhibition of its production, perhaps through dose-dependent ligand binding [90–92].

Methotrexate is also known to suppress TNF activity by suppressing TNF-induced nuclear factor- κ B activation *in vitro*, in part related to a reduction in the degradation and inactivation of an inhibitor of this factor, I κ B α , and probably related to the release of adenosine [93]. The generation of TNF- α by peripheral blood mononuclear cells is suppressed by an adenosine kinase inhibitor, by virtue of its ability to limit adenosine uptake and metabolism and thereby enhance extracellular adenosine concentration [94]. TNF- α synthesis in T cells and macrophages is suppressed [95]. In the murine collagen-induced arthritis model, *in vivo* intraperitoneal methotrexate treatment reduced TNF serum levels and diminished TNF production by splenic T cells and macrophages [96]. Methotrexate suppresses the production of both TNF and IFN- γ by T-cell-receptor-primed T lymphocytes from both healthy human donors and RA patients [97]. In early RA, in which the disease duration is less than 6 months, methotrexate treatment is associated with a significant decrease of TNF- α -positive CD4⁺ T cells, while the number of T cells expressing the anti-inflammatory cytokine IL-10 increased [98]. Methotrexate is also known to suppress the IL-6-induced generation of reactive oxygen species in the synovocytes of RA patients [99]. Serum IL-6 levels have also declined after methotrexate treatment in RA patients in some studies [100]. Constantin *et al.* reported that *ex vivo* treatment of peripheral blood monocytes with methotrexate increased expression of IL-4 and IL-10 while IL-2 and interferon- γ expression were decreased, suggesting that the immunoregulatory role of methotrexate is also targeted at adjusting the balance between Th1 proinflammatory and Th2 anti-inflammatory cytokines [101]. Again, the molecular mechanism of these changes is unclear.

Conclusion

Our search for mechanisms governing the inflammatory response has uncovered many facets relevant to the pathogenesis of arthritic diseases. The success of methotrexate as an antirheumatic agent rests on its many actions that affect a wide variety of pathogenic mechanisms, many of which are mediated by the release of adenosine. The molecular mechanism for many of these phenomena is related to the enhanced release of adenosine into the extracellular space, where it can activate its receptors on relevant cell types. In this respect, methotrexate is an excellent example of how knowledge and continuing research in molecular biology and pharmacology can be employed in the refinement of existing medications originally used on an observational basis. Such understanding will form the basis for the development of new and more effective therapy for the treatment of rheumatic diseases.

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