Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis

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Abstract

Objective. The mechanism of anti-inflammatory effects of methotrexate (MTX) at low dose may relate to a decrease in availability of the purine precursor or it may depend on accumulation of 5-aminoimidazole-4-carboxamide (AICAR) and the anti-inflammatory nucleoside adenosine. The aim of this study was to evaluate the possible mechanism of action by analysis of changes in blood concentrations of purine and pyrimidine metabolites during MTX treatment.

Methods. Venous blood samples were collected from rheumatoid arthritis patients before and at different times for up to 7 days after the start of MTX treatment. Whole blood concentrations of adenosine, uridine, hypoxanthine, uric acid and erythrocyte nucleotides were measured by HPLC.

Results. The initial blood adenosine concentration was \(0.073 \pm 0.013 \mu M\) and no differences were observed during MTX treatment. However, a decrease in uric acid concentration was observed from \(205.5 \pm 13.5\) to \(160.9 \pm 13.5 \mu M\) \((P < 0.05)\) within 24 h after MTX administration. The hypoxanthine concentration decreased in parallel with uric acid, while the uridine concentration decreased 48 h after MTX administration. No accumulation of AICAR-triphosphate (ZTP) was observed in the erythrocytes.

Conclusions. MTX decreases circulating purine and pyrimidine concentrations, and their availability for DNA and RNA synthesis, which may affect immune cell proliferation and protein (cytokine) expression. The absence of adenosine concentration changes and lack of ZTP formation is evidence against an AICAR/adenosine mechanism, although localized adenosine concentration changes cannot be excluded.

Key words: Methotrexate, Rheumatoid arthritis, Adenosine, Uric acid, Purines, Pyrimidines.

Methotrexate (MTX), originally applied for the treatment of cancer \([1]\), is now widely used as an anti-inflammatory and immunosuppressive agent in the treatment of rheumatoid arthritis (RA) and other chronic inflammatory disorders \([2]\). The mechanism of action of MTX treatment in RA requires detailed re-examination since the dose used is several orders of magnitude lower than in the treatment of cancer. A well-established anti-proliferative effect of MTX on cells involved in autoimmune reactions due to inhibition of dihydrofolate reductase and folate-dependent transformations was proposed initially to explain the mechanism of action \([3]\). According to this concept, inhibition of mononucleotide precursors of nucleic acids, particularly at the step of methylation of dUMP into dTMP by thymidylate synthetase, caused disruption of DNA synthesis and inhibition of the proliferation of cells involved in the inflammatory process in the joints. However, it has been noted in some experimental models that changes induced by MTX in \textit{de novo} purine synthesis included considerable accumulation of intermediates such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) resulting from inhibition of the AICAR transformylase reaction \([4]\). This in turn, via inhibition of adenosine deaminase and AMP deaminase or activation of ecto-5′-nucleotidase, could lead to an increased production of the anti-inflammatory and immunosuppressive adenosine \([5–7]\) which has been suggested as an alternative explanation. A number of other important observations have been made regarding the biochemical mechanisms of action of MTX, such as changes in interleukin levels during MTX treatment \([8]\), changes in T-lymphocyte, monocyte and synovial cell...
surface antigen expression [9], increase in blood homocysteine levels [10], decreased polyamine production in lymphocytes [11], induction of apoptosis and clonal deletion of activated peripheral T lymphocytes [12]. All these changes could be vital for the anti-inflammatory effect of MTX, but may be also secondary to changes in nucleotide metabolism of the cell. Since clinical data concerning the biochemical events associated with MTX administration are limited, the aim of the present study was to evaluate possible mechanisms of action of low-dose MTX by the determination of changes in blood nucleotide metabolite levels in RA patients treated with MTX at the initial phase of chronic treatment.

**Methods**

**Patients**

Seven patients (age 30–60 yr, all women) diagnosed with RA stage II/III (according to ARA criteria) and qualified for MTX treatment were recruited to the study. Venous blood samples were collected 1 day before, immediately (1 h) before and at different times (2 h, 24 h, 48 h, 1 week) after a first 7.5 mg oral dose of MTX starting prolonged weekly treatment with this drug. Blood cysteine levels [10], decreased polyamine production in surface antigen expression [9], increase in blood homo-

**Analysis of nucleotides, nucleosides and bases by HPLC**

Samples were thawed, mixed and centrifuged for 3 min at 13 000 g in a microcentrifuge. Supernatants were collected and neutralized with 3 m \(\text{K}_2\text{PO}_4\). After a subsequent centrifugation step (13 000 g, 1 min), potassium perchlorate precipitate was removed and the supernatant was injected onto the chromatograph. Concentrations of adenosine, hypoxanthine, uric acid and uridine were evaluated using reversed-phase HPLC as described in detail previously together with information about the reproducibility and recovery of the procedure [13, 14]. The reproducibility of sample extraction and the analysis procedure in the current series of analyses, expressed as the coefficient of variation, was 15.8% for adenosine and <5% for all other metabolites studied. Erythrocyte nucleotide concentrations [ATP, UTP, GTP, CTP, AICAR-triphosphate (ZTP)] were measured by anion-exchange HPLC [15]. UV-absorbing peaks in both the reversed-phase and anion-exchange systems were quantified from the absorbance at 254 nm relative to standards of known concentrations. Peak identity was confirmed by retention time coupled with analysis of diode array detector spectra.

**Discussion**

This study demonstrates a significant decrease in purine and pyrimidine metabolite concentrations immediately after low-dose MTX administration in patients with RA. The marked decreases in uric acid and hypoxanthine concentrations observed could be an indication of inhibited purine synthesis, which is a well-established mechanism of action of MTX in cancer. On the other hand, no change in adenosine concentration and the absence of ZTP formation in the erythrocytes shown in this study do not support the recently proposed AICAR/adenosine role in the mechanism of action of MTX.

Inhibition of purine nucleotide precursor synthesis by MTX is a primary effect of this drug, but it has been clearly confirmed only after high-dose MTX administration in the treatment of cancer [16]. Our study provides evidence that inhibition of purine synthesis and the consequent fall in its blood concentrations could also be important after low-dose MTX administration in patients with RA. However, the degree of purine synthesis inhibition or specific processes affected after low-dose MTX treatment must be different. Unlike high-dose MTX in malignancies, no significant cytotoxic effect occurred and blood uric acids were lowered not elevated. The gross elevation of uric acid which can occur due to excessive nucleic acid degradation from dead cells after high-dose MTX in cancer is well documented [17]. An alternative explanation for the uric acid-lowering effect we noted in RA could be its increased urinary excretion, but parallel falls in hypoxan-
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Fig. 1. Adenosine (A), uric acid (B), hypoxanthine (C) and uridine (D) concentrations in venous blood during methotrexate (MTX) treatment in patients with rheumatoid arthritis. Values are means ± s.e.m., n = 7. *P < 0.05 in comparison to 24 h pre-, 1 h pre- and 1 week post-MTX administration; #P < 0.05 in comparison to 1 h pre- and 1 week post-MTX administration; &P < 0.05 in comparison to 1 h pre-MTX administration; §P < 0.05 in comparison to the value 24 h pre-, 1 h pre- and 2 h post-MTX administration.

thine concentration support the inhibition of purine synthesis as the mechanism. However, a decrease in hypoxanthine concentration could also be caused by its increased salvage, but this again indicates inhibition of de novo synthesis by MTX which would be the most likely reason for that. Further studies focused on uric acid clearance in RA patients on low-dose MTX are necessary to clarify this problem.

An alternative explanation for our proposal of nucleotide precursor starvation by low-dose MTX focuses on adenosine as the effector metabolite in RA [6, 7]. It was suggested that this increase in adenosine production is mediated by accumulation of the purine de novo intermediate AICAR and its dephospho product 5-aminomimidazole-4-carboxamide ribonucleoside (AICAr). If the animal model used is applicable to humans, irrespective of in which part of the body AICAR or AICAr accumulation occurs, we should have been able to observe formation of the AICAR triphosphate ZTP in the circulating erythrocytes. Excessive AICAR accumulation in inherited hypoxanthine-guanine phosphoribosyltransferase deficiency (Lesch–Nyhan syndrome) is detectable in the erythrocytes as ZTP [18]. No accumulation of ZTP was observed at any stage of MTX treatment in our patients, nor were we able to show any direct effects of MTX on blood adenosine concentration. These findings argue against the AICAR/adenosine-mediated hypothesis of action of MTX which was developed based on animal and cell culture studies [6, 7]. However, the possibility cannot be excluded that localized changes in adenosine concentration may occur in the joint, not mediated by AICAR and without systemic changes being noted. A decrease in synovial fluid adenosine has been described in patients with RA, suggesting that this increase in adenosine production is mediated by accumulation of the purine de novo intermedi-ate AICAR and its dephospho product 5-aminomimidazole-4-carboxamide ribonucleoside (AICAr). If the animal model used is applicable to humans, irrespective of in which part of the body AICAR or AICAr accumulation occurs, we should have been able to observe formation of the AICAR triphosphate ZTP in the circulating erythrocytes. Excessive AICAR accumulation in inherited hypoxanthine-guanine phosphoribosyltransferase deficiency (Lesch–Nyhan syndrome) is
Fig. 2. Anion-exchange HPLC chromatograms of blood extracts from a patient with rheumatoid arthritis at different times after methotrexate (MTX) administration and from a patient with hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency. An accumulation of ZTP, typical for HGPRT deficiency, was not observed after MTX administration.
of recent experimental data [7]. Clarification of the role of adenosine in the mechanism of action of MTX, if any, is of prime importance as there are several alternative means to control adenosine concentrations [20–22]. A number of adenosine receptor agonists have also been developed [23]. These two approaches may provide new avenues for the treatment of RA avoiding the frequent toxic effects of MTX [24] if the adenosine hypothesis is correct.

It is difficult to relate observed changes in purine and pyrimidine levels directly to pharmacokinetics of MTX, since metabolic effects of MTX could be attributed predominantly to its polyglutamated derivatives which are formed inside the cells. MTX clearance from blood is rapid [25], but its polyglutamate derivatives can accumulate to a various extent in different types of cells and may interfere with purine and pyrimidine metabolism for a prolonged time following the initial dose. Some metabolic or physiological changes in the course of MTX treatment in RA were already observed 1 day after the first dose, while the other changes were observed only after several weeks. Clinical effects are typically noted within a month of treatment [26].

Consideration must be given to the possible role of decreased levels of uric acid and hypoxanthine in the mechanism of action of MTX. An association of increased uric acid concentration with joint inflammation is well known in gout [27]. A decrease in uric acid concentration after MTX administration may thus contribute to the anti-inflammatory effect of MTX, but is unlikely to be a primary mechanism. Marked differences in the clinical course of RA and gouty arthritis make any such link unlikely.

In summary, our results suggest that inhibition of nucleotide precursor synthesis and decreased uric acid concentrations are involved in the mechanism of action of MTX in patients with RA. We have not found any evidence supporting the adenosine-mediated mechanism, but localized changes in adenosine concentration were not evaluated. Further studies are thus necessary to establish the effect of MTX on local metabolite levels and on metabolic pathways in humans, including changes in enzyme expression, to clarify the mechanism of action of low-dose MTX in vivo.

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References