Fibroblasts from methotrexate-sensitive mice accumulate methotrexate polyglutamates but those from methotrexate-resistant mice do not

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ABSTRACT

Objective. *We and others have previously demonstrated that methotrexate (MTX) mediates its anti-inflammatory effects through an increase in cellular release of adenosine. Consistent with this observation, there is no increase in adenosine from exudates of mouse strains resistant to MTX. Because intracellular MTX polyglutamates inhibit AICAR transformylase (ATIC) activity and thereby promote adenosine release we determined whether there is any difference in the capacity of cells from MTX-resistant mice to accumulate MTX polyglutamates.*

Methods. *Dermal fibroblasts (DF) from BALBc, MTX-sensitive, and DBA/1J, MTX-resistant, mice were cultured in the presence or absence of MTX. Adenosine concentration in the supernatant and intracellular MTX polyglutamate (MTXPG1-5) concentrations were measured by liquid chromatography. ATIC activity in DF was monitored spectrophotometrically by the formation of formytetrahydrofolate.*

Results. *MTX (1 μM) increased adenosine production by DF from BALBc sensitive-mice from 269±40 nM to 446±4 nM. No adenosine production was found in supernates of cultured DF from DBA/1J mice regardless of MTX treatment. Intracellular MTX polyglutamates (MTXPG2-4) were detected only in BALBc DFs, not in DBA/1J DF. Further investigation demonstrated that ATIC activity was inhibited following MTX treatment in DF from BALBc mice.*

Conclusions. *These data suggest that resistance to the anti-inflammatory effects of MTX could be due to diminished MTX polyglutamate accumulation resulting in diminished ATIC inhibition and adenosine accumulation.*

Introduction

Methotrexate (MTX) is the base therapy for treatment of rheumatoid arthritis (RA). Despite the introduction of a number of effective biological agents as powerful alternatives to traditional disease modifiers in the past decade, MTX remains one of the most effective and commonly used therapies for RA (1). Increasing evidence indicates that MTX promotes extracellular adenosine accumulation (1) at sites of inflammation and adenosine mediates the anti-inflammatory effects of MTX (1).

At low doses MTX is taken up by cells and then converted to MTX polyglutamates (MTXPG) by the catalytic action of folylpolyglutamate synthetase (FPGS) (2). MTXPG are long-lasting metabolites which inhibit 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase (ATIC), an enzyme in the *de novo* purine synthetic pathway, and thus enhances the intracellular accumulation of AICAR (3). AICAR is a competitive inhibitor of AMP deaminase leading to accumulation of AMP which is exported from cells leading to enhanced adenosine concentrations in the extracellular fluid (4). Adenosine binds to specific adenosine receptors expressed on the surface of various types of cells; adenosine receptor occupancy modulates a large array of physiological functions as well as mediating adenosine anti-inflammatory actions (5).

We have previously demonstrated that MTX promoted extracellular adenosine accumulation at sites of inflammation and adenosine subsequently suppressed inflammation in the air pouch model of inflammation in C57BL/6 and BALBc, but not in DBA/1J mice (6). In this study, we explored the differences in MTX metabolism between inbred strains of mice that might lead to the differences in the anti-inflammatory response.

Materials and methods

Mouse dermal fibroblasts from BALBc and DBA/1J mice were isolated from adolescent abdominal skin. Briefly, the dermis was separated from the epidermis with forceps. The dermis sheet was then cut and seeded into 75-cm^2 tissue culture flasks in DMEM supplemented with penicillin G (100 kU/L), streptomycin (100 mg/L), and foetal bovine serum (10%). The cells were cultured at 37° C in a humidified CO_2 (5%) incubator and passaged with 1:3 dilutions. Homogenous DFs at passage 3 were used for the experi-

ments that consisted of treatment with or without MTX at a final concentration of 10^{-6}M. Adenosine concentration was determined by reverse-phase high-performance liquid chromatography (HPLC) (2, 9) while intracellular MTXPG concentrations were measured (in a blinded fashion) using an HPLC-fluorometry procedure with a post-column photooxidation technique (7). MTXPG results were normalised to $5x10^6$ cells with all samples analysed in duplicate. MTXPG with two to four glutamic residues ($MTXPG_{2-4}$) constituted the MTXPG polyglutamates pool. Adenosine concentration was determined by HPLC, as previously described (8). ATIC activity in dermal fibroblasts was determined as described by Baggott and colleagues (4). For animal experiments, all procedures were reviewed and approved by the Institutional Animal Care and Use Committee of NYU Medical Center and were performed under the supervision of the facility veterinary staff. Differences between the BALBc group and DBA1J group in terms of adenosine levels, MTXPGn concentrations, and AICAR TF activity were tested using independent *t*-tests.

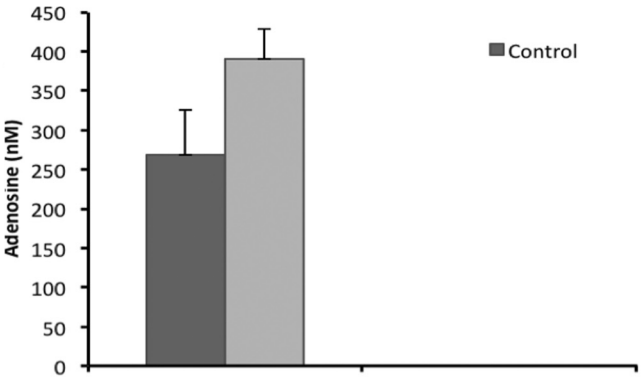
Results

MTX treatment increases adenosine production by fibroblasts from BALBc (MTX-sensitive) mice, not DBA/1J (MTX-resistant) mice

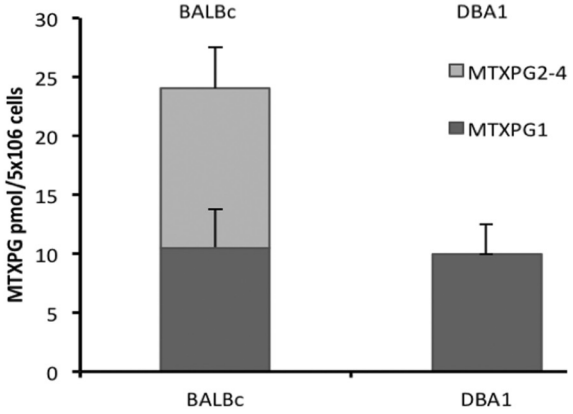
We first measured the adenosine levels produced by MTX-treated DF from MTX-resistant and MTX-sensitive mice. Our results indicated that MTX treatment (10^{-6} M) increased adenosine production by fibroblasts from BALBc (MTX-sensitive) mice (1.66-fold increase from 269±40 nM to 446±4 nM, Fig. 1A). In contrast, adenosine was undetectable in supernatant collected from cultured DF from MTX-resistant DBA/1J mice ($p<0.01$ *vs*. BALBc). This result is in accordance with previous *in vivo* studies in the air-pouch model of acute inflammation in which a marked MTX-induced decrease in leukocyte counts and increase in adenosine concentrations was observed in the exudates from BALBc mice but not in those from DBA/1J mice.

Fig. 1

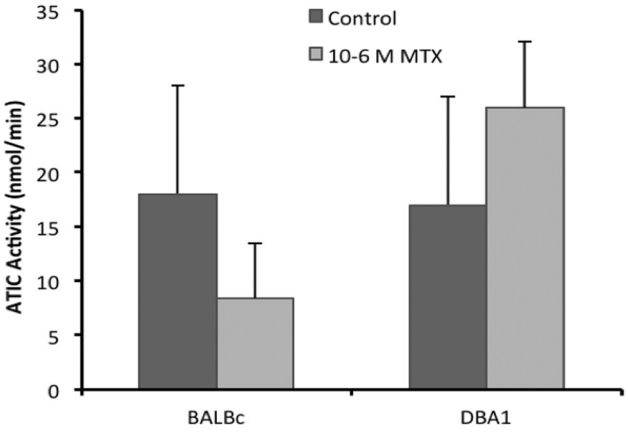
A. Supernatant adenosine levels in DF from BALBc and DBA1J mice with or without treatment with MTX for 48 hours. Supernates from DBA/1J mouse do not accumulate adenosine.



B. MTX polyglutamates in DF from BALBc and DBA/1J mice following 48h incubation with MTX. DBA/1J mice do not accumulate methotrexate polyglutamates.



C. ATIC activity in DF from BALBc and DBA1J mouse following MTX. ATIC activity in DBA/1J was not inhibited by MTX.



MTXPG accumulated in fibroblasts from BALBc (MTX-sensitive) mice, not DBA/1J (MTX-resistant) mice

MTX polyglutamates ($MTXPG_{2-4}$) following MTX treatment (10^{-6} M) for 48 hours (Fig. 1B) were detectable in lysates of BALBc dermal fibroblasts following incubation with MTX but only $MTXPG_1$ was found in DBA/1J fibroblast lysates. There was no difference in intracellular $MTXPG_1$ concentration at MTX 10^{-6}M between BALBc and DBA/1J DF lysates (13±3 *vs*. 13±3 pmol/$5x10^6$ cells, $p>0.05$) while significant $MTXPG_{2-4}$ was accumulated in BALBc fibroblasts compared to DBA/1J fibroblasts ($p<0.05$).

MTX suppressed ATIC activity in DF from BALBc (MTX-sensitive) mice, not DBA/1J (MTX-resistant) mice

To test whether MTX treatment regulates the activity of a key enzyme function for the production of adenosine we measured ATIC activity in DF of MTX-sensitive mice and MTX-resistant mice (Fig. 1C). MTX treatment of DF for forty-eight hours inhibited ATIC activity in DF from BALBc mice in a dose-dependent manner from 18±5

nmol/min to 8±4 nmol/min, $p<0.05$. In contrast, MTX treatment did not diminish ATIC activity in DF from DBA/1J mice but may even have enhanced the activity from 17±5 nmol/min, to 28±6 nmol/min.

Discussion

Prior studies have demonstrated that adenosine mediates many of the anti-inflammatory effects of MTX (1). We have previously reported that adenosine concentrations are higher in the inflammatory exudates of MTX-treated mice and that elimination of adenosine or antagonism or deletion of adenosine receptors reverses the anti-inflammatory effects of MTX treatment. Nonetheless, many patients do not respond to MTX treatment and we have previously reported that at least one strain of mice, DBA/1J (the strain which develops collagen-induced arthritis, a condition which does not respond to pharmacologically relevant methotrexate doses), is also resistant to the anti-inflammatory effects of MTX. The poor anti-inflammatory effects of MTX in this strain of mice results from the inability of MTX to induce adenosine release at inflamed sites (9). The experiments reported here provide evidence that in these resistant mice MTX polyglutamates do not accumulate intracellularly resulting in less inhibition of ATIC and adenosine release.

Numerous studies have demonstrated that in order for MTX to mediate its pharmacologic effects it must accumulate in cells and tissues. MTX is taken up by cells via the reduced folate carrier (RFC1) whereupon it is polyglutamated and the MTX polyglutamates, long-lived metabolites, mediate the anti-inflammatory effects of the drug (10, 11). The enzymatic step best inhibited by MTX polyglutamates is the formylation of AICAR by ATIC leading to intracellular accumulation of AICAR (4). We observed that the absence of MTX polyglutamates in the cells from the DBA/1J mice was associated with undiminished ATIC activity in contrast to fibroblasts from the susceptible strain in which ATIC is inhibited by MTX treatment in a dose-dependent fashion. AICAR, which accumulates when ATIC is inhibited, competitively inhibits AMP deaminase leading to adenine nucleotide release and extracellular adenosine formation (4). The results reported here, therefore, provide a biological explanation for the poor anti-inflammatory effects of MTX in DBA/1J mice and provide further indirect support for the hypothesis that adenosine mediates the anti-inflammatory effects of MTX therapy.

In summary, we report the critical association of intracellular MTX metabolism with resistance to the anti-inflammatory effects of the drug. The correlation between the absence of MTX polyglutamate accumulation, poor adenosine release, diminished MTX-mediated suppression of AICAR tranformylase and diminished anti-inflammatory effects of MTX treatment provide further evidence that adenosine mediates the anti-inflammatory effects of MTX therapy in rheumatoid arthritis and other inflammatory diseases.

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