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Modulation of Orphan Nuclear Receptor NURR1 Expression by Methotrexate in Human Inflammatory Joint Disease Involves Adenosine A2A Receptor-Mediated Responses

Jennifer A. Ralph,* Alice N. McEvoy,* David Kane,‡ Barry Bresnihan,† Oliver FitzGerald,† and Evelyn P. Murphy2*

Modulation by proinflammatory mediators indicate that NURR1 induction represents a point of convergence of distinct signaling pathways, suggesting an important common role for this transcription factor in mediating multiple inflammatory signals. The present study identifies NURR1 as a molecular target of methotrexate (MTX) action in human inflammatory joint disease and examines the mechanism through which MTX modulates NURR1 expression. MTX significantly suppresses expression of NURR1 in vivo in patients with active psoriatic arthritis (n = 10; p < 0.002) who were prescribed low-dose MTX for management of peripheral arthritis. Importantly, reduction in NURR1 levels correlate (n = 10; r = 0.57; p = 0.009) with changes in disease activity score (both clinical and laboratory parameters). MTX selectively modulates NURR1 levels induced by inflammatory stimuli and growth factors in resident cell populations of synovial tissue. In primary human synoviocytes and microvascular endothelial cells, we observe dose-dependent differential effects of MTX on steady-state and inducible NURR1 levels. Our data confirms that adenosine, and its stable analog 5′-N-ethylcarboxamidoadenosine, can mimic the differential effects of MTX on NURR1 transcription. In addition, we verify that the inhibitory effect of low-dose MTX on NURR1 activation is mediated through the adenosine receptor A2. More specifically, our data distinguishes the selective involvement of the A2A receptor subtype in these responses. In summary, these findings establish the nuclear orphan receptor NURR1 as a molecular target of MTX action in human inflammatory joint disease and demonstrate that the immunomodulatory actions of MTX on NURR1 expression are mediated through adenosine release. The Journal of Immunology, 2005, 175: 555–565.

Methotrexate (MTX)3 was developed as a chemotherapeutic agent in the treatment of childhood acute lymphocytic leukemia in the late 1940s (1). The antiproliferative properties of high-dose MTX are due to inhibition of dihydrofolate reductase and other folate-dependent enzymes, which inhibit both purine and pyrimidine nucleotide biosynthesis (2–5). At high levels, MTX inhibits cells in the S phase of cell cycle and slows the entry of cells from the G1 phase into S phase (6, 7).

Since 1985, low-dose intermittent MTX has been demonstrated to be an effective therapy for rheumatoid arthritis (RA) and is the most commonly used disease-modifying antirheumatic drug in the treatment of this disease (4, 8). Currently, its use is increasing in other immune-mediated pathologies including psoriatic arthritis (PsA), Crohn’s disease, and graft-vs-host disease (9–13). In human inflammatory joint disease, MTX acts by reducing the levels of soluble mediators of inflammation such as metalloproteases and cytokines (14–16). MTX also significantly reduces cellular infiltration through down-regulation of cell surface adhesion molecules (17, 18). Several lines of evidence now indicate that the immunosuppressive and anti-inflammatory actions of low-dose MTX are not due to inhibition of folic acid metabolism but are mediated by its capacity to increase extracellular adenosine levels (16, 19–22). Adenosine, acting at one or more of its membrane receptors, is responsible for the anti-inflammatory actions of MTX in animal models of both acute and chronic inflammation (20, 21, 23). Despite a growing body of evidence to indicate that the mechanisms of MTX action are closely related to enhanced release of adenosine, the molecular targets modulated by MTX or adenosine action in vivo remain to be elucidated.

NURR1, together with NOR-1 and NUR77, constitute the NURR subfamily of orphan receptors within the steroid/thyroid receptor superfamily (24, 25). Unlike most nuclear receptors, the NURR subfamily are products of immediate early genes, the expression of which can be induced in response to a variety of extracellular stimuli (26–32). All three proteins bind to the same cis-acting consensus sequence (NBRE) to regulate target gene expression, and can function as constitutively active transcription factors (33–35). These genes are involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic stimuli in different cell types (28, 30, 31, 36–40). NURR1 is involved in certain diseases involving dopamine transmission including Parkinson’s disease (41). The NOR-1 gene was...
identified originally through its involvement in the chromosomal rearrangement associated with extraskeletal myoid chondrosarcoma (42). Recent studies identify the NURR subfamily as effector proteins of cytokine action (32, 39, 43, 44). Data from our laboratory and others (39, 45–47) propose that the NURR subfamily could serve as novel targets in the treatment of inflammatory arthritis, atherosclerosis, and lung cancer.

Inflammation and hyperplasia of the synovium are hallmarks of RA and PsA (48, 49). The normal synovium is a delicate tissue lining the joint capsule: however, in arthritis, the synovium transforms into an aggressive, tumor-like structure called pannus (48, 49).

Angiogenesis, the formation of new blood vessels, is one of the earliest histopathological findings in disease pathogenesis and is required for pannus development (49, 50). The endothelium lining the blood vessels is recognized as playing an active and pivotal role in the recruitment of leukocytes to the inflamed synovium (50, 51). Macrophage-derived cytokines are potent mitogens for proliferating synoviocytes (fibroblast-like) in the vicinity of the affected cartilage that produce matrix-degrading molecules (48, 49).

Synoviocytes and macrophages within the synovium orchestrate a self-perpetuating inflammatory response via the autocrine actions of cytokines (e.g., TNF-α and IL-1β), growth factors (e.g., basic fibroblast growth factor [bFGF] and vascular endothelial growth factor; VEGF), and prostaglandins (e.g., prostaglandin E2; PGE2) (48, 49, 52). It is the persistent invasive and destructive growth of synovial tissue (ST) that ultimately leads to joint erosion (48, 49).

Markedly enhanced expression of NURR1 is observed in ST of patients with RA and PsA as compared with normal subjects (32). Enhanced NURR1 expression in the synovial lining layer (LL), subsynovial synoviocytes, and vascular endothelium suggests that NURR1 is expressed primarily in cells believed to be at the leading edge of invasive pannus (32). Modulation by proinflammatory mediators in primary and normal synoviocytes suggests that PGE2, TNF-α, and IL-1β markedly enhance NURR1 mRNA and protein levels in contrast to other family members, NUR77 and NOR-1 (39). NURR1 induction represents a point of convergence of distinct signaling pathways, suggesting an important common role for this transcription factor in mediating multiple inflammatory signals (39).

In this study, we report that MTX significantly suppresses NURR1 expression in vivo in patients with PsA (n = 10) who were prescribed low-dose MTX for management of peripheral arthritis. Importantly, reduction in NURR1 levels correlate with changes in both clinical and laboratory parameters. We investigated whether MTX modulates endogenous NURR1 levels induced by inflammatory stimuli and growth factors in resident cell populations of ST. In primary synoviocytes and microvascular endothelial cells, we observe dose-dependent differential effects of MTX on NURR1 levels. At pharmacologically relevant doses, MTX differentially modulates induced NURR1 mRNA and protein levels. Our data confirms that adenosine can mimic the differential effects of MTX on steady-state and inducible NURR1 expression. Finally, we confirm that the inhibitory effects of low-dose MTX on NURR1 activation are mediated through the adenosine receptor type A2A.

Materials and Methods

Patient details and tissue collection

All ST biopsies were obtained from patients with inflammatory arthritis and active knee synovitis who were undergoing knee arthroscopy. PsA was defined according to established criteria (53). All patients attended the Early Arthritis Clinic at St. Vincent’s University Hospital (Dublin, Ireland), and ethical permission was obtained from the Ethics Committee in accordance with the Declaration of Helsinki principles. MTX was commenced at a dose of 7.5 mg/wk P.O. and could be increased by the supervising rheumatologist to a maximum of 15 mg/wk, depending on the degree of clinical response and/or side effects. Folic acid supplementation was also prescribed to all patients (54). Patients were continued on stable nonsteroidal anti-inflammatory drugs. Intra-articular steroids were contraindicated for the duration of the study.

Biopsies were taken before MTX treatment and between six and 12 mo during MTX treatment, either when a clinical response had been obtained or a maximal dose of 15 mg of MTX had been prescribed. At the time of the first biopsy, all patients (n = 10) had active knee synovitis (median, 18 mo; range, 0.25–168 mo). Patients received a weekly dose of MTX (median, 13.75 mg/wk) and were not receiving any other disease-modifying agents, including prednisolone. Patients were assessed on the day of each arthroscopy. Assessments included the Ritchie articular index (RI) (55), the European League Against Rheumatism swollen joint count for the involved knee (56), and the erythrocyte sedimentation rate (ESR) (measured by standard Westergren technique), the C-reactive protein (CRP) level (measured by standard nephelometry), and the 3-variable Disease Activity Score (DAS) (56).

Arthroscopic synovial biopsy of the knee was performed on patients under local anaesthesia using a 2.7-mm Storz arthrooscope and 1.5-mm guiding sheaths. Biopsies of the knee were processed in a similar manner (32, 48, 49). ST obtained from the knee was treated for 4 h with 1 mg/ml collagenase (type I; Worthington Biochemical) in RPMI 1640 at 37°C in 5% CO2. Dissected synovium was stored in RPMI 1640 supplemented with 10% FCS (Invitrogen Life Technologies), penicillin (100 U/ml), and streptomycin (100 U/ml) (52). To eliminate nonadherent mononuclear cells from synovial cell populations, the plated cells were cultured for at least 24 h. The cells were then washed extensively with PBS. Primary ST synoviocyte cultures were used between the third and seventh passages for subsequent experiments (32, 52). Primary ST synoviocyte cultures were then treated with 1 mg/ml dexamethasone, adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), alloxazine, VEGF, bFGF, PGE2, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 3,7-dimethyl-1-propargylxanthine (DMPX; Sigma-Aldrich), recombinant human TNF-α, IL-1β (Calbiochem), 4-[(2-amino-2-[(2-furyl)-1,2,4]triazolo[2,3-a][1,3,5]triazin-8-y]methyl)phosphonylethanol (ZM-241385), and N-[9-chloro-2-(2-furyl)-1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylbenzene (MBI-1220; Tocris Cookson) were resuspended according to the manufacturer’s guidelines and included at concentrations as indicated. The concentration of MTX and duration of exposure used had no effect on cell viability (data not shown) (22, 59).

Immunohistochemistry

ST immunohistochemistry was conducted on matched tissue sections from biopsies taken before and during MTX treatment (n = 10). The primary Ab (anti-NURR1; Santa Cruz Biotechnology) was used at 1:100 dilution. ST synoviocytes were identified by Ab mapping to the amino terminus of human and rat NURR1 (32, 39). Tissue sections were incubated in diluted normal goat serum (Vector Laboratories) for 1 h. The primary Ab for NURR1 was diluted (1:100) in normal human serum and incubated on the tissue sections for 1 h. Following the addition of biotinylated anti-rabbit secondary Ab (1:500) (Vector Laboratories), sections were incubated with the avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). For negative controls, isotype-matched nonimmune IgG were included, and the primary Ab was preabsorbed with its specific synthetic peptide (blocking peptide; Santa Cruz Biotechnology) (32, 39).

Immunocytochemistry on primary PsA synoviocytes was conducted using identical procedures as that used for ST immunohistochemistry. Primary cells were grown in serum-free medium for 24 h before stimulation. MTX, the knee joint, embedded in TissueTek OCT compound (Sakura Finetek), snap frozen, and stored in liquid nitrogen until used. Cryostat sections (7 μM) were mounted on 3-amino-propyltriethoxysilane-coated glass slides, air dried overnight, wrapped in foil, and stored at −80°C until immunohistochemical analysis was performed.

Cell culture

ST obtained from the knee by arthroscopy was treated for 4 h with 1 mg/ml collagenase (type I; Worthington Biochemical) in RPMI 1640 at 37°C in 5% CO2. Dissociated cells were plated in RPMI 1640 supplemented with 10% FCS (Invitrogen Life Technologies), penicillin (100 U/ml), and streptomycin (100 U/ml) (52). To eliminate nonadherent mononuclear cells from synovial cell populations, the plated cells were cultured for at least 24 h. The cells were then washed extensively with PBS. Primary ST macrophages were used between the third and seventh passages for subsequent experiments (32, 52). Primary PsA synoviocyte and macrophage cell lines were then cultured using EGM-MV BulletKit (BioWhittaker) (58). All cells were grown in serum-free medium for 24 h before stimulation. MTX, dexamethasone, adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), alloxazine, VEGF, bFGF, PGE2, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 3,7-dimethyl-1-propargylxanthine (DMPX; Sigma-Aldrich), recombinant human TNF-α, IL-1β (Calbiochem), 4-[(2-amino-2-[(2-furyl)-1,2,4]triazolo[2,3-a][1,3,5]triazin-8-y]methyl)phosphonylethanol (ZM-241385), and N-[9-chloro-2-(2-furyl)-1,2,4]triazolo[2,3-a][1,3,5]cquinazolin-5-ylbenzene (MBI-1220; Tocris Cookson) were resuspended according to the manufacturer’s guidelines and included at concentrations as indicated. The concentration of MTX and duration of exposure used had no effect on cell viability (data not shown) (22, 59).

Microscopic analysis

Only ST sections in which the LL was identifiable were included in the analysis. Two people, who were blinded to the identities of the sections at
the time of scoring, evaluated all tissue sections randomly. The results of the analysis were scored on a 0–5 scale: 0, <1% positive cells; 1, 1–10%; 2, 11–25%; 3, 26–50%; 4, 51–75%; and 5, 76–100%. Scoring was performed in randomly selected high-power fields (hpf) at ×400 magnification; a minimum of 17 hpf from three separate sections and the mean score were calculated. For each hpf, a separate score for percentage positive cells was made for LL, sublining (SL) layer, and blood vessels. Percentage degree of localization of NURR1 (cytoplasmic and nuclear) was also evaluated (data not shown). These techniques have been validated previously and reported (60–62).

**Northern blot analysis**

Cell cultures grown in 25-cm² tissue culture flasks were maintained in serum-free RPMI 1640 for 24 h before treatment. Total RNA was isolated using RNeasy (Qiagen) after specific treatments as indicated. RNA was quantified by UV absorption, and 10 µg total RNA was electrophoresed on a standard Northern gel and transferred to nylon membrane (Bio-Rad). NURR1 cDNA, spanning the amino-terminal region to avoid cross-hybridization, and GAPDH cDNA were radiolabeled to a high specific activity using [α-32P]dCTP and a random primer labeling system (Promega) (31). Blots were exposed to film at −80°C using intensifying screens, and autoradiographic intensity was quantitated using an imaging densitometer. Densitometric values provided are representative of at least three separate experiments and are expressed as fold induction over basal levels relative to GAPDH expression ± SEM.

**Western blot analysis**

Cell cultures grown in 25-cm² tissue culture flasks were maintained in serum-free RPMI 1640 for 24 h before treatment. Cells were harvested after specific treatments, and nuclear protein extracts were prepared as described previously (31, 58). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels using concentrations of nuclear lysates as indicated. The resolved proteins were transferred onto nitrocellulose sheets, which were immunoblotted overnight at 4°C with anti-NURR1 mouse mAb (1:1000) (BD Biosciences). Secondary Abs to mouse IgG, conjugated to HRP, were used for NURR1 detection and identified using SuperSignal West Dura Extended Duration Substrate (Pierce) according to the manufacturer’s instructions.

**Synthesis of cDNA and PCR**

Complementary DNA was prepared by reverse transcription of 1 µg of each total RNA sample from cultured synoviocytes as described previously (32). For PCR, the sense (5′-CGA CAT TTC TGC CTT CTC C-3′) and antisense (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′) primer pair was used to amplify human NURR1, yielding a 277-bp product. The sense (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′) and antisense (5′-TCT AGA CGG CAG GTC AAG TCC ACC-3′) primer pair was used to amplify GAPDH, yielding a 465-bp product. Both primer pairs flanked intron sequences. The conditions for amplification were as follows: an initial denaturation step at 97°C for 2 min, followed by denaturation at 97°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. PCR was performed in 50-µl volumes, and each PCR sample underwent a 30-cycle amplification, which ensured that the reactions had not reached the plateau phase of amplification. PCR products were electrophoresed on a 1.5% agarose gel (ImageMaster VDS; Amersham Biosciences).

**Statistical analysis**

Analysis of the data was performed using nonparametric analysis in InStat software (GraphPad). Mann-Whitney U test was performed to compare groups. Spearman rank correlation coefficient was used to test for correlation of variables.

**Results**

**MTX suppresses NURR1 expression in vivo**

Previous findings from our laboratory demonstrated that inflamed RA and PsA ST produces markedly increased levels of NURR1 compared with normal ST (32, 39). To determine the effects of MTX treatment on NURR1 expression in vivo, ST was obtained before and during treatment from 10 patients with active disease who were beginning MTX therapy. All patients presented with active synovitis of one or more knees with a median of 7.5 swollen joints at presentation. The median duration of disease at the point of entry to the study was 18 mo (range, 0.25–168 mo). Patients were treated with a mean MTX dose of 13.75 mg/wk at the time of follow-up biopsy. During treatment, there was a significant improvement in clinical measures of disease activity including DAS (p = 0.014) and swollen joint count (p = 0.018), with improvements in laboratory measures of ESR and CRP also observed (Table I). All patients experienced a significant reduction (p = 0.009) in their DAS, which is a composite of both clinical and laboratory measurements (Table I).

At baseline arthroscopy, all patient ST (n = 10) demonstrated macroscopic features of synovial inflammation. Microscopic analysis confirmed inflammatory cell infiltrate, increased LL, thickness, and extensive SL vascularization (Fig. 1, A–F) (61, 62). Consistent with previous reports on the immunomodulatory effects of MTX in inflammatory joint disease (18, 63), all patient tissue demonstrated significant reduction in LL thickness and inflammatory cell infiltrate (Fig. 1, G–L).

Before treatment, NURR1 immunostaining was extensive in all samples (n = 10), with abundant staining observed in synoviocyte and macrophage cell populations of the synovial LL and SL regions (Fig. 1, A–F). Synovial vascular endothelium was also a site of NURR1 expression in all inflamed ST studied. This pattern of NURR1 staining is consistent with patterns of NURR1 expression in RA ST (32, 39, 64). During MTX treatment, cells positive for NURR1 immunostaining in the LL and SL regions (Fig. 1, G–L) decreased significantly (Table I). Reduction in NURR1 levels correlated significantly with changes in DAS (n = 10; r = 0.57; p = 0.009). Vascular endothelial expression of NURR1 was significantly reduced but not eliminated during MTX treatment. Importantly, most NURR1 immunostaining that remained during treatment was predominately cytoplasmic (Fig. 1, G–L) compared with dominant nuclear localization in inflamed ST (Fig. 1, A–F). The specificity of NURR1 staining was verified by loss of staining when NURR1 Ab was preincubated with an excess of Ag (blocking peptide) and tissue incubation with isotype-matched nonimmune IgG (data not shown) (32, 39, 64).

**MTX selectively inhibits inducible NURR1 mRNA levels in primary human synoviocyte and microvascular endothelial cells**

Proinflammatory mediators (PGE2, IL-1β, and TNF-α) and growth factors (bFGF and VEGF) associated with joint inflammation (48, 49, 58) modulate NURR1 mRNA expression in normal human synoviocytes, primary RA and PsA synoviocytes, and vascular endothelium (32, 39, 64). Characteristic of immediate early response

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Before MTX</th>
<th>During MTX</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>RI</td>
<td>10.0 ± 2.5 (9.0)</td>
<td>3.0 ± 1.2 (1.5)</td>
<td>0.014</td>
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<tr>
<td>Swollen joint count</td>
<td>9.6 ± 3.0 (7.5)</td>
<td>2.7 ± 0.8 (1.5)</td>
<td>0.018</td>
</tr>
<tr>
<td>ESR, mm/hr</td>
<td>29.1 ± 12.3 (19)</td>
<td>9.5 ± 3.6 (4)</td>
<td>0.103</td>
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<tr>
<td>CRP mg/l</td>
<td>46.5 ± 23.5 (15.5)</td>
<td>7.7 ± 3.8 (1.5)</td>
<td>0.268</td>
</tr>
<tr>
<td>DAS (variable)</td>
<td>3.3 ± 0.48 (2.9)</td>
<td>1.6 ± 0.3 (1.4)</td>
<td>0.009</td>
</tr>
<tr>
<td>Synovial membrane analysis of NURR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>4.6 ± 0.2 (5.0)</td>
<td>2.1 ± 0.4 (1.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>SL layer</td>
<td>4.3 ± 0.2 (4.5)</td>
<td>2.0 ± 0.3 (2.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Values are the mean ± SEM (median). ST findings were scored semiquantitatively on a scale of 0–5, where 0 = <1% positive cells, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100%. p values were determined by Mann-Whitney U test.

*NURR1, nuclear receptor-related protein 1; MTX, methotrexate; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS, disease activity score (28); RA, rheumatoid arthritis; PsA, psoriatic arthritis; SEM, standard error of the mean.*
MTX at higher concentrations (100 \( \times \) H9262) had no significant effect on PGE2-induced NURR1 mRNA levels at 50 \( \times \) H9262. The potent effect of MTX was mediated at 50 \( \times \) NURR1 mRNA levels in both cell types. Interestingly, the most significant decrease in NURR1 expression occurred within all regions of the synovium. Vascular endothelial NURR1 expression was reduced but not eliminated. The majority of NURR1 immunostaining that remained during MTX treatment is predominantly cytoplasmic compared with the predominant nuclear localization before treatment.

To determine the effects of MTX alone on endogenous NURR1 transcription, human synoviocyte cells (Fig. 3A) and endothelial cells (data not shown) were treated with increasing concentrations of MTX (\( n = 3 \)). Low doses of MTX had no effect on endogenous NURR1 mRNA turnover; however, at 100 \( \mu \)M MTX, levels of PGE2-induced NURR1 mRNA were reduced from 35.9 \( \pm \) 5.5-fold to 28.4 \( \pm \) 6.8-fold induction. The extent of 50 \( \mu \)M MTX inhibition on NURR1 mRNA levels was comparable to the anti-inflammatory actions of dexamethasone (32). In the presence of dexamethasone (10\(^{-8}\) M), PGE2-induced NURR1 mRNA levels were reduced from 35.9 \( \pm \) 5.5-fold to 17.7 \( \pm \) 2.5-fold induction. Modest effects of MTX (50 \( \mu \)M) on IL-1\( \beta \) and TNF-\( \alpha \)-induced NURR1 mRNA levels in primary endothelial cells and synoviocytes were observed (Fig. 2, C, D, and G). In contrast, MTX, at the concentrations tested, had no inhibitory effect on bFGF and VEGF-dependent NURR1 expression (Fig. 2, E–G).

MTX modulates endogenous and PGE2-induced NURR1 expression in a dose-dependent manner

To further analyze the differential inhibitory effects of MTX on PGE2-dependent NURR1 expression, synoviocytes and endothelial cells were preincubated with increasing concentrations of MTX (5–200 \( \mu \)M) and maintained at 50 \( \mu \)M and 100 \( \mu \)M levels (Fig. 3, A–C). Low doses of MTX had no effect on endogenous NURR1 mRNA levels; however, at 100 \( \mu \)M MTX, levels of PGE2-induced NURR1 mRNA were reduced from 35.9 \( \pm \) 5.5-fold to 14.8 \( \pm \) 0.6-fold induction. MTX at higher concentrations (100 \( \mu \)M) had no significant effect on PGE2-induced NURR1 mRNA levels.

### FIGURE 1. MTX suppresses NURR1 expression in vivo. Immunohistochemical staining for NURR1 in ST sections obtained from patients with PsA. Representative ST sections from patients with PsA (#1, #2, and #3) were stained with NURR1-specific antiserum before commencing MTX treatment (A–F) and during treatment (G–L).

- **Pre-MTX**
  - Patient #1: X100, X400
  - Patient #2: A, B, C, D
  - Patient #3: E, F, G
- **Post-MTX**
  - Patient #1: X100, X400
  - Patient #2: H, I, J
  - Patient #3: K, L

Positive immunoreactivity appears as brown/black. Nuclei are counterstained with hematoxylin (blue/purple). Before treatment, specific nuclear NURR1 staining was extensive throughout the lining (LL) and SL layers and vascular endothelium (→). During treatment, cells positive for NURR1 decreased significantly within all regions of the synovium. Vascular endothelial NURR1 expression was reduced but not eliminated. The majority of NURR1 immunostaining that remained during MTX treatment is predominantly cytoplasmic compared with the predominant nuclear localization before treatment. Original magnification, \( \times 100 \) in A–C and G–I; and \( \times 400 \) in D–F and J–L.
of 100 μM MTX failed to significantly modulate induced NURR1 levels. Interestingly, higher MTX concentrations (200 μM) augmented the effects of PGE$_2$ on NURR1 transcription, which is consistent with the stimulatory effects of 100–200 μM MTX on endogenous NURR1 gene transcription (Fig. 3A).

In human synoviocytes, basal levels of NURR1 protein are induced, and maximum levels were observed within 1–2 h following PGE$_2$ stimulation (data not shown). Immunohistochemical studies using isolated primary PsA synoviocytes confirm that MTX can potently inhibit PGE$_2$-induced NURR1 protein levels (Fig. 4). NURR1 is primarily localized to the nucleus in PGE$_2$-stimulated synoviocytes. Following incubation with MTX (n = 3), PGE$_2$-induced levels are inhibited, and, comparable to its cellular localization during MTX treatment in vivo, NURR1 is primarily confined to the cytoplasm (Fig. 4A).

Cells were pretreated with increasing concentrations of MTX (10–200 μM), exposed to PGE$_2$ (10$^{-6}$ M) for 1 h, and then examined for nuclear NURR1 protein levels by Western blot analysis (n = 3) (Fig. 4B). Consistent with our mRNA analysis, inhibition of NURR1 protein levels was observed following incubation with 10 μM MTX; inhibited levels were maintained with 25–50 μM MTX and began to increase, reaching PGE$_2$-dependent levels at 100–200 μM MTX (Fig. 4, B and C).

MTX suppresses PGE$_2$-induced NURR1 expression through adenosine release

Recent reports provide convincing evidence that the nucleoside adenosine mediates the anti-inflammatory and immunoregulatory effects of MTX in in vivo models of acute and chronic inflammation (19–23). To determine whether adenosine alone can modulate
steady-state NURR1 mRNA levels, synoviocyte cells (Fig. 5A) were treated with increasing concentrations of adenosine (0.1–500 μM). Our results indicate that following incubation with 0.1–10 μM adenosine, steady-state levels of endogenous NURR1 mRNA remain unchanged. At 100 μM adenosine, levels of NURR1 mRNA begin to increase, and, at 500 μM adenosine, modulation of NURR1 transcription is comparable to levels reached by PGE₂ stimulation (Fig. 5A). Thus, confirming that adenosine can mimic the effects of MTX on endogenous NURR1 transcription.

To determine whether adenosine modulates PGE₂-mediated NURR1 transcription, human synoviocytes were pretreated with different amounts of adenosine (0.5–500 μM) and then examined for effects on PGE₂-induced NURR1 transcription. Our results indicate that preincubation with adenosine for 2 h abrogates PGE₂-induced NURR1 mRNA levels in a dose-dependent manner at all concentrations tested (Fig. 5B). The stimulatory effects of higher concentrations of adenosine on steady-state NURR1 mRNA levels were not detected in the presence of PGE₂ (10⁻⁶ M) (Fig. 5B). Therefore, we decided to extend the dose range of adenosine from 10 μM to 1500 μM and examine the effects of higher adenosine levels on PGE₂-induced NURR1 levels (Fig. 5C). In the presence of PGE₂, the inhibitory effect of adenosine on PGE₂-induced NURR1 transcription was maintained over an extended concentration range (Fig. 5C).
Adenosine, acting at one or more of its receptors (A1, A2A, A2B, and A3), can exert a number of actions on a variety of cell types relevant to the anti-inflammatory actions of MTX (4, 66–68). In support of this mechanism of adenosine action, in vivo studies suggest that adenosine receptor antagonists can reverse or prevent the anti-inflammatory effects of MTX (21). To further investigate whether the inhibitory effects of MTX on NURR1 activation is mediated through adenosine, we examined the effect of type A1 and type A2 adenosine receptor antagonists. As shown in Fig. 6, MTX suppressed PGE2-induced NURR1 mRNA, and the selective A2 receptor antagonist (DMPX) reversed this effect in a concentration-dependent manner. In contrast to the potent effects of DMPX, the selective A1 receptor antagonist (DPCPX) had no effect at any of the concentrations tested (Fig. 6).

Adenosine bioavailability for signaling at its receptor sites is determined by production, release, and cellular uptake. Because adenosine is metabolized efficiently, the “effective” concentration necessary for adenosine receptor-mediated modulation of NURR1 gene transcription may be significantly lower than that observed in our experiments (Fig. 5). Therefore, a stable adenosine analog, NECA, was used to further examine adenosine effects on NURR1 gene transcription in primary human synoviocytes. Our results indicate that following incubation with NECA alone (0.01–1 μM), endogenous NURR1 mRNA levels remain unchanged (Fig. 7A). At the higher concentration of NECA (10 μM), steady-state NURR1 gene transcription begins to increase (Fig. 7A), a trend similar to that observed with higher adenosine concentrations (100 μM) (Fig. 5A). NECA potently abrogates PGE2-induced NURR1 transcription in a concentration-dependent manner (0.01–1 μM) (Fig. 7B). The concentrations of adenosine (0.5–100 μM) eliciting similar responses as NECA (0.01–1 μM) are ~50- to 100-fold higher. Therefore, the effective concentrations of NECA required to inhibit PGE2-induced NURR1 transcription are analogous to appreciably higher concentrations of adenosine (Fig. 5B).

Having established that the effects of low-dose MTX on NURR1 gene expression are mediated through the adenosine receptor A2 (Fig. 6), we sought to determine the selective involvement of A2 receptor isoforms in modulating adenosine responses in our system. The adenosine receptor subtype, A2A, is a critical player in a physiological negative feedback mechanism involved in the limitation and termination of inflammatory responses (67, 68). Thus, the consequences of A2A receptor inhibition were tested using the selective A2A receptor antagonist, ZM-241385. As shown in Fig. 7C, ZM-241385 reversed the inhibitory effects of NECA (1 μM), confirming that adenosine effects on PGE2-induced NURR1 mRNA levels can be mediated through this receptor subtype.

Finally, the involvement of A2B and A3 receptors on NURR1 gene expression following stimulation by high doses of adenosine was examined. The specific effects of antagonizing the A2B and A3 receptors were analyzed using the selective A2B receptor antagonist alloxazine and the A3 receptor antagonist MRS 1220. NECA at high concentrations (>10 μM) induces NURR1 gene transcription in a dose-dependent manner (Fig. 8). The presence of alloxazine or MRS 1220 with high concentrations of NECA had no significant effect on NECA-induced NURR1 gene transcription (Fig. 8). These data suggest that the A2B and A3 receptors are not involved in the modulation of NURR1 expression by high-dose adenosine.
adenosine and lends further support to our conclusion that modulation of NURR1 expression by MTX involves adenosine A2A receptor-mediated responses.

Discussion

Members of the NURR subfamily of nuclear orphan receptors are expressed aberrantly in human lung cancer cells, atherosclerotic lesions, and inflamed ST compared with normal tissue (32, 39, 45–47). Prolonged or inappropriate inflammatory responses contribute to the pathogenesis of these diseases (69, 70). Consistent with our earlier findings that transcription of the NURR1 gene is activated by inflammatory mediators associated with inflamed ST, recent reports also identify NURR receptors as effector molecules of cytokine signaling (32, 39, 43, 44). In this present study, we describe our observation that NURR1 is a molecular target of MTX action in vivo in human inflammatory joint disease. Furthermore, our data establishes that the immunomodulatory effect of MTX on endogenous and cytokine-induced NURR1 expression can be mimicked by adenosine and mediated through adenosine receptor-mediated responses.

In the five decades since Farber et al. (1–5, 8, 66) first described clinical remissions in children with acute leukemia after treatment with the folate antagonist aminopterin, antifolate drugs dominated by MTX have been used to treat millions of patients with malignant and immune-mediated diseases. It is estimated that MTX is now prescribed to at least 500,000 patients with RA and other forms of inflammatory arthritis (71). As demonstrated by several studies, the potent anti-inflammatory efficacy of low-dose intermittent MTX cannot be due entirely to folate antagonism and diminished purine synthesis. The effect of MTX treatment on lymphocyte and monocyte proliferation and apoptosis in patients is diminished purine synthesis. The effect of MTX treatment on lymphocyte and monocyte proliferation and apoptosis in patients is transient and is reversed by administration of folic acid (8, 66, 71). Significantly, folic acid or folic acid supplementation (1–5 mg/day) during MTX treatment of patients with inflammatory joint disease moderates MTX toxicity with no consequences on the anti-inflammatory efficacy of the drug (54, 72). MTX reduces ST inflammation by decreasing infiltration of lymphocytes and monocytes, possibly through down-regulation of cytokine levels including IL-1β and TNF-α and consequently a reduction in cytokine-induced expression of adhesion molecules (18, 63, 66). The molecular mechanisms by which MTX decreases cytokine levels in inflamed ST remain to be elucidated (4, 66).

Enhanced NURR1 expression in the synovial LL, subsynovial synoviocytes, and vascular endothelial cells of inflamed ST confirms that NURR1 is expressed primarily in cells believed to be at the leading edge of invasive tumor-like synovium (pannus) into adjacent cartilage and bone (32, 39, 49). During MTX treatment, NURR1 expression is significantly reduced in cells of the lining (p = 0.002) and SL regions (p = 0.001). The significant changes in NURR1 levels correlate with clinical response and improvements in patients’ DAS (r = 0.57; p = 0.009). The principal site of NURR1 expression during treatment is limited to the vascular endothelium; importantly, this pattern of expression is identical with NURR1 cellular localization in normal ST (32, 39). We have demonstrated previously that NURR1 is primarily confined to the nucleus of cells in inflamed tissue compared with a predominant cytoplasmic localization in normal tissue (32, 39). Comparison of NURR1 cellular localization in ST before and during MTX treatment reveals a distinct adjustment from nuclear to cytoplasmic staining in response to MTX. Taken together, these data suggest that MTX may modulate the transcriptional regulatory capacity of NURR1 in vivo.

Inflamed ST produces large quantities of proinflammatory mediators and growth factors. PGE2, IL-1β, and TNF-α occupy a prominent place in the hierarchy of proinflammatory mediators associated with inflammatory, immune, and arthritic diseases (48, 49). Peptide growth factors, including bFGF and VEGF, enhance synoviocyte and endothelial cell migration and proliferation and promote extracellular matrix protein turnover, processes which play an important role in synovitis (49). The pathological importance of NURR1 was verified by establishing the ability of these proinflammatory agonists, produced locally by inflamed synovium, to stimulate NURR1 transcription in synoviocytes (32, 39). Moreover, the NURR1 promoter responds to these same immunological stimuli in a manner similar to the response of endogenous NURR1 expression (39). Consistently, PGE2 has the most potent effect in stimulating NURR1 mRNA in these cells (39). In this study, we have established that low-dose MTX selectively inhibits NURR1 mRNA levels induced by PGE2 and other inflammatory agents. The potency of low-dose MTX inhibition is comparable to dexamethasone, a powerful anti-inflammatory agonist previously shown to inhibit cytokine and PGE2-induced NURR1 expression (32). Importantly, with increasing MTX concentrations, we observe a molecular switch of MTX signaling. MTX, at concentrations >50 μM, fails to inhibit cytokine-induced NURR1 gene transcription and has significant stimulatory effects on endogenous NURR1 mRNA and protein levels. The immunomodulatory actions of MTX are not confined to specific cell types because analogous responses were observed in primary synoviocyte and microvascular endothelial cells.

In RA, activated synoviocytes demonstrate increased proliferative and invasive activity, and they release proteolytic enzymes, cytokines, and prostanooids leading to cartilage erosion (49). MTX suppresses synoviocyte invasion and ameliorates cartilage destruction in the SCID mouse model for human RA (73). Cytokine-stimulated release of resorptive agents, such as matrix metalloproteinases, occurs in association with a change from a fibroblast-like to a stellate morphology (74). This study and our previous analysis demonstrate that cytokine and PGE2 induction of NURR1 also occurs in parallel with a transformation of synoviocytes from a fibroblast-like to a stellate morphology (32, 39). In primary synoviocytes, MTX can amend this induced stellate morphology and reduce cytokine-induced NURR1 protein levels. The predominant cytoplasmic localization of NURR1 initiated by MTX in vitro is comparable to NURR1 cellular location observed during MTX treatment in vivo. These data identify NURR1 as a molecular target of MTX signaling and verify that MTX effects on NURR1 levels observed in vivo are direct and not solely due to diminished infiltration of lymphocytes and monocytes and a reduction in cytokine levels.

The observation that the in vitro action of adenosine and its stable analog, NECA, mimic the effects of low-dose MTX on NURR1 in vivo supports several reports that the anti-inflammatory effects of MTX are due in large part to its capacity to enhance extracellular adenosine at sites of inflammation. Adenosine regulates inflammation via interaction with one or more of its four known receptors (A1, A2A, A2B, and A3) (67). Stimulation of A2 and A3 type adenosine receptors has been shown to alter the cytokine milieu by decreasing inflammatory cytokine secretion by macrophage cells in vitro (59, 75–77). In animal models of acute and chronic inflammation, nonselective adenosine receptor antagonists can reverse the anti-inflammatory effects of MTX treatment (17, 20, 21). A recent report using the murine air-pouch model of acute inflammation confirms that MTX fails to suppress inflammation in the air pouches of A2A and A3 receptor-deficient mice (23). Our demonstration of A2A receptor involvement in mediating MTX effects is consistent with the role of the A2A receptor as a regulator of inflammatory events. A2A receptor signaling is as...
thought to comprise an endogenous immunosuppressive loop capable of distinguishing beneficial and harmful inflammatory responses, whereby accumulated extracellular adenosine signals through cAMP-elevating A2A receptors in a delayed negative feedback manner (67, 68). Ohta and Sítkovsky (68) have demonstrated the involvement of the A2A receptor in the down-regulation of inflammation and inhibition of tissue damage in vivo. In this study, subthreshold doses of an inflammatory stimulus that caused minimal tissue damage in wild-type mice were sufficient to induce extensive tissue damage, more prolonged and higher levels of proinflammatory cytokines, and death of animals deficient in the A2A receptor.

Adenosine exerts autocrine and paracrine responses when released from inflamed tissue (67, 78). Adenosine A2 receptor-mediated inhibition of inflammation is dominant when cells are exposed to adenosine concentrations at ≥0.5 μM (79). In both syngeneic and endothelial cells, adenosine inhibition of PGE2-stimulated NURR1 expression was dose dependent and maximal at concentrations >0.5 μM and sustained, in the presence of PGE2, over a wide concentration range. A novel function of PGE2 reveals that this prostanooid may exert its effect by coupling the chemokine CCR7 receptor to signal transduction modules (80). In the presence of PGE2, stimulation with CCR7 ligands led to enhanced phosphorylation of protein kinases and subsequent downstream effects. Thus, it is possible that PGE2 may influence adenosine receptor-mediated signaling in a similar manner. Both PGE2 and adenosine have been shown to signal, at least in part, via cAMP/protein kinase A-dependent and -independent pathways. However, our preliminary analysis reveals no alterations in protein kinase A-dependent CREB phosphorylation in cells exposed to adenosine in the presence of PGE2 (J.A. Ralph, unpublished observations). This data may be consistent with the observation that engagement of multiple adenosine receptors, capable of stimulating and inhibiting intracellular cAMP levels, contribute to discordant gene expression in the amelioration of inflammatory disease (21, 23, 81–83).

In the absence of PGE2, and similar to the effects of MTX on steady-state NURR1 mRNA turnover, adenosine with increasing concentration displays stimulatory effects on endogenous NURR1 mRNA levels. Recent findings indicate this nuclear orphan receptor may play a role in mediating the intracellular effects of the purine antimetabolite 6-methylpurine (6-MP) (84). Ordentlich et al. (84) showed that 6-MP, only at concentrations ≥50 μM, is a potent regulator of NURR1 transcriptional activity. Significantly, the effect of 6-MP on NURR1 transcriptional activity is fully inhibited in the presence of 15 μM adenosine. Taken together, these data suggest that the cellular content of purine nucleotides may modulate both the expression and transcriptional activity of this nuclear orphan receptor.

In summary, these findings provide new insights into signaling pathways through which MTX can mediate its effect in human inflammatory arthritis. The identification of NURR1 as a target of MTX and adenosine action may facilitate the development of new and more effective therapies for the treatment of inflammatory disease. The clinical potential of nuclear receptors as drug targets is enhanced by the direct action of natural and synthetic compounds that modulate orphan receptors for drug discovery even in the absence of known natural ligands. In the case of NURR1, the further characterization of molecular signaling cascades both regulating and modulated by NURR1 may provide new approaches for intervention using this transcription factor as a molecular target.

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References


