Expression of Methotrexate Transporters and Metabolizing Enzymes in Rheumatoid Synovial Tissue

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ABSTRACT. ***Objective.*** To determine whether methotrexate (MTX) affects the expression of genes involved in the transport [*SLC19A1* (*RFC1*), *ABCB1* (MDR1), *ABCC1* (multidrug resistance proteins 1), *ABCG2* (BCRP)], metabolism [γ-glutamyl hydrolase (GGH), folylpolyglutamate synthetase (FPGS)], and mechanism of action of MTX [thymidylate synthase, MTR, MTRR] in rheumatoid synovium.

Methods. Synovial tissue samples were obtained from 20 patients with rheumatoid arthritis (RA). Gene expression was undertaken using quantitative real-time PCR.

Results. All the genes examined were expressed in all samples. Expression of *SLC19A1*, GGH, FPGS, *ABCC1*, and *MTRR* was significantly higher in patients receiving MTX compared to those not receiving MTX ($p < 0.05$). The ratio of FPGS:GGH gene expression was 2.7 ± 0.51 ng/ml GAPDH (range 0.67–9.58).

Conclusion. Genes involved in the transport, metabolism, and mechanism of action of MTX are expressed in rheumatoid joint synovium. These data provide evidence that MTX has the potential to be polyglutamated within the joint. The higher expression of FPGS compared to GGH in synovial tissue might favor production of long-chain MTX polyglutamates. Thus MTX has the potential to exert its therapeutic effects at the primary site of the inflammatory process in RA. (First Release July 15 2013; J Rheumatol 2013;40:1519–22; doi:10.3899/jrheum.130066)

Key Indexing Terms:
METHOTREXATE GENES RHEUMATOID ARTHRITIS SYNOVIUM

Methotrexate (MTX) is the anchor drug in the management of rheumatoid arthritis (RA). After absorption, MTX is taken up into cells by transporters including the reduced folate carrier 1 (RFC1; *SLC19A1*) and the folate receptors. Within cells, additional glutamate residues are added by folylpolyglutamate synthetase (FPGS), resulting in formation of MTX polyglutamates ($MTXGlu_n$). Terminal glutamates are removed by γ-glutamyl hydrolase (GGH), returning MTX to its monoglutamate form, which is transported out of cells by multidrug resistance proteins (MRP) including MDR1/*ABCB1*, MRP1/*ABCC1*, and BCRP/*ABCG2*. MTX polyglutamates inhibit a number of intracellular enzymes in the folate pathway including dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC). This results in accumulation of AICAR and increased adenosine concentrations, which inhibits the production of the proinflammatory cytokines tumor necrosis factor, interferon-γ, and interleukin 1β

The site at which MTX has its therapeutic action remains unknown. We have reported the expression of the adenosine receptor (ADOR) genes in RA synovium including $ADOR_{2A}$ and $ADOR_{2B}$ that show increased expression in patients receiving MTX[1]. The expression of other genes involved in the transport, metabolism, and mechanism of action of MTX within RA synovial tissue is unknown.

We hypothesized that genes regulating the transport [*SLC19A1* (*RFC1*), *ABCB1* (MDR1), *ABCC1* (MRP1), *ABCG2* (BCRP)], metabolism (GGH, FPGS), and mechanism of action of MTX (*TYMS*, *MTR*, *MTRR*) will be expressed within RA synovium.

MATERIALS AND METHODS

Synovial tissue samples from 20 patients with RA as defined by the 1987 American Rheumatism Association criteria[2] were obtained at the time of joint replacement surgery. Ethical approval was obtained from the New Zealand Multi-Regional Ethics Committee. All patients gave written informed consent.

Patient demographics. Mean age of patients was 66.4 years (range 48–80 yrs); 90% were female. Rheumatoid factor was positive in 85% and anti-cyclic citrullinated peptide antibody was positive in 73.3%. Twelve patients (60%) had rheumatoid nodules and 19 (95%) had radiographic erosions. Eleven patients (55%) were receiving MTX at a mean dose of 16.4 mg/week (range 7.5–20 mg/wk), 3 (15%) were receiving anti-tumor necrosis factor (anti-TNF) therapy, 4 salazopyrin, 3 hydroxychloroquine, and 3 leflunomide. Two patients were not receiving any disease-modifying antirheumatic drug (DMARD). Mean disease duration was 21.1 years

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(range 3.6–47 yrs). Mean erythrocyte sedimentation rate (ESR) was 41.4 mm/h (range 14–74 mm/h; n = 10) and mean C-reactive protein (CRP) was 18.9 mg/l (range 3–79 mg/l; n = 17).

PCR and quantitative real-time PCR. Total RNA was extracted from synovial tissue using Qiagen RNeasy mini kits and reverse transcribed as described[1]. Expression of the selected genes was determined by quantitative real-time PCR using commercial TaqMan gene expression assays (Applied Biosystems): *SLC19A1* (*RFC1*; Hs00953345), *ABCB1* (MDR1; Hs00184599), *ABCC1* (MRP1; Hs00219905), *ABCG2* (BCRP; Hs01053790), GGH (Hs00914163), FPGS (Hs00191956), *TYMSI* (Hs00426586), *MTR* (Hs00165188), and *MTRR* (Hs00985015). Gene expression was determined in triplicate for all samples and controls and the mean value normalized to the mean value for GAPDH expression (Hs999999905).

Statistical analysis. Data were analyzed using GraphPad Prism 4. All data are presented as mean ± SEM. The statistical significance of differences between groups was determined by Mann-Whitney U test and correlation was tested using the Spearman test.

RESULTS

Expression of genes in MTX transport and metabolism. All the genes examined were expressed in all 20 synovial tissue samples (Table 1). There was a significant negative correlation between ESR and the expression of *SLC19A1*, *ABCC1*, and *ABCB1* (encoding transporters), GGH (metabolizer), and TYMS, MTRR, and MTR (mechanism of action; $p < 0.05$ for all). However, there was no correlation between CRP and expression of any of the genes examined (data not shown).

Association of MTX therapy with gene expression. Expression of the transporters *SLC19A1* and *ABCC1*, the metabolizing enzymes GGH and FPGS, and of the reductase *MTRR*, which is involved in the mechanism of action, was significantly higher (~2-fold) in patients receiving MTX (n = 11) compared to those not receiving MTX (n = 9; Table 1). There was no relationship between MTX dose and the amount of gene expression for any of the genes examined (data not shown).

The ratio of FPGS:GGH gene expression was 2.7 ± 0.51 ng/ml GAPDH (range 0.67–9.58 ng/ml). There was no significant difference in the ratio of FPGS:GGH between those receiving and those not receiving MTX.

DISCUSSION

MTX is used as the first-line DMARD in RA unless there are specific contraindications. Adequate suppression by MTX of the inflammatory process that drives synovial inflammation is critical to prevention of joint erosion and functional impairment. The expression of genes involved in MTX transport, metabolism, and mechanism of action in the synovial membrane provides evidence that MTX has the potential to be polyglutamated within the synovium, and thus to exert its antiinflammatory effects locally at the primary site of the inflammatory process in RA.

MTX is taken up into cells by *SLC19A1* and the folate receptor. It has been reported that the folate receptor is selectively elevated in synovial macrophages from patients with RA with no expression of *SLC19A1* (RFC1)[3]. We have shown some low expression of the *SLC19A1* gene in synovial tissue from RA patients. This suggests that *SLC19A1* may be expressed by cells other than synovial macrophages, such as the synovial fibroblast-like cells or the T and B lymphocytes recruited as part of the inflammatory process in RA.

Genes encoding those ABC transporters responsible for exporting of MTX out of cells [*ABCB1* (*MDR1*), *ABCC1* (*MRP1*), and *ABCG2* (BCRP)] were also expressed in synovial tissue. This is in keeping with a report of *ABCG2* (BCRP) and *ABCC1* (MRP1) expression in RA synovial tissue[4]. Van der Heijden, *et al* demonstrated that the expression of *ABCG2* (BCRP) was constant before and after 4 months of treatment with MTX. However, there was a higher number of BCRP-positive cells in those patients who failed to respond to MTX, suggesting that poorer response may be due to enhanced transport of MTX out of cells[4]. It has also been reported that *ABCC1* (*MRP1*) expression in peripheral blood mononuclear cells (PBMC) is downregulated after treatment with MTX and folic acid[5].

Table 1. Expression of genes involved in the transport, metabolism, and mechanism of action of methotrexate (MTX) in rheumatoid synovium. All data are mean gene expression (ng/ml; normalized to GAPDH) ± SEM for all 20 patients or for subgroups receiving or not receiving MTX.

Gene	Synovial Gene Expression			
	Sample Group, n = 20	No MTX, n = 9	MTX, n = 11	p
MTRR	4.21 ± 0.73	2.66 ± 0.60	5.48 ± 1.13	0.04
MTR	4.06 ± 0.90	2.66 ± 0.66	2.88 ± 0.49	0.59
ABCB1	2.78 ± 0.39	2.38 ± 0.53	5.4 ± 1.48	0.15
FPGS	1.59 ± 0.36	1.2 ± 0.59	1.89 ± 0.44	0.027
ABCC1	1.38 ± 0.18	0.86 ± 0.18	1.81 ± 0.23	0.02
ABCG2 (BCRP)	0.71 ± 0.18	0.45 ± 0.15	0.93 ± 0.30	0.22
GGH	0.62 ± 0.09	0.43 ± 0.09	0.79 ± 0.11	0.048
TYMS	0.42 ± 0.06	0.31 ± 0.06	0.51 ± 0.08	0.09
SLC19A1 (RFC1)	0.37 ± 0.04	0.28 ± 0.04	0.45 ± 0.05	0.037

FPGS: folylpolyglutamate synthetase; GGH: γ-glutamyl hydrolase; TYMS: thymidylate synthase.

FPGS is responsible for the addition of glutamate moieties to MTX. Decreased expression or activity of FPGS, which results in decreased production of the longer-chain MTX polyglutamates, has been suggested as a mechanism of resistance to MTX in human leukemic cells[6]. However, in a study of 141 patients with RA, only 55% had expression of FPGS mRNA in PBMC[7]. Patients without FPGS had significantly higher response rates to MTX compared to those with FPGS (57% vs 33%; p = 0.005)[7]. Interestingly, in patients with RA, mononuclear cell MTX polyglutamate concentrations were not significantly different between responders and nonresponders to MTX[8]. There has been a similar lack of association between disease activity and red blood cell (RBC) MTX polyglutamate concentrations in some, but not all studies[9,10]. However, we have shown that the FPGS gene is expressed in synovial tissue and there was higher expression in patients receiving MTX at the time of the study. Similar differences are associated with GGH expression, ensuring the FPGS:GGH ratio is maintained in the presence of MTX. One possibility is that the higher expression of FPGS compared to GGH observed in our study might favor production of long-chain MTX polyglutamates in synovial tissue. These data raise the possibility that local synovial MTX polyglutamate concentrations may be associated with response, even if RBC concentrations are not.

Intracellular folate concentrations have an influence on the expression of enzymes involved in MTX transport and metabolism. Studies in cancer cells show low intracellular folate concentrations result in upregulation of BCRP, RFC-1, and FPGS expression[11]. In patients with RA, higher RBC folate concentrations are associated with poor response to MTX, and conversely, low RBC folate concentrations are associated with improved response[9,10]. At least part of the explanation for these observations may be the alterations in expression of genes involved in the MTX pathway.

We observed a negative correlation between ESR and expression of some genes (*SLC19A1*, *ABCC1* and *ABCB1*, GGH, TYMS, MTRR, and MTR). One might expect this negative association with higher expression of genes responsible for MTX uptake (*SLC19A1*) and MTX mechanism of action. Indeed, experimental evidence indicates inflammatory components such as interleukin 6 have the potential to downregulate *SLC19A1* expression[12], consistent with the negative association with ESR that we observed. However, a positive correlation would be expected with genes responsible for MTX efflux (*ABCC1*, *ABCB1*) and removal of glutamate residues (GGH). The clinical relevance of this finding is uncertain, particularly because a similar relationship was not observed with CRP. We also observed that expression of a number of genes was higher in synovium from those patients receiving MTX. The significance of this remains uncertain. Patients who respond to MTX may have higher expression of genes relevant to its uptake and metabolism, and would be more likely to respond and to remain on MTX therapy. Alternatively, expression of the genes may be influenced by MTX therapy. Further investigation of synovial gene expression pre- and post-MTX therapy is required to determine whether either of these possibilities can occur and which might be predictive of MTX response. Any influence from polymorphisms known to affect gene expression also remains unknown. That these might be particularly relevant to synovial gene expression is a further avenue requiring investigation.

Our study is limited by the use of synovial samples from rheumatoid patients with longstanding disease requiring joint surgery. Whether these findings are the same in patients with early disease remains to be determined. Given that our study was cross-sectional, we could not determine whether the increased expression of genes in those patients receiving MTX was a result of MTX therapy or might be predictive of response to MTX. In addition, the inflamed synovium is characterized by infiltrating T cells, B cells, macrophages, and resident fibroblast-like cells, each with their own differential folate pathway gene expression profile. This may in turn be influenced by the local environment within the inflamed synovium, such as hypoxia, acidic milieu, and cytokines. Further studies are required to determine expression of the genes and encoded proteins in specific infiltrating and resident cell types and the effects of the local synovial environment on gene expression.

Our data provide evidence that MTX has the potential to exert its antiinflammatory effects at the primary site of the inflammatory process in RA. The higher expression of FPGS compared to GGH in synovial tissue might favor production of long-chain MTX polyglutamates. The results suggest that a longitudinal study to determine enzyme levels before and after therapy and the relationship to synovial MTX polyglutamate levels and clinical response is indicated.

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