Involvement of Breast Cancer Resistance Protein Expression on Rheumatoid Arthritis Synovial Tissue Macrophages in Resistance to Methotrexate and Leflunomide

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Objective. To determine whether multidrug-resistance efflux transporters are expressed on immune effector cells in synovial tissue from patients with rheumatoid arthritis (RA) and compromise the efficacy of methotrexate (MTX) and leflunomide (LEF).

Methods. Synovial tissue biopsy samples obtained from RA patients before treatment and 4 months after starting treatment with MTX (n = 17) or LEF (n = 13) were examined by immunohistochemical staining and digital image analysis for the expression of the drug efflux transporters P-glycoprotein, multidrug resistance–associated protein 1 (MRP-1) through MRP-5, MRP-8, MRP-9, and breast cancer resistance protein (BCRP), and the relationship to clinical efficacy of MTX and LEF was assessed.

Results. BCRP expression was observed in all RA synovial biopsy samples, both pretreatment and posttreatment, but not in control noninflammatory synovial tissue samples from orthopedic patients. BCRP expression was found both in the intimal lining layer and on macrophages and endothelial cells in the synovial sublining. Total numbers of macrophages in RA patients decreased upon treatment; in biopsy samples with persistently high macrophage counts, 2-fold higher BCRP expression was observed. Furthermore, median BCRP expression was significantly increased (3-fold) in nonresponders to disease-modifying antirheumatic drugs (DMARDs) compared with responders to DMARDs (P = 0.048). Low expression of MRP-1 was found on synovial macrophages, along with moderate expression in T cell areas of synovial biopsy specimens from one-third of the RA patients.

Conclusion. These findings show that the drug resistance–related proteins BCRP and MRP-1 are expressed on inflammatory cells in RA synovial tissue. Since MTX is a substrate for both BCRP and MRP-1, and LEF is a high-affinity substrate for BCRP, these transporters may contribute to reduced therapeutic efficacy of these DMARDs.

The disease-modifying antirheumatic drugs (DMARDs) methotrexate (MTX), leflunomide (LEF), sulfasalazine, and (hydroxy)chloroquine have an established place in the treatment of patients with rheumatoid arthritis (RA), either as single agents or in combination with other DMARDs or biologic agents (1,2). Despite the potent disease-modifying effects of these DMARDs, it is well recognized that long-term treatment is often accompanied by a gradual loss of efficacy, requiring dosage escalation up to a stage where treatment has to be discontinued for reasons of inefficacy and/or toxicity (3–5). From oncology research, it has been established that specific cell membrane–associated drug efflux transporters belonging to the family of ATP-binding cassette
(ABC) proteins, notably P-glycoprotein (ABCB1), multi-drug resistance–associated protein 1 (MRP-1) through MRP-5 (ABCC1 through ABCC5), and breast cancer resistance protein (BCRP; ABCG2), can be induced upon therapy with various anticancer drugs to confer a phenomenon known as acquired (multi)drug resistance (6–9). Studies conducted in our laboratory have shown that acquired resistance to the DMARDs sulfasalazine and chloroquine could be mediated by the increased expression of the ABC transporters BCRP and MRP-1, respectively (10–12). Furthermore, it was very recently demonstrated that LEF is a high-affinity substrate for BCRP (13).

A number of studies have examined the expression of P-glycoprotein and MRP-1 (messenger RNA levels, protein levels, functional activity) on peripheral blood cells of RA patients. Maillefer et al (14) observed that P-glycoprotein levels were increased on peripheral blood cells from patients with RA who were treated with prednisolone compared with untreated healthy individuals, which may be compatible with the known role of steroids as substrates for P-glycoprotein. Likewise, Llorente et al (15) showed that RA patients had functionally active P-glycoprotein on peripheral blood lymphocytes, which was markedly higher in RA patients in whom disease was refractory to treatment than in RA patients with a good clinical response to DMARD therapy.

More recently, 2 studies evaluated the expression of MRP-1 (by functional activity and immunohistochemical staining) as a potential MTX drug efflux pump in peripheral blood cells of RA patients. In a cross-sectional study, Wolf et al (16) demonstrated that, counterintuitively, patients with functional MRP-1 expression who were treated with MTX had better response rates based on the European League Against Rheumatism response criteria (17) than did patients with low MRP-1 activity. Hider et al (18) showed that MRP-1 expression on peripheral blood cells declined from baseline to 6 months with MTX therapy. These results suggest that beyond drug-induced changes in MRP-1 expression during dosage escalations, basal levels of MRP-1 expression in immune effector cells may also be induced by disease activity/inflammation status (9). In contrast to studies on peripheral blood cells from RA patients, little is known about expression levels of ABC transporters on inflammatory cells in RA synovial tissue.

In the present study, we assessed the expression of a panel of ABC transporters with an established role in drug resistance in RA synovial tissue biopsy samples that were obtained prior to and after 4 months of therapy with MTX and LEF. Beyond P-glycoprotein, we focused on the more recently identified ABC transporters MRP-1 through MRP-5, MRP-8, and MRP-9, which have MTX among their substrates (8), and BCRP, for which both MTX and LEF are known substrates (13), thereby allowing correlation analysis with response to these drugs.

PATIENTS AND METHODS

Patients. We analyzed synovial tissue biopsy specimens derived from the same knee joints of 17 patients with active RA before and after 4 months of treatment with MTX (starting dosage 7.5 mg/week; increased stepwise to 15 mg/week over 12 weeks) and 13 patients with RA before and after 4 months of treatment with LEF (20 mg/day; loading dose 100 mg/day for the first 3 days). Active disease was defined as ≥6 swollen or tender joints and levels of moderate or worse on the physician’s and patient’s assessments of disease activity. All patients had at least 1 clinically involved knee joint. Concomitant treatment with low-dose prednisone (<10 mg/day) and stable doses of nonsteroidal antiinflammatory drugs was allowed. None of the patients had ever received MTX or LEF before enrolling in the study. Treatment with other DMARDs was terminated following a washout phase of 28 days. Before treatment and after 4 months of treatment, the Disease Activity Score in 28 joints (DAS28) (19) was determined. Arthroscopies were performed as described previously (20), as part of a joint study that was approved by the medical ethics committees of the Leiden University Medical Center (Leiden, The Netherlands), and the Leeds University Medical Center (Leeds, UK) (21). As noninflammatory control synovial tissue, we included 7 samples from patients with mechanical joint injury (kindly provided by Dr. B. J. van Royen, VU University Medical Center).

For statistical analysis, RA patients were divided into a group of patients who responded to DMARDs and a group of patients who did not respond to DMARDs, according to the DAS28 response criteria (19). Responders were patients with a significant decrease in DAS28 score (>1.2) after treatment and patients with a moderate change in DAS28 score (≥1.2 and <1.6) and low/moderate disease activity (DAS28 score ≤5.1). The remaining patients were classified as nonresponders. Additional analysis was performed based on the C-reactive protein (CRP) level. Patients who had a normal CRP level (<10 mg/liter) after 4 months of treatment were considered to be responders.

Immunohistochemical analysis. Immunohistochemical staining of cryostat sections (5 μm) of synovial tissue from RA patients and controls was performed using a 3-step immunoperoxidase method as described previously (22). Sections were stained with the following monoclonal antibodies (5–10 μg/ml): C219 (anti–P-glycoprotein; Alexis, Lausen, Switzerland), MRPr1 (anti–MRP-1), M2II16 (anti–MRP-2), M3II9 (anti–MRP-3), M4II10 (anti–MRP-4), M5II1 (anti–MRP-5), M8II16 (anti–MRP-8), M9II27 (anti–MRP-9), and BXP-21 (anti-BCRP), as previously described (22,23). T cells and macrophages were stained with phycoerythrin (PE)–conjugated...
anti-CD3 (Dako, Glostrup, Denmark) and 3A5 (24), respectively. Synovial tissue fibroblasts were visualized using PE-conjugated anti-CD90 (BD PharMingen, San Diego, CA).

**Analysis of multidrug resistance–associated protein expression.** Sections stained for BCRP and 3A5 (macrophages) (24) were analyzed by digital image analysis, as described previously (25). Briefly, for each marker representative regions were used for image acquisition, using 400× magnification. These regions were divided into 6 high-power fields (hpf) with a 3-pixel overlap. Positive cells were evaluated by analyzing 18 consecutive hpf and scoring the numbers of positive cells per square millimeter in the intimal lining layer and synovial sublining.

**Double-labeling immunofluorescence.** ABC transporter protein expression was detected with species-specific horseradish peroxidase (HRP)–labeled secondary reagents, and development was with rhodamine/thryamine (red fluorescence) according to the instructions of the manufacturer (Dako). T cells and macrophages (primary antibodies anti-CD3 and anti-CD68, respectively) were detected with biotin-labeled anti-mouse secondary antibodies (Dako), with streptavidin–Alexa 488 used as substrate (green fluorescence; Molecular Probes, Eugene, OR). Slides were mounted with Vectashield, containing 1 µg/ml 4′,6-diamidino-2-phenylindole (for staining of nuclei) (Vector, Burlingame, CA). Cells were examined using a fluorescence microscope (Leica, Rijswijk, The Netherlands).

**Statistical analysis.** Statistical analysis was performed using nonparametric tests. Correlation of BCRP expression in individual patients before treatment and after 4 months of treatment with MTX or LEF was calculated using Spearman’s rho. The Mann-Whitney U test was used to assess significant differences in macrophage counts before and after treatment with MTX and LEF, for BCRP expression between RA patients compared with healthy controls and DMARD-responsive patients compared with patients whose RA was not responsive to DMARDs. P values less than 0.05 were considered significant.

**RESULTS**

Expression of drug resistance–related transporters on macrophages, T cells, and endothelial cells in RA synovial tissue. Immunohistochemical staining for the drug resistance–related transporters P-glycoprotein, MRP-1 through MRP-5, MRP-8, MRP-9, and BCRP was performed on synovial biopsy specimens obtained from RA patients prior to and after 4 months of treatment with MTX (n = 17) or LEF (n = 13). Negative staining was observed for all isotype controls (Figure 1A). Staining with 3A5 showed that synovial tissue samples were highly enriched for infiltrated macro-
phages in the intimal lining layer and synovial sublining (Figure 1B). The most abundantly expressed drug efflux transporter in RA synovial tissue, both in the intimal lining layer and the synovial sublining, appeared to be BCRP (Figure 1C). Only a few BCRP-positive cells could be identified in “noninflammatory” synovial tissue from orthopedic patients (Figure 1F), which also displayed low levels of infiltrated macrophages (3A5 staining) (Figure 1E) as compared with RA synovium (Figure 1B). More detailed analysis by fluorescence microscopy demonstrated the colocalization of BCRP and CD68 in the intimal lining layer and synovial sublining, confirming that BCRP is expressed on RA macrophages. BCRP was also found to be expressed on endothelial cells (Figures 2A–H), and weak expression was observed on perivascular CD3-positive T cells (results not shown).

With light microscopy, weak MRP-1 staining was observed in synovial tissue biopsy specimens from approximately one-third of the RA patients. With more sensitive detection methods (immunofluorescence; rhodamine/thyramine development), clear MRP-1 expression was observed on CD3-positive lymphocytes, being most abundant in lymphocytic aggregates and less pronounced on neighboring T cells (Figures 3A–D). With this detection method we also observed moderate MRP-1 expression on CD68-positive macrophages in biopsy specimens from the same patients (Figures 3E and F). No colocalization of MRP-1 was found with CD90, a marker for synovial tissue fibroblasts (results not shown). Expression levels of P-glycoprotein, MRP-2 through MRP-5, MRP-8, and MRP-9 in synovial tissue from RA patients were below the immunohistochemical detection limit.

**BCRP expression and response to MTX and LEF therapy.** In synovial tissue from 7 healthy controls, consistent with very low macrophage counts, only a few BCRP-positive cells were identified. BCRP expression in synovial tissue from RA patients was significantly higher than that in controls ($P < 0.001$) (Figure 4A). BCRP expression in individual patients appeared to be rather constant over time, since a significant correlation was found between BCRP expression before treatment and after 4 months of treatment with MTX or LEF ($R = 0.57; P < 0.001$) (Figure 4B). The median number of 3A5-positive macrophages decreased after treatment
with MTX or LEF, from 295 cells/mm² (range 11–821) before treatment (mean DAS28 6.2) to 160 cells/mm² (range 12–853) after treatment (mean DAS28 5.0) \( (P = 0.06) \) (Figure 4C). Within the latter group, those patients with a persistently high macrophage count in the synovial tissue after 4 months of treatment with MTX or LEF had a 2-fold higher median number of BRCP-positive cells (200 cells/mm²) compared with patients with a low macrophage count (101 cells/mm²) \( (P = 0.082) \) (Figure 4D).

Since both MTX and LEF are BCRP substrates \( (8,13) \) and thus can be extruded from synovial tissue macrophages via this transporter, expression levels of BCRP in the synovial sublining were correlated with the response to MTX and LEF therapy. In patients whose RA did not respond to therapy, the median number of BCRP-positive cells in nonresponders was 3-fold higher using the DAS28 response criteria \( (P = 0.048) \) (Figure 5A) and 4-fold higher using CRP level \( (P = 0.035) \) (Figure 5B). Analysis of MTX and LEF separately also showed higher BCRP expression in nonresponders (using either the DAS28 or CRP level) compared with responders, although this difference only reached statistical significance for MTX in the analysis of CRP level \( (P = 0.023) \) (Figure 5B). The low incidence of MRP-1 expression in the synovial samples from RA patients did not allow correlation analysis with disease activity or response to MTX therapy.

**DISCUSSION**

This is the first detailed study investigating RA synovial tissue expression of a panel of multidrug transporter proteins from the family of ABC transporters, which are known to confer resistance to multiple anticancer drugs as well as DMARDs \( (9,26) \). Immunohistochemical detection of P-glycoprotein, MRP-2 through MRP-5, MRP-8, and MRP-9 in RA synovial tissue was not observed, in contrast to the drug transporters MRP-1 and BCRP, the latter being markedly expressed on RA synovial tissue macrophages in the intimal lining layer and the synovial sublining and on endothelial cells.
Importantly, BCRP expression was observed in the synovial tissue from RA patients prior to therapy. This finding suggests that BCRP expression may be intrinsically associated with the inflammatory status of RA synovial tissue, rather than being a therapy-induced phenomenon as is commonly observed in anticancer treatment (27–30).

It should, however, be noted that some patients had received sulfasalazine as initial treatment prior to a washout period and initiation of MTX or LEF treatment in the current study. Previous studies from our laboratory have shown that prolonged in vitro exposure of T cells to sulfasalazine may provoke induction of BCRP (10), but this induction was transient and reversed upon discontinuation of sulfasalazine exposure (11). Thus, it seems unlikely that the marked expression levels of BCRP on synovial macrophages observed at the beginning of MTX or LEF treatment may be the result of any previous sulfasalazine treatment.

BCRP expression and functional activity in cancer cells is highly variable (27). There may be epigenetic reasons for this, since aberrant promoter methylation inactivates the BCRP gene (31,32) or alternatively, some alterations in environmental conditions may up-regulate BCRP expression as observed under hypoxic conditions (30) or after folate supplementation to sustain folate homeostasis (33). One pathophysiologic condition in RA synovial tissue that could be implicated in BCRP expression is its hypoxic environment. It is well recognized that synovial infiltrating cells suffer from hypoxic stress (34). Given the notion that BCRP transcription is induced by hypoxia via the hypoxia-inducible transcription factor complex hypoxia-inducible factor 1α (HIF-1α) (35), and that HIF-1α is particularly expressed in synovial tissue...
macrophages (36,37), this may underlie the expression of BCRP on these cells.

The results of biodistribution and functional studies have indicated that BCRP plays important physiologic and pharmacologic roles, particularly in the clearance of toxic agents from the gastrointestinal tract, blood–brain barrier, liver, placenta, and mammary glands (22,38,39). With respect to hematologic cells, BCRP has been identified in hematopoietic stem cells (40) and dendritic cells (41). From a treatment perspective, BCRP has been recognized to mediate the cellular export of several anticancer drugs (8,27–29), and its expression has been associated with poor clinical outcome in the treatment of acute myeloid leukemia (42). Beyond this, DMARDs, such as MTX, sulfasalazine, and LEF, can also be extruded from cells via BCRP (8,10,13,43). Of note, BCRP can export MTX as well as its diglutamate and triglutamate metabolites, which could thus lead to diminished efficacy of MTX (43).

The present study revealed BCRP expression on synovial tissue macrophages from all tested patients with RA. High BCRP expression was associated with the persistence of infiltrated synovial tissue macrophages after treatment with MTX or LEF (Figures 4C and D), which might be related to the BCRP-mediated efflux of these drugs in macrophages. Accordingly, a statistically significant association was found between BCRP expression in RA synovial tissue and a diminished clinical response to DMARD therapy, assessed by attenuated improvement in DAS28 scores after 4 months of treatment, or persistent high CRP levels.

In addition to BCRP expression on synovial tissue macrophages, moderate expression of MRP-1 was found on macrophages in synovial tissue from one-third of the RA patients, while more prominent expression of MRP-1 could be noted on CD3-positive T cells in lymphocytic aggregates from these patients, by more sensitive staining methods. MTX and the antimalarial agent chloroquine are among the DMARD substrates of MRP-1, and expression of this transporter may thus confer resistance to these drugs (6,12,44). However, since MRP-1 levels in synovial T cells are rather moderate, a major contribution to a reduced response to MTX or antimalarial drugs remains elusive. Apart from its putative pharmacologic role in MTX extrusion, MRP-1 has an important physiologic function; it exports mediators of inflammation such as leukotriene C4, which triggers antigen-presenting dendritic cell migration to lymph nodes via chemotraction by CCL19 (45,46). Since high levels of CCL19 have been identified in RA synovial tissue (47,48), MRP-1 may be instrumental in the homing of inflammatory cells in RA synovial tissue.

Current experimental strategies to reverse drug resistance associated with efflux transporters include...
the use of specific inhibitors of ABC transporters. Small molecule inhibitors of BCRP have been tested in the laboratory setting (27,29). Whether such an approach may also be of therapeutic benefit in RA remains to be established. Recently, Zaher et al (49) reported that Iressa, an epidermal growth factor receptor antagonist and BCRP transport inhibitor, could markedly enhance the bioavailability of the DMARD sulfasalazine (a BCRP substrate [10,11]) by blocking intestinal extrusion of sulfasalazine via BCRP. Consistent with these findings, specific polymorphisms in the BCRP gene were identified as determining factors in sulfasalazine absorption and disposition (50). Based on this proof of principle, BCRP expression in RA synovial tissue may also be an interesting target for intervention aimed at increasing MTX, LEF, or sulfasalazine levels in RA macrophages. Such an approach, however, should also take into account possible negative effects on potential physiologic properties of BCRP in inflamed RA synovial tissue.

In conclusion, along with an established role in the efficacy of cancer therapy, this study revealed that multidrug resistance–related transporters, notably BCRP and to a lesser extent MRP-1, may play a role in the efficacy of specific DMARDs in treating RA. Determination of their precise pharmacologic and physiologic effects warrants further evaluation and should be helpful in improving the efficacy of DMARDs such as MTX, LEF, sulfasalazine, and (hydroxy)chloroquine, which are substrates for these drug efflux transporters.

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AUTHOR CONTRIBUTIONS

Dr. Jansen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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