CONCISE REPORT

Down regulation of multidrug resistance protein-1 expression in patients with early rheumatoid arthritis exposed to methotrexate as a first disease-modifying antirheumatic drug

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Background: Methotrexate (MTX) is the current gold standard conventional disease-modifying antirheumatic drug (DMARD) and is effluxed from cells by several transmembrane proteins, including multidrug resistance protein-1 (MRP1). It is hypothesised that the overexpression of these proteins may mediate reduced efficacy of MTX. To date, it is unclear how expression of these proteins changes over time or after exposure to drugs.

Aims: To compare MRP1 expression in newly diagnosed patients with DMARD-naïve rheumatoid arthritis with that in healthy controls and to investigate how MRP1 expression changed after exposure to MTX.

Methods: 18 newly diagnosed patients with DMARD-naïve rheumatoid arthritis and 14 healthy controls were recruited. Peripheral blood mononuclear cell counts were taken at baseline and after 6 months' treatment with MTX. Cells were separated by density gradient centrifugation and MRP1 expression was measured using the QCRL-1 monoclonal antibody.

Results: MRP1 expression in patients did not seem to be up regulated compared with that in healthy controls. In patients who were positive for MRP1 at baseline (61%), treatment with MTX and folic acid led to a marked down regulation of MRP1 expression at 6 months.

Conclusion: In patients with rheumatoid arthritis expressing MRP1, treatment with MTX and folic acid led to down regulation of MRP1 expression. Further studies are required to determine the mechanism behind this observation and whether MRP1 expression mediates altered efficacy to MTX.

outcome in terms of Disease Activity Score 28 (DAS28). This was, however, a cross-sectional study on patients with established rheumatoid arthritis, measuring MRP function at a single time point. As transporter expression may vary at different stages of disease or after exposure to different drugs, we hypothesised that measuring expression in patients with newly diagnosed DMARD-naïve rheumatoid arthritis may be of relevance. We therefore sought to (a) compare expression of MRP1 between patients with rheumatoid arthritis and healthy controls; (b) investigate how MRP1 expression changed after exposure to MTX; and (c) assess if there was any association between baseline transporter expression and clinical response to MTX.

METHODS

In all, 18 patients with newly diagnosed DMARD-naïve rheumatoid arthritis (14 women; mean age 52.3; SD 14.12 years) were compared with 14 healthy controls (6 women; mean age 32.9; SD 6.7 years). The median symptom duration was 5 (interquartile range (IQR) 3–12) months. Of the 18 patients, 12 had previously been treated with non-steroidal anti-inflammatory drugs. None of the patients in the study had received steroids by any route before inclusion. All patients underwent a standard clinical examination, including a DAS28, and provided a blood sample at baseline (before starting MTX) and at 6 months. They gave written informed consent, and the study was approved by the local research ethics committee. The patients were started on MTX 7.5 mg/week and folic acid 5 mg/week, and the dose of MTX was increased according to the level of disease activity.

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation. MRP1 expression was determined by modification of a previously reported method.⁸ This method was validated using MDCKII cells that overexpressed MRP1 (data not shown), and we used this method to quantify surface MRP1 in PBMCs from healthy volunteers and patients positive for HIV⁹ (B Chandler, personal communication, 2005). Briefly, cells were fixed with CellFIX (30 min; 4°C), permeabilised with saponin (0.1 mg/ml in Hank's balanced salt solution) and labelled with the MRP1-specific mouse anti-human primary antibody, QCRL-1 (200 µl; 2.5 µg/ml; Calbiochem, San Diego, California, USA). Detection was achieved with fluorescein isothiocyanate-conjugated IgG secondary antibody (200 µl; 5.0 µg/ml). Fluorescence was measured with a Coulter FACScan cytometer (Fullerton California, USA).

Abbreviations: DAS28, Disease Activity Score 28; DMARD, disease-modifying antirheumatic drug; IQR, interquartile range; MRP, multidrug resistance protein; MTX, methotrexate; P-gp, P-glycoprotein; PBMCs, peripheral blood mononuclear cells; RFC, reduced folate carrier

Methotrexate (MTX) is currently accepted as the gold standard conventional disease-modifying antirheumatic drug (DMARD) for use in rheumatoid arthritis. Clinical efficacy partly depends on intracellular retention of the drug. MTX enters cells through the reduced folate carrier (RFC) and is effluxed by several transmembrane proteins, including multidrug resistance proteins 1–4 (MRP1–4)^{1,2} and breast cancer resistance protein.³ It is hypothesised that overexpression of these transporters may be a cause of suboptimal response to MTX. Previous studies have suggested that overexpression of another transporter, P-glycoprotein (P-gp), may be associated with non-response to DMARD,^{4,5} although it is increasingly accepted that MTX is not a substrate for P-gp.⁶ To date, just one study has examined the effect of MRP function on outcome of treatment with MTX. Wolf *et al*⁷ found that concordance in functional MRP and RFC status led to a better therapeutic

Figure 1 (A) Multidrug resistance protein-1 (MRP1) expression over time in patients ($n = 11$) positive for MRP1 at baseline compared with healthy controls. Results are expressed as mean fluorescence intensity (FL2) between MRP1 cells and isotype control (IC) antibody. All samples were tested in duplicate and the result was expressed as a mean of the two samples. (B) Representative flow cytometry traces from this study showing highest expression, lowest expression and negative expression of MRP1. The final panel shows expression of MRP1 in MDCKII cells that overexpress MRP1.

Results are expressed as a fold increase in fluorescence, calculated by subtracting the median fluorescence intensity value observed in cells incubated with QCRL-1 from that observed with the isotype control antibody. Each sample was tested in duplicate and the mean of the two samples was taken as the results. The cut-off for MRP1 positivity was defined as >0 . As the data were non-parametric, the paired patient data were examined using a Wilcoxon's signed rank

test and the data of the patients and controls were compared using Mann-Whitney test.

RESULTS

In all, 11 of the 18 (61%) patients and 8 of the 14 (57%) controls were positive for MRP1 expression at baseline. The values obtained for MRP1 expression in this study were in the range obtained in our previous studies⁹ on healthy

volunteers, patients with HIV and cell lines⁹ (Chandler, personal communication). Median MRP1 expression was higher in patients (0.013, IQR 0–0.235) than in controls (0.003, IQR 0–0.0595), although this difference was not significant ($p = 0.38$). Of the patients who were positive for MRP1 at baseline, the level of MRP1 expression was significantly down regulated at 6 months (baseline median 0.139 (IQR 0.005–0.25), follow-up 0 (IQR 0–0.051); $p = 0.04$). Figure 1 shows the MRP1 expression at the two time points in 11 patients who were positive for MRP1 expression at baseline.

On the European League Against Rheumatism Scale, 6 patients achieved a good response, 7 achieved a moderate response and the remaining 5 a poor response.¹⁰ The MTX dose was increased in 10 patients with persistent disease activity and the final median (IQR) dose MTX in good responders was 7.5 mg/week (7.5–10 mg) compared with 15 mg (10–17.5 mg/week) in those not attaining a good response. The baseline expression of MRP1 in those achieving a good response was lower than in those not achieving a good response, median (IQR) MRP1 expression in responders was 0.011 (0–0.035) versus 0.047 (0–0.236) in non-responders, although this was not significant ($p = \text{NS}$). MRP1 expression did not correlate with erythrocyte sedimentation rate or DAS28 at either time point, or with final MTX dose. Exposure to intramuscular or intra-articular steroids ($n = 13$) after the assessment at baseline did not influence subsequent MRP1 expression.

DISCUSSION

This is the first study to examine MRP1 expression longitudinally in patients with DMARD-naïve early rheumatoid arthritis and as such provides a valuable opportunity to study this unique population before any DMARD exposure, which may modulate the expression of MRP1. Although MRP1 expression was not markedly different from that in healthy controls, in those patients (61%) who had detectable MRP1 expression at baseline, treatment with MTX and folic acid resulted in a marked down regulation of MRP1 expression at 6 months. Further studies are required to clarify the relationship between MRP1 expression and subsequent response to MTX, although it may be hypothesised that increased levels of MRP1 at baseline could be associated with a poorer response if more of the MTX is effluxed from the cells. Importantly, however, we did not find that MRP1 expression was induced during 6 months of MTX treatment, suggesting that this is not a mechanism for later drug inefficacy.

Wolf *et al*⁷ suggested that there was little difference in response to MTX (measured as reduction in DAS28) in patients who were MRP positive compared with those who were MRP negative. Indeed, in their study, the greatest change in DAS28 was seen in patients who were positive for both MRP function and RFC expression. The method used in their study with probenecid as an inhibitor, however, would inhibit all subgroups of MRP rather than just MRP1. The authors agree that their results are somewhat counter-intuitive and require replication in an independent cohort.

Some limitations to this study are as follows. Firstly, our numbers are small, although the group was homogeneous, comprising patients with newly diagnosed DMARD and steroid-naïve rheumatoid arthritis. A trend towards up regulation of MRP1 in patients with rheumatoid arthritis was observed as compared with healthy controls, although the small numbers may be responsible for the lack of statistical significance. As MRP1 expression may be modifiable by treatment, we thought it important to recruit patients before drug exposure. All the patients followed a similar treatment protocol, including 5 mg folic acid a week. We cannot exclude a modifying effect of folic acid as some

studies suggest that folate levels may influence MRP1 function.¹¹ As all the patients were exposed to the same dose of folate, however, our data, if anything, would suggest that low-dose folic acid may enhance sensitivity to MTX by reducing its cellular efflux by MRP1. Alternatively, it may be that MRP1 expression reflects higher levels of inflammation, which are controlled after DMARD treatment, although in this study we found no association between MRP1 expression and erythrocyte sedimentation rate or DAS28. Further studies are required to clarify the relationship between inflammation, treatment and MRP1 expression.

Secondly, this study examined only MRP1 expression, as this is best characterised with respect to MTX transport. Other efflux transporters, including MRP2–4 and breast cancer resistance protein, may be important and similar studies will be needed to clarify the role of these proteins, as it may be the combination of expression of several transporters that determines treatment efficacy rather than any single one. We also studied the whole PBMC population and so the differences found may be attributed to the expansion or depletion of specific lymphocyte subsets. Further studies may help to determine whether specific cell subtypes differentially express MRP1 and influence MTX responses.

In summary, this study shows that in patients with early DMARD-naïve rheumatoid arthritis, expression of MRP1 changes with treatment and that levels of expression of MRP1 may mediate altered efficacy to MTX. Larger prospective studies are required to further study the kinetics of MRP1 and other drug efflux proteins in predicting response and non-response to MTX.

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