Leflunomide, a novel immunomodulating drug, inhibits homotypic adhesion of peripheral blood and synovial fluid mononuclear cells in rheumatoid arthritis

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Abstract. Objective and Design: A novel immunomodulating drug, leflunomide has been shown recently to be effective and well tolerated in patients suffering from rheumatoid arthritis (RA). The present study evaluated the effect of the drug on cell adhesion in RA.

Material and Treatment: Peripheral blood and synovial fluid mononuclear cells were obtained from a clinical trial, undertaken primarily to evaluate the efficacy and pharmacokinetic profile of multiple-dose pulsing leflunomide therapy in RA patients. PB MNC and corresponding synovial fluid (SF) MNC for in vitro homotypic aggregation (HA) assay were obtained from healthy volunteers and RA patients with active disease not treated with leflunomide in vivo.

Methods: Expression of activation antigens (CD25, CD54, CD69, CD71, HLA-DR) on peripheral blood mononuclear cells (PB MNC), as well as ex vivo ability of cells to aggregate spontaneously were determined in patients before entering into the clinical trial and at the end of 6 months treatment. HA was measured by aggregation in vitro. Data were compared by Student's t-test.

Results: There was a decreased expression of activation antigens and decreased spontaneous MNC clustering after leflunomide therapy. We found in the in vitro study that HA of PB and SF MNC was mainly mediated through β2-integrin molecules. The active metabolite of leflunomide, A77 1726, effectively suppressed both spontaneous and phorbol-ester (PMA)-induced HA. Disruption of cell aggregates by A77 1726 was dose-dependent and, most likely, unrelated to the quantitative modulation of integrin receptors.

Conclusions: Results from this study support the idea that leflunomide elicits its immunomodulatory action, at least partially, by modulating the adhesion process.

Key words: Leflunomide – Homotypic adhesion – Rheumatoid arthritis

Introduction

Leflunomide, a novel immunomodulatory drug, has been shown to be effective not only in preventing, but in the therapy of several autoimmune animal diseases [1–4] and reactions leading to transplant rejection [5]. Moreover, we have demonstrated recently that the drug is very efficacious and well tolerated in patients suffering from rheumatoid arthritis (RA) [6]. Although the mode of action of leflunomide is not fully known, it seems that several sites of action contribute to the drug's anti-inflammatory and immunomodulatory properties [2, 7–11]. Leflunomide has been reported to interfere with lymphocyte activation, proliferation and differentiation [2]. Antiproliferative effects of the drug appear to be mediated predominantly by the inhibition of de novo pyrimidine biosynthesis [12], specifically dihydroorotate dehydrogenase (DHO-DH) [13]. In addition to this, the active metabolite of leflunomide, A77 1726, has been demonstrated to inhibit certain tyrosine kinases [10, 11]. However, molecular targets other than DHO-DH, or tyrosine kinases cannot be excluded.

It is well known that migration, retention in situ and persistent activity of the emigrating immune/inflammatory cells within the synovial tissue are responsible for the chronic inflammation in RA [14, 15]. Adhesive interactions are of crucial importance for initiation and perpetuation of such an inflammatory process. They facilitate the stabilised binding of leukocytes to the blood vessel wall, subsequent transendothelial migration of these cells into the site of the inflammation [16–18] and communication of leukocytes with surrounding cells and extracellular matrix. Therefore, the aim of the present study was to evaluate whether the therapeutic efficacy of leflunomide, demonstrated in RA patients [6] and the potent antiinflammatory and immunomodulatory effect [7, 8] is due, at least in part, to the drug's influence on mononuclear cell adhesion. Our interest was focused on β2-integrins (CD11/CD18 heterodimer), a family of adhesion molecules broadly distributed on leukocytes.
clonal antibodies were added at appropriate concentrations and in blocked with 0.5% (v/v) hydrogen peroxide in methanol. Mono-

Materials and methods

Separation of MNC

Peripheral blood (PB) samples for this study were obtained from 31 RA patients included in the randomised single-blind, multiple-dose, pulsing (100 mg/week or 200 mg/week) clinical trial of the safety and pharmacokinetic profile of leflunomide (Hoechst AG, Wiesbaden, Germany). Samples were taken at baseline and at the end of the 6-month-treatment period. For the additional in vitro study, PB and corresponding SF samples (therapeutic aspiration of knee joints) were obtained from seven other RA patients, not treated with leflunomide. All patients were recruited according to the revised ARA criteria for the classification of RA [22]. The same inclusion criteria as for the phase II clinical study [6] were applied. Control PB samples were taken from 10 healthy volunteers comparable to RA patients concerning age and sex. MNC were isolated from PB and SF by density gradient centrifugation on Lymphoprep (Nycemed, Oslo, Norway), extensively washed in phosphate buffered solution (PBS) pH 7.4 and checked for viability by trypan blue exclusion test. Cell viability was more than 95%.

Flow cytometry analysis

Two-colour flow cytometry was performed using a dual laser FACScan system (Becton Dickinson, CA, USA). Cell pellets (1 x 10^6) were labelled using a direct or indirect immunofluorescence technique. MNC were first incubated with FITC-labelled monoclonal antibody (or unlabelled monoclonal antibody followed by FITC-conjugated goat anti-mouse antibody), then washed and incubated with PE-conjugated second monoclonal antibody (mAb). After incubation, cells were washed in PBS and finally resuspended in PBS containing 1% paraformaldehyde. Percentage of cells stained for each antibody and the percentage of double stained cells were calculated by counting 5000 MNC.

Cytocentrifuge preparations

Cytospin smears of PB MNC from RA patients were prepared before and after leflunomide treatment. For cytological and immunocytochemical analysis, several staining techniques were performed. May Grünwald-Giemsa staining was used for morphological characterisation of cells. ANAE staining was applied for detection of α-naphthyl acetate esterase (ANAE) activity of monocytes [23]. Immunoperoxidase staining was performed using the procedure and reagents developed by Amersharm International, UK. In brief, cytospin smears were thoroughly dried at room temperature, fixed in cold acetone for 5 min, washed in 0.1 M Tris buffer solution (TBS) pH 7.6, and endogenous peroxidase was blocked with 0.5% (v/v) hydrogen peroxide in methanol. Monoclonal antibodies were added at appropriate concentrations and in sufficient amounts to cover the smears. After incubation for a minimum of 60 min, samples were washed in TBS, and biotinylated sheep anti-mouse Ig antibody (RPN 1001, Amersham, UK) diluted 1 : 200 was added to the specimens for 45 min at room temperature. Smears were additionally washed in TBS and after that incubated for 30 min with biotinylated peroxidase streptavidin complex (RPN 1051, Amersham, UK) diluted 1 : 100. Peroxidase reaction was developed with 0.05% diaminobenzidine (DAB) in 0.01% H_2O_2 for 10 min. The colour development was stopped by washing the slides in running water. Finally, samples were lightly counterstained with hematoxylin and mounted in gelatine/glycerol medium. Double immunofluorescent staining was used for detection of two antigens on the single cell. This included combination of two mAb of different specificity and different isotype. Either direct, or indirect immunofluorescence staining was performed. Acetone-fixed smears were stained first with one labelled mAb, washed in PBS pH 7.4 to remove unbound antibody, thereafter stained with another differently labelled mAb. Slides were mounted with buffered glycerol and analysed under the Olympus BH2 microscope (Tokyo, Japan) with u.v. epi-illumination by using different combinations of filters. Specificity of immunocytochemical staining was checked using appropriate controls, including single staining, omitting first or second antibody, as well as their replacement with the irrelevant negative control antibody.

Monoclonal antibodies

Monoclonal antibodies used for flow cytometry and immunocytochemical staining are listed in Table 1. Secondary antibodies were obtained from Ortho Diagnostic, NJ, USA, Becton Dickinson, CA, USA (FITC goat anti-mouse Ig antibody), and Amersharm International, UK (biotinylated sheep anti-mouse Ig antibody).

Statistical analysis

Results were expressed as mean±SD. Statistical significance (p<0.05) was determined by Student's t-test for paired RA samples before and after the therapy. Comparison of population means (control vs. RA patients) was carried out using the two-sample t-test.