CONCISE REPORT

Methotrexate (MTX) and albumin coupled with MTX (MTX-HSA) suppress synovial fibroblast invasion and cartilage degradation in vivo

C Fiehn, E Neumann, A Wunder, S Krienke, S Gay, U Müller-Ladner

Objective: To investigate the effect of methotrexate (MTX) and albumin coupled with methotrexate (MTX-HSA) on cartilage invasion and induction of perichondrocytic degradation by rheumatoid arthritis synovial fibroblasts (RA SF) in vivo.
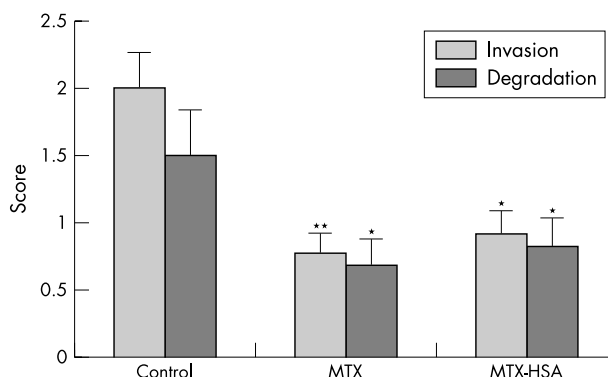
Methods: Human cartilage and RA SF were co-transplanted in three groups of severe combined immunodeficient mice (SCID), which received 1 mg/kg free MTX (n = 9), 1 mg/kg MTX-HSA (n = 6), or 0.9% NaCl (n = 5), respectively, intraperitoneally twice a week. After 4 weeks' treatment, the mice were killed and the implants analysed histologically.

Results: The control group had a mean (SEM) score for cartilage invasion of RA SF of 2.0 (0.26) and for perichondrocytic cartilage degradation of 1.5 (0.34). In contrast, mice which received MTX showed a significantly reduced invasion (0.78 (0.14), p<0.01) and a reduction in perichondrocytic cartilage degradation scores (0.69 (0.2), p<0.05) in comparison with the control group. Mice treated with MTX-HSA also had significantly reduced scores for RA SF invasion into the cartilage (0.92 (0.41), p<0.05) and for cartilage degradation (0.83 (0.44), p<0.05) compared with controls. The effects of MTX and MTX-HSA were not significantly different between these two groups.

Conclusion: Treatment with MTX or MTX-HSA significantly ameliorates cartilage destruction in the SCID mouse model for human RA.

Figure 1 Treatment with MTX or albumin coupled MTX (MTX-HSA) suppresses cartilage invasion and perichondrocytic cartilage degradation in the SCID mice model of human RA. SCID mice were co-implanted subcutaneously with human cartilage and RA SF. Thereafter, they were treated with intraperitoneal injections twice weekly of 0.9% NaCl (control, n = 5), or 1 mg/kg MTX (n = 9), or 1 mg/kg MTX-HSA (n = 6). After 4 weeks of treatment, the implants were analysed histologically (fig 2), and cartilage invasion by SF as well as perichondrocytic cartilage degradation were scored (*p<0.05; **p<0.01; error bars represent SEM).

A prominent feature of rheumatoid arthritis (RA) is the progressive destruction of the joints, characterised by synovial hyperplasia, synovial cell activation, and articular inflammation associated with subsequent cartilage and bone destruction. In this pathophysiological process, activated RA synovial fibroblasts (SF) appear to have a dominant role.[1–4]

Methotrexate (MTX) is the most commonly used disease modifying drug for RA.[5] It has been shown that MTX induces apoptosis[6] and inhibits the proliferation of SF in vitro.[7] Moreover, recently it was stated that the in vitro growth rate of RA SF is reduced in patients receiving MTX treatment in comparison with those without MTX.[8] Unfortunately, progression of joint destruction cannot be inhibited completely by MTX treatment in most patients with RA. This lack of efficacy is due to the fact that large amounts of the administered MTX are rapidly eliminated by the kidneys, resulting in a short plasma half life and low drug concentration in the targeted tissue. To overcome these disadvantageous pharmacokinetic properties, we recently introduced albumin coupled MTX (MTX-HSA) as a new drug conjugate, which consists of albumin and MTX covalently coupled to

each other in a 1:1 molecular ratio.[9–13] MTX-HSA has a substantially prolonged half life in the circulation owing to increased extravasation of albumin in inflamed tissue and metabolisation of albumin by cells with a high protein turnover, such as cells of the rheumatoid synovial tissue.[14] MTX-HSA accumulates also in inflamed joints of mice with collagen induced arthritis (CIA). We have shown that in comparison with equivalent concentrations of MTX, MTX-HSA is significantly more efficient than MTX in the prevention of arthritis in mice with CIA.[13] Moreover, human RA SF metabolised albumin conjugates in vitro and accumulated them intracellularly.[13]

In this study, we provide new data showing that MTX and MTX-HSA can reduce the invasion of RA SF into cartilage, and the RA SF dependent stimulation of perichondrocytic degradation, in an in vivo model for human RA using severe combined immunodeficient (SCID) mice which were co-transplanted with human cartilage and SF from patients with RA.[15]

METHODS

The SCID mice model for human RA was used as described previously.[14–16] Briefly, synovial tissue was obtained from two patients with RA undergoing joint replacement surgery. The

Abbreviations: CIA, collagen induced arthritis; HAS, human serum albumin; IL, interleukin; MTX, methotrexate; RA, rheumatoid arthritis; SCID, severe combined immunodeficient; SF, synovial fibroblasts
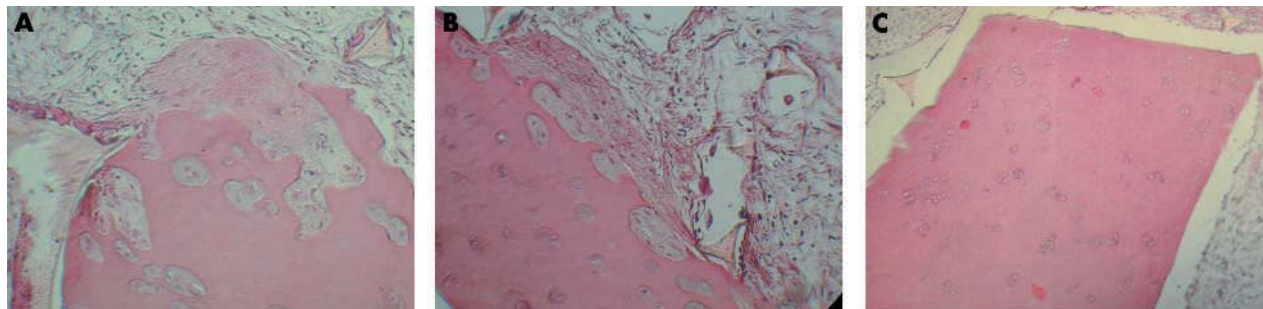
Figure 2 H&E staining of co-transplanted RA SF and normal cartilage after an implantation time of 4 weeks from control mice (A; original magnification ×800) and mice treated with either MTX (B; original magnification ×1600) or MTX-HSA (original magnification ×1600). In the control mice a deep invasion of the co-implanted RA SF into the cartilage can be seen (A), but in the mice which received treatment with either MTX or MTX-HSA the invasion is reduced (B) or even absent (C).

synovial membrane was dissected and RA SF were isolated, cultured, and characterised.[14] Twenty female Crl-scidBR 4 week old SCID mice were co-transplanted with RA SF and normal human cartilage using the ''inverse wrap'' technique.[16]

MTX-HSA was prepared according to the protocol performed in our laboratory[10] using DCC = N,N'-dicyclohexyl-carbodiimide and HSI = N-hydroxysuccinimide (Aldrich, Steinheim, Germany) for activation and linking MTX (Deisenhofen, Germany) to HSA (Pharma Dessau, Dessau, Germany). For separation of the compounds, ultrafiltration units (exclusion size 30 kDa) from Millipore (Eschborn, Germany) were used.

To examine the effect of MTX and MTX-HSA in vivo, 20 SCID mice were distributed randomly into three groups. Six days after implantation, 1 mg/kg free MTX, 1 mg/kg MTX covalently coupled with albumin (MTX-HSA), or 0.9% NaCl, respectively, were administered to the mice of the different groups intraperitoneally twice a week. The dose of MTX and MTX-HSA was chosen in accordance with previous studies, which had evaluated the maximum tolerable doses of MTX in SCID mice.[17] Both drugs were given in the same dose of 1 mg/kg in order to examine the effect of the different pharmacokinetic properties of MTX and MTX-HSA in this model. After 4 weeks of treatment (eight injections) the mice were killed and the implants were removed and cut into 5 µm sections. After staining of the sections with haematoxylin and eosin (H&E), the grade of cellular invasion into the cartilage by the co-implanted SF and the grade of perichondrocytic cartilage degradation was scored independently by four observers (CF, S K , E N, and UM-L) following an established protocol.[18] The p values were calculated using Student's t test with the WinStat statistics program.

RESULTS
The mice treated with 0.9% NaCl (control group, n = 5) had a mean (SEM) score for cartilage invasion of RA SF of 2.0 (0.26) and a mean (SEM) score for perichondrocytic cartilage degradation of 1.5 (0.34). In contrast, the mice which received eight injections of 1 mg/kg MTX twice weekly (n = 9) showed not only a significantly reduced invasion (0.78 (0.14), $p<0.01$) but also a reduction of perichondrocytic cartilage degradation scores (0.69 (0.2), $p<0.05$) when compared with the control group (fig 1).

In addition, the mice, which were treated with eight injections of 1 mg/kg MTX-HSA twice weekly (n = 6), also had significantly reduced scores for RA SF invasion into the cartilage (0.92 (0.41), $p<0.05$) and for cartilage degradation (0.83 (0.44), $p<0.05$) in comparison with the control group.

Notably, the effects of MTX and MTX-HSA on the invasion of RA SF into cartilage and on the perichondrocytic cartilage degradation did not differ significantly between the two treatment groups.

DISCUSSION
The results of the study show that treatment with either MTX or the new conjugate MTX-HSA significantly ameliorates cartilage destruction in the SCID mouse model for human RA. The experiments demonstrate also that these inhibitory effects are not only due to a direct effect on RA SF, which is illustrated by their reduced invasivity, but most likely also due to a reduction of the interleukin (IL)1 mediated effects of RA SF on chondrocytes.[18 19] Therefore, indirect effects of MTX by modulation of the IL1/IL1 receptor antagonist ratio as outlined previously,[20 21] might additionally be responsible for the observed effects of MTX in this model.

Most interestingly, the data demonstrate that MTX-HSA and MTX both inhibit fibroblast dependent invasion and degradation of cartilage in the SCID mouse model of RA. Therefore, it can be speculated that MTX-HSA may combine the effectiveness of MTX in the treatment of rheumatoid joint destruction with improved pharmacokinetic properties—namely, a prolonged half life and a potential accumulation of the drug in the inflamed joints, as recently demonstrated.[13]

In summary, MTX and MTX-HSA inhibit both aspects of RA SF mediated destruction of cartilage in vivo. With the use of MTX-HSA, a treatment specifically targeted to the destructive compartments might be feasible, which might also lower the rate of side effects of MTX treatment.

. . . .
Authors' affiliations
C Fiehn, S Krienke, Department of Haematology, Oncology and Rheumatology, Clinic of Internal Medicine V, University of Heidelberg, Germany
E Neumann, U Müller-Ladner, Division of Rheumatology and Clinical Immunology, Department of Internal Medicine I, University of Regensburg, Germany
A Wunder, Department of Radiochemistry and Radiopharmacology (E0300), German Cancer Research Centre (DKFZ), Heidelberg, Germany
S Gay, WHO Collaborating Centre for Molecular Biology and Novel Therapeutic Strategies for Rheumatic Diseases, University of Zürich, Switzerland

Correspondence to: Dr U Müller-Ladner, FJS-Allee 11 Regensburg, 93042 Germany; ulf.mueller-ladner@klinik.uni-regensburg.de

REFERENCES
1 **Gay S**, Gay RE, Koopman WJ. Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction? *Ann Rheum Dis* 1993;**52**(suppl 1):S39–47.

2 **Firestein GS**. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? *Arthritis Rheum* 1996;**39**:1781–90.

3 **Pap T**, Müller-Ladner U, Gay RE, Gay S. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res* 2000;**2**:361–7.

4 **Müller-Ladner U**, Gay RE, Gay S. Molecular biology of cartilage and bone destruction. *Curr Opin Rheumatol* 1998;**10**:212–19.

5 **Furst DE**. The rational use of methotrexate in rheumatoid arthritis and other rheumatic diseases. *Br J Rheumatol* 1997;**36**:1196–204.

6 **Nakazawa F**, Matsuno H, Yudoh K, Katayama R, Sawai T, Uzuki M, et al. Methotrexate inhibits rheumatoid synovitis by inducing apoptosis. *J Rheumatol* 2001;**28**:1800–8.

7 **Meyer FA**, Yaron I, Mashiah V, Yaron M. Methotrexate inhibits proliferation but not interleukin 1 stimulated secretory activities of cultured human synovial fibroblasts. *J Rheumatol* 1993;**20**:238–42.

8 **Lories RJ**, Derese I, De Bari C, Luyten FP. In vitro growth rate of fibroblast-like synovial cells is reduced by methotrexate treatment. *Ann Rheum Dis* 2003;**62**:568–71.

9 **Stehle G**, Wunder A, Sinn H, Schrenk HH, Schutt S, Frei E, et al. Pharmacokinetics of methotrexate-albumin conjugates in tumor-bearing rats. *Anticancer Drugs* 1997;**8**:835–44.

10 **Stehle G**, Sinn H, Wunder A, Schrenk HH, Schutt S, Maier-Borst W, et al. The loading rate determines tumor targeting properties of methotrexate-albumin conjugates in rats. *Anticancer Drugs* 1997;**8**:677–85.

11 **Wunder A**, Stehle G, Schrenk HH, Hartung G, Heene DL, Maier-Borst W, et al. Antitumor activity of methotrexate-albumin conjugates in rats bearing a Walker-256 carcinoma. *Int J Cancer* 1998;**76**:884–90.

12 **Stehle G**, Wunder A, Schrenk HH, Hartung G, Heene DL, Sinn H. Methotrexate-albumin conjugate causes tumor growth delay in Dunning R3327 HI prostate cancer-bearing rats. *Anticancer Drugs* 1999;**10**:405–11.

13 **Wunder A**, Müller-Ladner U, Stelzer EH, Funk J, Neumann E, Stehle G, et al. Albumin-based drug delivery as novel therapeutic approach for rheumatoid arthritis. *J Immunol* 2003;**170**:4793–801.

14 **Müller-Ladner U**, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, et al. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. *Am J Pathol* 1996;**149**:1607–15.

15 **Geller T**, Kriegsmann J, Keyszer GM, Gay RE, Gay S. A new model for rheumatoid arthritis generated by engraftment of rheumatoid synovial tissue and normal human cartilage into SCID mice. *Arthritis Rheum* 1994;**37**:1664–71.

16 **Judex M**, Neumann E, Fleck M, Pap T, Mountz R, Gay RE, Scholmerich J, et al. ''Inverse wrap'': an improved implantation technique for virus-transduced synovial fibroblasts in the SCID mouse model for rheumatoid arthritis (RA). *Mod Rheumatol* 2001;**11**:145.

17 **Hosler GA**, Bash R, Scheuermann RH. Kinetics of early therapeutic response as measured by quantitative PCR predicts survival in a murine xenograft model of human T cell acute lymphoblastic leukemia. *Leukemia* 2000;**14**:1215–24.

18 **Müller-Ladner U**, Evans CH, Franklin BN, Roberts CR, Gay RE, Robbins PD, et al. Gene transfer of cytokine inhibitors into human synovial fibroblasts in the SCID mouse model. *Arthritis Rheum* 1999;**42**:490–7.

19 **Neumann E**, Judex M, Kullmann F, Grifka J, Robbins PD, Pap T, et al. Inhibition of cartilage destruction by double gene transfer of IL-1Ra and IL-10 involves the activin pathway. *Gene Ther* 2002;**9**:1508–19.

20 **Seitz M**, Zwicker M, Loetscher P. Effects of methotrexate on differentiation of monocytes and production of cytokine inhibitors by monocytes. *Arthritis Rheum* 1998;**41**:2032–8.

21 **Chang DM**, Weinblatt ME, Schur PH. The effects of methotrexate on interleukin 1 in patients with rheumatoid arthritis. *J Rheumatol* 1992;**19**:1678–82.