Effects of methotrexate on normal articular cartilage in vitro and in vivo

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Abstract

Objective—Methotrexate (MTX) has become the disease modifying drug of choice for the treatment of rheumatoid arthritis (RA). Direct effects of MTX on articular cartilage in vivo and in vitro were studied to determine possible adverse effects of the drug.

Methods—For in vitro experiments, adult bovine articular cartilage explants were cultured in the presence of MTX (0 to 100 μM), and effects on DNA and matrix metabolism were studied. For in vivo studies, 48 adult female rabbits were treated with MTX (30 mg/kg/week intramuscularly) or placebo, respectively, for up to 12 weeks, and effects on the cartilage of the femoral condyles were assessed.

Results—In vitro, MTX dose dependently increased the uptake of [³H]-thymidine, and decreased incorporation of [³H]-d-uridine into chondrocytes with a half maximal effect at 0.03 μM, suggesting inhibition of thymidylate-synthetase activity by the drug. MTX also dose dependently reduced the proportion of chondrocytes in S-phase, as determined by flow cytometry. MTX did not affect LDH release from chondrocytes or the proportion of viable cells, nor did it change the rate of protein synthesis, proteoglycan synthesis, proteoglycan breakdown, or the hydrodynamic size of newly synthesised proteoglycans. In vivo, MTX did not appreciably affect proteoglycan synthesis of the chondrocytes, proteoglycan content of the cartilage matrix, density of the chondrocyte population, or histological integrity of the cartilage.

Conclusions—The data suggest the absence of major adverse effects by MTX on articular cartilage proteoglycan metabolism. Chondrocyte DNA metabolism seems to be changed by MTX only in concentrations and exposition periods clearly exceeding those found in synovial fluid of RA patients receiving the commonly prescribed doses of the drug.

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This paper has been
respectfully dedicated to
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Rheumatoid arthritis (RA) is a chronic inflammatory joint disease often leading to destruction of articular cartilage, periarticular bone, ligaments, and tendons. Treatment of RA has changed over recent years toward an earlier and more aggressive application of disease modifying anti-rheumatic drugs (DMARDs), and increased use of these drugs has been shown to positively influence the disability index of RA patients.[1] Among DMARDs, methotrexate (MTX) is probably the one most widely used today, and seems to have in many clinics replaced injectable gold salts as the drug of choice after non-steroidal anti-inflammatory drugs (NSAIDs).[2–4]

Clinical studies demonstrate a relatively rapid onset of action of MTX in RA compared with other DMARDs,[5] and double blind trials have proved the efficacy of MTX in RA in both the short and the long term.[2 3] The mode of action of MTX in RA is not yet fully understood. High dose MTX seems to suppress cell replication in general and tumour growth in particular by inhibiting the activity of dihydrofolate reductase, an enzyme involved in DNA and purine biosynthesis. In contrast, weekly pulse therapy with MTX in low doses of 7.5 to 25 mg, as used to treat RA, probably does not significantly interfere with DNA metabolism but may rather exert an anti-inflammatory effect in RA and related disorders.[6] While the mechanisms underlying this presumed anti-inflammatory effect are still unclear, interference by MTX with leucocyte migration, expression of proteinases, and with the activity of cytokines may be involved.[7–10]

Widespread use of methotrexate for the treatment of RA has raised interest in possible direct effects of MTX on articular cartilage. Few data, however, have as of yet been published on this topic. Initial degenerative cartilage changes have been described in patients receiving high doses of MTX in combination with other anti-cancer drugs for malignancy,[11] and also in experimental animals treated with low doses of the drug.[12] In contrast, articular cartilage from MTX treated RA patients undergoing total knee replacement contained less metalloproteinase activity than cartilage from a control group of RA patients who did not take MTX.[13] Moreover, low dose MTX had limited therapeutic effects in a rabbit model of osteoarthritis (OA).[12] Finally, the rate of proteoglycan synthesis of cultured cartilage was found to be unaffected by MTX.[14]

While in RA patients, cartilage in inflamed joints presumably benefits from reduction of synovitis by MTX, negative MTX effects on cartilage from joints unaffected by RA cannot be excluded. In this study, we therefore investigated the effects of MTX on normal articular cartilage in vitro and in vivo.

Methods

MATERIALS

Methotrexate was purchased from Medac, (Hamburg, Germany). [³⁵S]-methionine,

$Na_2[^{35}S]O_4$, $[^3H]$-thymidine, and $[^3H]$-d-uridine were obtained from Amersham (Braunschweig, Germany). T61 was from Hoechst (Frankfurt aM), and dimethyl-methylene-blue from Polysciences (Philadelphia, PA). Dulbecco's modified Eagles medium (DMEM) and all other substances were from Sigma (Deisenhofen, Germany), unless otherwise specified.

ARTICULAR CARTILAGE EXPLANT CULTURES
Bovine articular cartilage was cultured as described previously,[15] in the presence of various concentrations of MTX for the periods of time indicated. Briefly, metacarpophalangeal joints of adult Holstein cows were obtained immediately after slaughter from a local abbatoir. The joints were opened in the laboratory under sterile conditions, and cartilage discs representing all cartilage layers but, as far as possible, no subchondral bone, prepared with a cork-borer and scalpel. The discs (wet weight approximately 25 mg) were halved, and each half incubated separately in 180 μl culture medium in a 96 well tissue culture plate at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Culture media were supplemented with 100 IU/ml penicillin G, 50 μg/ml streptomycin, 1 μg/ml amphotericin B, 100 μg/ml ascorbic acid, and 5% (vol/vol) heat inactivated fetal calf serum. Each treatment group consisted of eight cartilage samples, unless otherwise specified. A resting period of 16 to 24 hours was allowed before experimental incubation with MTX containing media was started. Media were replaced every two days in all experiments, keeping MTX concentrations constant at the required levels for the respective treatment groups.

In this tissue culture model, the effects of MTX on a variety of parameters were studied as described below, including LDH release from chondrocytes, chondrocyte protein synthesis, proteoglycan synthesis, size of newly synthetised proteoglycans, release of proteoglycans, DNA synthesis, proportion of chondrocytes in S-phase, and proportion of viable chondrocytes.

ANIMALS
After approval of the study by the local ethics committee, adult female New Zealand White rabbits (weight approximately 4 kg) were obtained from a professional breeder (Wenzel, Detmold, Germany). The animals were kept in standard cages under the supervision of a veterinarian, and were fed dry food and tap water ad libitum.

PHARMACOKINETICS OF MTX IN RABBITS
MTX (30 mg/kg body weight) was administered intravenously (n=3), or intramuscularly (n=5). Serial plasma samples were then obtained from the animals during the next 24 hours, and MTX concentrations determined fluorometrically, using the TDX assay kit from Abbott (Wiesbaden, Germany). The MTX concentrations in the synovial fluid in the knee joints of the animals were determined 24 hours after intramuscular injection of 30 mg MTX/kg

body weight (n=5), as follows. Normal saline solution (0.5 ml) was injected intra-articularly in one knee joint, the joint flexed 10 times, and the joint wash fluid aspirated (usually about 0.35 ml). In the wash-fluid, the concentration of MTX was determined as described above. In a separate experiment, the normal synovial fluid volume in a rabbit knee was determined by intra-articular injection of 1 ml aqueous Coomassie blue solution (E_{545} 1.8) in five animals. The knee joints were then flexed 10 times, joint fluid aspirated, and the dilution determined photometrically at 545 nm. The normal synovial fluid volume was found to be 20 (4) μl (mean (SEM), n=5). This compares well with a reported mean synovial fluid mass of 24 mg (approximately 23 μl) for normal rabbit knee joints.[16] On the basis of our data, MTX concentrations in synovial fluid could be calculated.

EFFECTS OF MTX ON ARTICULAR CARTILAGE IN VIVO
Rabbits were treated with MTX in weekly pulses of 30 mg/kg body weight for 6 or 12 weeks, respectively, and subsequently killed by intravenous injection of T61. Both knee joints were dissected free by femoral and crural osteotomies after removal of the cutis. Care was taken not to open the joints. The surface of the specimens was sterilised by immersing the joints in an aqueous povidon-iodine solution at 4°C for one hour. The subsequent preparation was performed under sterile conditions in a laminar air flow hood. Cartilage discs representing all cartilage layers, but, as far as possible, no subchondral bone, were obtained from the weight bearing area of the femoral condyles. Some of these samples were used to measure the proteoglycan content of the cartilage as described below. In the remaining samples, the ex vivo rate of proteoglycan synthesis of the chondrocytes was determined. Briefly, cartilage discs were incubated in DMEM culture medium in 96 well tissue culture plates for 16 hours, then labelled with $Na_2[^{35}S]O_4$ for four hours. The samples were washed repeatedly with PBS, weighed, and digested with papain as described below. Proteoglycans in the cartilage digest were then determined as reported below.

For histological analysis of the cartilage, the respective contralateral knee was used. Both femoral condyles were carefully separated from the distal femur, and fixed in formaline. After decalcification in EDTA for 4 to 6 weeks, the samples were embedded in paraffin wax, cut in the frontal plane, and stained with safranin-O and fast green or haematoxilin and eosin. To avoid observer bias, all slides were coded before microscopical analysis. To assess the degree of cartilage damage, histological grading of the samples was performed using the Mankin score[17] in the slightly modified form described by van der Sluijs.[18] The Mankin score reflects the structure of the cartilage, chondrocyte population, and proteoglycan content of the matrix, and the state of the tidemark between calcified and hyaline cartilage. Score values can
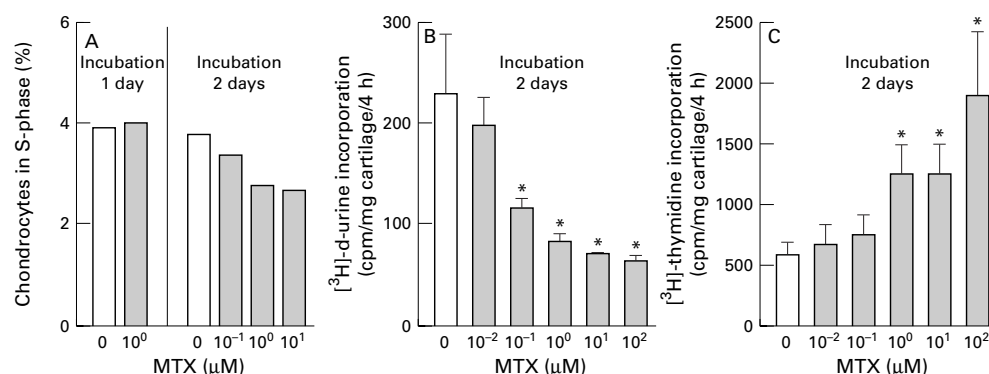
Figure 1 Effects of MTX on the DNA metabolism of cultured articular cartilage. (A) Reduction of the proportion of chondrocytes in S-phase by MTX in vitro. Bovine articular cartilage explants were cultured in the presence of various MTX concentrations, then isolated with collagenase, stained with propidium iodide, and analysed for DNA content by flow cytometry. The proportion of chondrocytes in S-phase was dose dependently reduced by MTX after 48 hours of culture (p<0.05; linear regression analysis), but not after 24 hours. Bars represent means of at least 10 000 events. (B) Inhibition of [³H]-d-uridine-incorporation into chondrocytes by MTX in vitro. Bovine articular cartilage explants were cultured in the presence of various MTX concentrations for two days, then labelled with [³H]-d-uridine for four hours, washed in PBS to remove free [³H]-d-uridine, digested with papain, and counted for [³H]. Bars represent means and SEM of eight cartilage samples per group; ★ p < 0.05 v control without MTX (t test). (C) Stimulation of [³H]-thymidine incorporation into chondrocytes by MTX in vitro. Bovine articular cartilage explants were cultured in the presence of various MTX concentrations for two days, then labelled with [³H]-thymidine for four hours, washed in PBS to remove free [³H]-thymidine, digested with papain, and counted for [³H]. Bars represent means and SEM of eight cartilage samples per group; ★ p<0.05 v control without MTX (t test).

range from zero points (normal cartilage) to 14 points (maximally damaged cartilage).

ANALYTICAL PROCEDURES
Determination of LDH
Concentrations of LDH in conditioned culture media were measured fluorometrically, using the TDX assay kit from Abbott (Wiesbaden, Germany).

Rate of chondrocyte protein synthesis
Cartilage explants were cultured in the presence or absence of MTX or cycloheximide, respectively, for two days, then labelled with [³⁵S]-methionine (20 µCi/ml) for four hours. Samples were washed with PBS, weighed, and hydrolysed with papain as described below. Hydrolysed cartilage (100 µl) was precipitated with 100 µl 6% trichloroacetic acid (TCA) on ice, then centrifuged at 2000 *g* for 10 minutes. The pellet was resuspended in PBS, and the precipitated material collected on 45 µm filters. Filters were counted for contained radioactivity, and results expressed as cpm/mg/ cartilage wet weight/4 hours.

Rate of chondrocyte proteoglycan synthesis
Cartilage explants were incubated in the presence of various MTX concentrations for the time indicated. At the end of each respective experiment, the specimens were labelled with [³⁵S]-sulphate (10 µCi/ml) for four hours, washed with PBS, weighed, and digested with papain in 0.1 M phosphate buffer (pH 6.5), containing 5 mM cysteine HCl and 5 mM EDTA, at 65°C. Aliquots of the hydrolysed cartilage were brought up to a final concentration of 30 mM cetylpyridinium chloride (CPC). Samples were agitated, and precipitates collected on 45 µm filters (Millipore, Eschborn, Germany). The filters were washed with 30 mM CPC and counted for [³⁵S] in an LKB Rackbeta liquid scintillation counter (Pharmacia-LKB, Freiburg, Germany). Re-

sults were expressed as cpm/mg/cartilage wet weight/4 hours. Others have shown that bovine articular cartilage explants in culture will incorporate [³⁵S]-sulphate almost exclusively into proteoglycans, and not into other macromolecules.[19]

Hydrodynamic size of newly synthesised proteoglycans and total proteoglycans
Proteoglycan monomers were analysed as described by Tyler,[20] with minor modifications. Briefly, cartilage explants were cultured in the presence or absence of MTX for the time indicated, then labelled with [³⁵S]-sulphate for six hours. The samples were then sliced, and the PGs extracted with 4 M guanidine-HCl in a 50 mM Na-acetate buffer (pH 5.8) at 4°C for 24 hours. The extracted PGs were dialysed against a 10 mM Na-acetate buffer (pH 5.8), and lyophilised. During extraction and dialysis, the following proteinase inhibitors were present: iodoacetic acid (1 mM), pepstatin (1 mg/l), phenanthroline (1 mM), and phenylmethanesulfonyl-fluoride (1 mM). For gel filtration, the samples were resuspended in 500 mM Na-acetate buffer (pH 7). Size exclusion chromatography was performed using the same buffer, on a Sephacryl-S-200-HR column (1.2 × 40 cm; V_0 15.5 ml; V_t 45.5 ml; flow rate 11 ml/h) under dissociative conditions (4 M guanidine-HCl), at room temperature. An aliquot of the fractions was counted for [³⁵S] in a liquid scintillation counter to determine the amount of newly synthesised PGs. The total PG content of the samples was determined as described in the next paragraph.

Proteoglycan content of cartilage
For this, cartilage explants were hydrolysed using papain as described above. The PG content of the samples was determined using a dimethyl-methyleneblue dye binding assay as reported by Farndale *et al*,[21] with chondroitin sulphate (CS) from bovine trachea as standard.
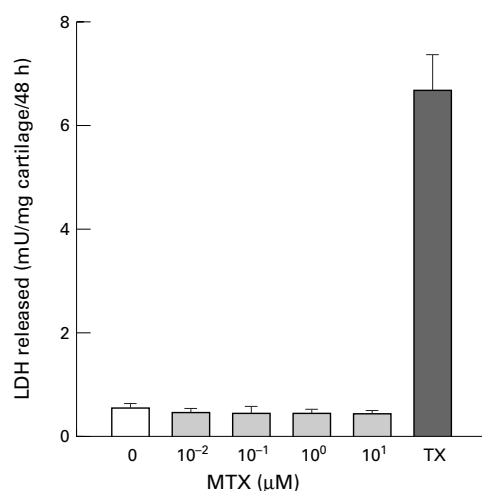
Figure 2   No effect of MTX on LDH release from cultured cartilage. Bovine articular cartilage was incubated for two days in the presence of MTX (0 to 10 μM), and the amount of lactate dehydrogenase (LDH) released into the culture medium determined fluorometrically. The detergent Triton-X 100 (TX) was used as a positive control. Bars represent means and SEM of eight cartilage explants per group.
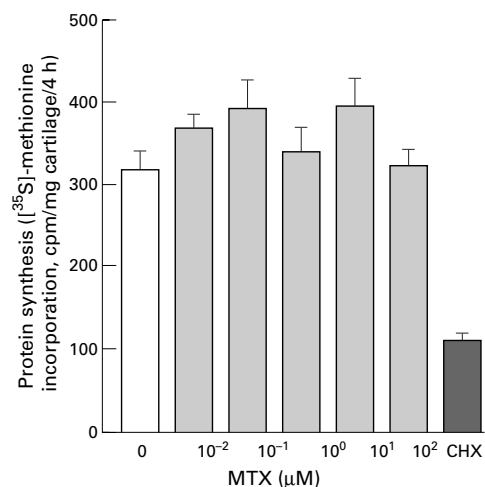

Figure 3   No inhibition by MTX of chondrocyte protein synthesis in vitro. Bovine articular cartilage was incubated for two days in the presence of MTX (0 to 100 μM), or cycloheximide (CHX) (10 mg/l). The samples were subsequently labelled with [^{35}S]-methionine for four hours, hydrolysed with papain, and incorporation of the tracer into trichloroacetic acid precipitable macromolecules was determined. Bars represent means and SEM of eight cartilage explants per group.

Results were expressed as μg CS/mg cartilage wet weight.

Release of proteoglycans from cultured cartilage

Cartilage explants were labelled with [^{35}S]-sulphate as described above, then washed with PBS, until the proportion of non-incorporated [^{35}S]-sulphate contained in the cartilage was below one per cent. This was validated in a separate experiment by size exclusion chromatography of papain digested cartilage on a Sephacryl-S-200 column (not shown). Incubation of the cartilage was continued in the presence of various concentrations of MTX for 10 days. Culture media were replaced every two days with media containing MTX in concentrations corresponding to the respective treatment group. The spent media were counted for [^{35}S] contained in CPC precipitable macromolecules as desribed above. At the end of the experiment, the remaining [^{35}S] in the cartilage samples was determined by liquid scintillation counting after papain digestion, as described above. The proportion of the released PGs was then calculated.

Chondrocyte DNA metabolism

Cartilage explants were incubated in DMEM with MTX in various concentrations for two days, then labelled with [^{3}H]-thymidine or [^{3}H]-deoxy-uridine, respectively, (10 μCi/ml) for four hours. The samples were subsequently washed repeatedly in ice cold PBS, until the radioactivity in the wash-fluid had reached background values. Incorporation of radionuclides into chondrocytes was then determined by liquid scintillation counting after papain digestion of the cartilage as described above. Results were expressed as cpm/mg/ cartilage wet weight/48 hours. Autoradiography of cartilage specimens demonstrated the presence of radioactivity almost exclusively within chondrocytes (not shown).

Flow cytometry of articular chondrocytes

Cartilage explants were exposed to MTX in culture for the time indicated, and chondrocytes subsequently isolated by collagenase treatment. Samples were digested in DMEM in spinner culture at 37°C overnight, using 0.08% (weight/volume) clostridial collagenase (Sigma). The cells were washed in PBS, centrifuged at 1500 *g* for 10 minutes, and this step repeated twice. Subsequently, nuclear DNA was stained with propidium iodide (Cycle Test, Becton Dickinson) according to the manufacturer's instructions. Cells were washed twice in 5 ml citrate buffer, centrifuged (300 *g*, 5 minutes), resuspended in citrate buffer, and adjusted to a concentration of 2.5 × 10^6 cells/ml. The cell suspension (200 μl) was added to 1.8 ml trypsin containing spermine tetrahydrochloride detergent buffer, and incubated for 10 minutes at room temperature under gentle agitation. Trypsin-inhibitor/ ribonuclease A-buffer solution (1.5 ml) was then added, and incubation continued while gently shaking the tubes. Subsequently, 1.5 ml of ice cold propidium iodide solution was added, and the tin-foil wrapped tubes incubated for another 10 minutes at room temperature under gentle agitation. The suspension was then filtered through a 50 μm nylon mesh, transferred to the FACScan measurement tubes, and stored in an ice bath under light protection. Cell cycle analysis was performed on a FACScan (Becton Dickinson) flow cytometer, equipped with an argon laser (15 mV, 488 nm) and a double discrimination module. The machine was equilibrated with DNA QC particles (Becton Dickinson) according to the manufacturer's instructions. A minimum of 10 000 events was acquired for each sample. Analysis of the data was performed using the CellFIT software (Becton Dickinson). The proportion of cells in the dif-
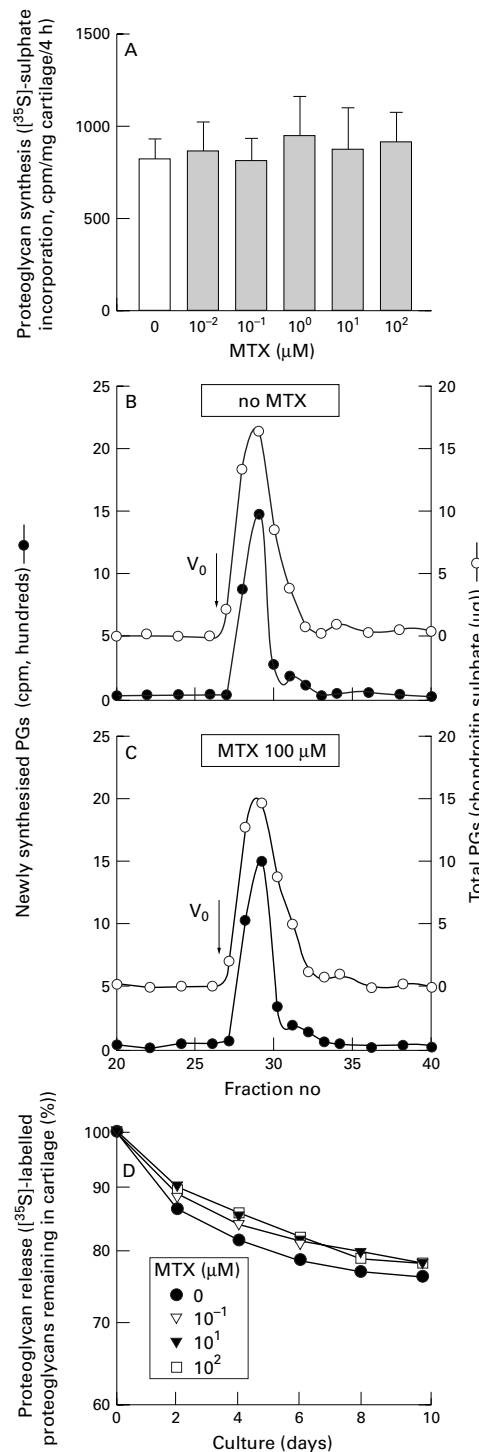
Figure 4 No effects of MTX on cartilage proteoglycan metabolism in culture. (A) No inhibition by MTX of chondrocyte proteoglycan synthesis in vitro. Bovine articular cartilage was incubated for two days in the presence of MTX (0 to 100 µM). The samples were subsequently labelled with [³⁵S]-sulphate for four hours, hydrolysed with papain, and incorporation of the tracer into cetyl-pyridiumchloride precipitable macromolecules was determined. Bars represent means and SEM of eight cartilage explants per group. Identical results were obtained, when cartilage was cultured for 4, 8, or 16 days in the presence of MTX (not shown). (B) and (C) No alteration by MTX of the hydrodynamic size of newly synthesised (solid circles) or total (open circles) proteoglycan monomers in cultured articular cartilage. Bovine cartilage explants were incubated for 16 days in the absence (B) or presence (100 µM) of MTX (C). Media were replaced every two days, keeping the respective MTX concentrations (that is, 0 or 100 µM) constant throughout the entire incubation period. The samples were then labelled with [³⁵S]-sulphate for four hours by addition of the tracer. The PGs were extracted from the cartilage using 4 M guanidine-HCl, and subjected to size exclusion chromatography on a Sephacryl-S-200-HR column under dissociative conditions. In the respective fractions, the newly synthesised PGs (as [³⁵S]; solid circles) were determined by liquid scintillation counting, and the total PGs (as chondroitin sulphate (CS); open circles) measured using a dimethyl-methyleneblue dye binding assay. The smooth lines are based on a peak analysis performed with the SIGMA-Plot software package for microcomputers (Jandel Scientific, Erkrath, Germany). The arrows indicate v_0 of the column, v_t corresponds to fraction no 77. (D) No MTX effect on the spontaneous PG release from articular cartilage in culture. Bovine articular cartilage explants were labelled with [³⁵S]-sulphate for four hours. Free sulphate was subsequently removed by repeatedly washing the samples in PBS (verified by size exclusion chromatography). Incubation was then continued for 10 days in the presence of various MTX concentrations. Media were harvested every two days and replaced with fresh media containing MTX in concentrations corresponding to the respective treatment group. Radiolabelled PGs contained in the harvested media, and PGs remaining in the cartilage samples, respectively, were determined by liquid scintillation counting as described in methods. The rate of PG release was then calculated.

ferent phases of the cycle was determined using the SOBR model provided with the program.

Proportion of viable chondrocytes
This was determined by flow cytometry (see above), and also by the trypan blue dye exclusion method.

DESCRIPTIVE AND ANALYTICAL STATISTICS
Score values were reported as median with percentiles, continuous variables as mean (SEM). Differences between group medians or group means, respectively, were tested as indicated. All tests were two tailed, and $p < 0.05$ was considered significant.

Results

EFFECTS OF MTX ON BOVINE ARTICULAR CARTILAGE IN EXPLANT CULTURE
MTX dose dependently reduced the proportion of chondrocytes in S-phase when cartilage was exposed to the drug for two days (fig 1A). No respective MTX effect was observed when incubation of the cartilage was carried out for only one day (fig 1A), or for one day in the presence of MTX, followed by another six days in the absence of the drug (latter data not shown). MTX also dose dependently decreased incorporation of [³H]-d-uridine into chondrocytes with a half maximal effect at 0.03 µM, and increased the uptake of [³H]-thymidine (fig 1B,C). The release of LDH from chondrocytes was not affected by MTX (fig 2), nor did the drug change the proportion of viable chondrocytes in cartilage explants cultured in the presence of 100 µM MTX for 16 days (MTX: 95% viable cells; control without MTX: 94% viable cells; determined by flow cytometry. Identical results were obtained with the trypan blue dye exclusion method).

According to further studies, MTX did not appreciably influence the rate of chondrocyte protein synthesis, proteoglycan synthesis, pro-
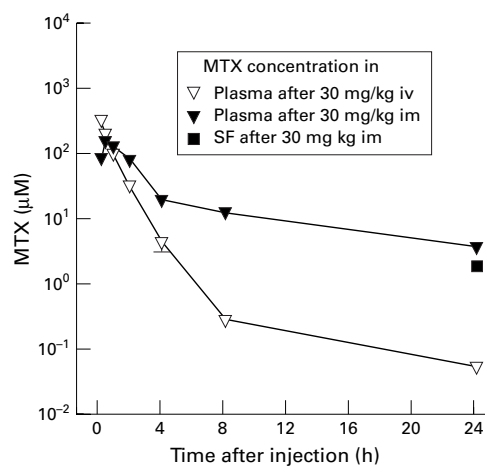
Figure 5   Pharmacokinetics of MTX in adult rabbits. MTX (30 mg/kg body weight) was administered intravenously (n=3), or intramuscularly (n=5). Serial plasma samples were then obtained, and MTX concentrations measured fluorometrically. The MTX concentrations in the synovial fluid (SF) of the knee joints of the animals were determined 24 hours after intramuscular injection of 30 mg MTX/kg body weight (n=5), as described in methods. Symbols represent means and SEM of the indicated number of animals. Most error bars do not exceed the size of their respective symbol.

teoglycan breakdown, or the hydrodynamic size of newly synthesised proteoglycans or total proteoglycans (figs 3 and 4).

EFFECTS OF MTX ON LAPINE ARTICULAR
CARTILAGE IN VIVO

Pharmacokinetic studies demonstrated a mean MTX concentration of 2 µM in the synovial fluid of rabbits, 24 hours after intramuscular injection of 30 mg MTX/kg (fig 5).

Systemic MTX treatment in weekly pulses (intramuscular 30 mg MTX/kg/week) for 6 or 12 weeks, respectively, had no significant effects on the articular cartilage of the animals, as determined by investigating the ex vivo rate of chondrocyte proteoglycan synthesis, proteoglycan content of the cartilage matrix,
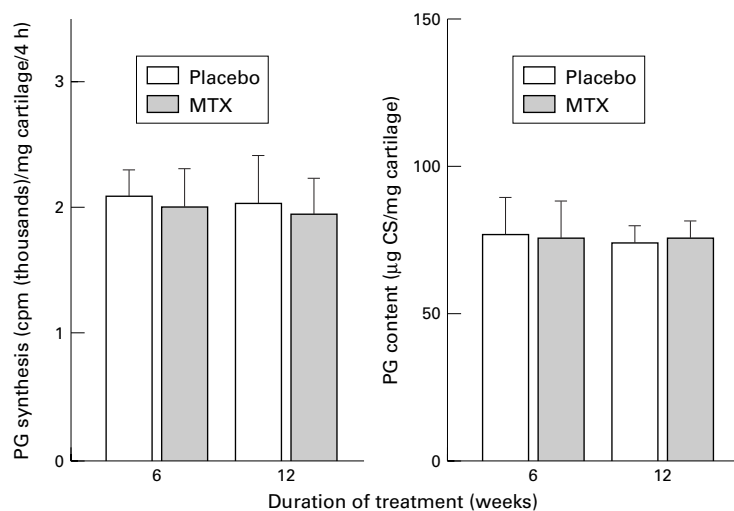

Figure 6   No significant effects of systemic MTX on PG synthesis or PG content of articular cartilage in adult rabbits. Rabbits were treated with MTX (30 mg/week/kg intramuscularly) or placebo, respectively, for 6 or 12 weeks, and effects on the PG metabolism of the cartilage of the femoral condyles were assessed as described in methods. Bars represent means and SEM of eight animals per group (treatment for six weeks), or 16 animals per group (treatment for 12 weeks). CS = chondroitin sulphate.

Table 1   Histological analysis (Mankin score) of articular cartilage (rabbits, femoral condyles), after weekly treatment with MTX (30 mg/kg body weight im), or placebo, respectively, for the time indicated

| Treatment | Mankin score (median (range)) | |
	6 weeks	12 weeks
MTX	1 (0–4)	1 (0–3)
placebo	1 (0–3)	1 (0–3)

Values represent median and range of eight animals per group (treatment for 6 weeks), or 16 animals per group (treatment for 12 weeks), respectively. The Mankin score reflects the structure of the cartilage, chondrocyte population, and proteoglycan content of the matrix, and the state of the tidemark between calcified and hyaline cartilage. Score values can range from zero points (normal cartilage) to 14 points (maximally damaged cartilage).

density of the chondrocyte population, and histological integrity of the cartilage matrix (fig 6, table 1).

Discussion

In RA, inflammatory cells from the blood invade the synovium and release cytokines and catabolic enzymes that induce destruction of articular cartilage and adjacent bone. Initial changes observed in RA cartilage are characterised by proteoglycan loss caused by suppression of PG synthesis and increase of PG breakdown.[15] With progressing disease, total cartilage loss may ensue.

Therapeutic effects of MTX in RA may be based on inhibition of leucocyte adherence, reduction of collagenase gene expression in inflamed synovium, a decrease of IL1 concentrations in synovial fluid, and changes in the activity of IL1.[7–10] In contrast, MTX cannot reverse the catabolic effects of exogenous IL1 on articular cartilage PG synthesis or PG breakdown, either in vitro,[14 22] or in vivo (Neidel, unpublished results).

While MTX effectively controls arthritis in a subset of RA patients,[2 3] and in animal models of RA,[22–25] little is known on direct effects of the drug on normal articular cartilage as in joints spared from RA.

In this study, we used MTX in a wide concentration range to study its effects on normal articular cartilage in vitro and in vivo. MTX concentrations used included levels and areas under the concentration curve exceeding those present in plasma or synovial fluid of RA patients receiving weekly pulses of 7.5 to 25 mg MTX.[26]

Our data suggest the absence of major MTX effects on cartilage proteoglycan synthesis or PG breakdown in vitro or in vivo. Neither did MTX significantly affect the size of newly synthesised PGs or total PGs, nor the anatomic integrity of the cartilage tissue. We also failed to observe general toxic MTX effects on chondrocytes, demonstrated by an unchanged proportion of viable cartilage cells after MTX exposure. As the release of the intracellular enzyme LDH from chondrocytes was unaffected by MTX, the drug does not seem to damage the membrane of these cells. Neither did MTX inhibit chondrocyte protein synthesis.

MTX, however, did affect chondrocyte DNA metabolism in a dose dependent way. An increased uptake of [³H]-thymidine in conjunc-

tion with a decreased uptake of [³H]-d-uridine into chondrocytes exposed to MTX suggests inhibition of thymidylate synthetase by the drug, demonstrated here to our knowledge for the first time for chondrocytes. This is in accordance with reduction by MTX of the proportion of chondrocytes in S-phase. Interestingly, chondrocyte DNA metabolism was only altered by MTX in concentrations and exposition periods clearly exceeding those found in SF or plasma of RA patients receiving the commonly prescribed dose range of the drug.[26]

Published data on direct effects of MTX on cartilage are limited. Three decades ago, Steinberg et al[27] administered MTX intra-articularly in rabbits in single or repeated doses and saw no adverse effects on the articular cartilage.

In a more recent study, the rate of proteoglycan synthesis of cultured human cartilage was found to be unaffected by MTX,[14] which is in accordance with our results, but no data were given on the size of the newly synthesised PGs.

Mild degenerative cartilage changes were observed, in contrast, in samples taken at necropsy from cancer patients who had been treated with a variety of drugs including MTX.[11] Nine patients of the study group had received MTX, however, all of them had simultaneously been treated with at least two other potent anticancer drugs, among them vincristine, adriamycin, and cyclophosphamide. Hence, the data do not permit conclusions regarding possible effects of MTX on cartilage.

Mannoni et al[12] demonstrated that MTX, in comparison with placebo treatment, could to a limited extent prevent cartilage degeneration in a rabbit model of osteoarthritis. At the same time, they found no significant MTX effects on macroscopic and microscopic appearance of normal cartilage taken from non-operated joints. These data were based on the histological analysis of normal cartilage in three animals per respective treatment group. Collagen content, in contrast, was found to be reduced in normal cartilage of MTX treated animals, but these results were erratic in as much as after MTX treatment the collagen content of OA cartilage was reportedly higher than the collagen content of normal cartilage while the opposite was the case in the placebo treated animals. Their data also did not show significant changes in the content of metalloproteinases or TIMP in normal cartilage under MTX treatment, which could have accounted for loss of collagen. Moreover, in another study, cartilage collagen content was reportedly normal in cancer patients receiving high doses of MTX.[11]

We failed to find significant degeneration of normal lapine articular cartilage by MTX when we administered MTX to an identical strain of fully grown rabbits in 300-fold higher weekly doses and over a longer period of time than in the study by Mannoni and coworkers.[12]

Martel-Pelletier et al[13] found that MTX given in combination with prednisone for the treatment of RA, reduced collagenase activity in the articular cartilage in comparison with

samples from RA patients receiving non-steroidal anti-inflammatory drugs, corticosteroids, or gold salts. These findings may reflect indirect MTX effects on cartilage, however, as MTX has been shown to reduce collagenase gene expression in RA synovium.[8] MTX also decreases raised IL1 concentrations in RA synovial fluid,[10] which could subsequently lower stromelysin synthesis by chondrocytes.[28] Stromelysin, in turn, is a potent activator of latent collagenase.[29]

In summary, data from the literature suggest, MTX may to some extent prevent cartilage breakdown in inflamed joints both in human RA, and in animal models of the disease.[13 22–25] This MTX effect is probably mediated via reduction of synovitis rather than by direct effects on cartilage.

Possible adverse effects of MTX on cartilage in joints spared from RA have been subject of concern. However, MTX in doses used for the treatment of RA was shown to lack significant toxic effects on articular cartilage under the conditions studied by us. Interpretation of these results should be performed with caution as data stemming from the study of animal tissue may only in part reflect the situation in humans.

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