Methotrexate Inhibits Rheumatoid Synovitis by Inducing Apoptosis

FUJIO NAKAZAWA, HIROAKI MATSUNO, KAZUO YUDOH, RIE KATAYAMA, TAKASHI SAWAI, MIWA UZUKI, and TOMOATSU KIMURA

ABSTRACT. ***Objective.*** To clarify the pharmacological action of methotrexate (MTX) on the synovium of patients with rheumatoid arthritis (RA) using severe combined immunodeficient (SCID) mice in which human RA synovial tissue had been grafted (SCID-HuRAg).

Methods. One month after engraftment of human RA tissue into SCID mice, MTX (0.3 mg/kg) was administered orally, then the appearance of apoptosis in the grafted tissue was examined by TdT mediated dUTP nick end labeling (TUNEL) staining and electron microscopy at various time points after MTX administration. In cultured synovial cells, synovial apoptotic changes after MTX treatment were studied by agarose gel electrophoresis and flow cytometric analysis. To compare the histological changes induced by MTX with those induced by other disease modifying antirheumatic drugs (DMARD) and a nonsteroidal antiinflammatory drug, histological examination of the grafted synovial tissues from SCID-HuRAg mice was conducted after 4 weeks of oral administration of MTX (0.3 mg/kg/week), salazosulfapyridine (30 mg/kg/day), auranofin (0.2 mg/kg/day), bucillamine (10 mg/kg/day), or indomethacin (2 mg/kg/day).

Results. A significant decrease in the number of inflammatory cells was observed in the grafted synovial tissue of MTX treated SCID-HuRAg. A similar antiinflammatory effect was not observed with the other DMARD. Induction of apoptosis was noted with MTX treatment but not with the others. The pro-apoptotic effect of MTX was also observed in synovial cell cultures.

Conclusion. MTX induces apoptosis in RA synovium that, in turn, may contribute to its antiinflammatory effect on RA synovitis. (J Rheumatol 2001;28:1800–8)

Key Indexing Terms:
METHOTREXATE RHEUMATOID ARTHRITIS SYNOVIUM
APOPTOSIS SCID MOUSE

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflamed synovial hyperplasia with excessive inflammatory cell infiltration. Several disease modifying antirheumatic drugs (DMARD) are used in the treatment of RA. Methotrexate (MTX) is considered one of the most effective DMARD[1]. Several pharmacological mechanisms for MTX have been reported: suppression of lymphocyte proliferation[2], reduction of immunoglobulin[3], inhibition of interleukin 1 (IL-1) production[4], suppression of neutrophil chemotaxis[5], inhibition of vascular endothelial cell proliferation[6], and increased adenosine release[7]. These pharmacological actions are not unique to MTX[8] and have been described after the administration of other DMARD or nonsteroidal antiinflammatory drugs (NSAID). However, the antirheumatic effect of MTX is more potent and more rapid than other DMARD and NSAID[9]. This led us to hypothesize that a mechanism different from those reported previously might be involved in the antirheumatic effect of MTX.

We have reported[10,11] the induction of apoptosis in RA synovitis by Fas ligand gene transfer or local administration of an anti-Fas monoclonal antibody. Fujisawa, *et al* reported that local administration of an anti-Fas Mab to a mouse RA model led to improvement in the arthritis[12]. A report on leukemia cells indicated that MTX induced apoptosis of cancer cells[13]. If MTX could induce apoptosis in RA synovial tissue, this would provide insight into the pharmacological action of MTX for treatment of RA synovitis. Thus we investigated the histological changes and induction of apoptosis after MTX administration using severe combined immunodeficient (SCID) mice in which human RA synovial tissue had been grafted (SCID-HuRAg)[14].

MATERIALS AND METHODS

Preparation of SCID-HuRAg mice. SCID-HuRAg mice were prepared as described[10,11,14]. Male SCID mice (CB.17/lcr; Clea Japan Inc., Tokyo, Japan) that were 6 to 7 weeks old and bred under specific pathogen-free conditions at our university animal center were used. Pannus tissue from synovial membrane, cartilage, and bone, collected as a mass from patients with RA at surgery, was used for implantation. The antirheumatic drugs

From the Department of Orthopedic Surgery, Toyama Medical and Pharmaceutical University, Toyama; and the Department of Pathology, Iwate Medical School, Iwate, Japan.

F. Nakazawa, MD; H. Matsuno, MD, PhD; K. Yudoh, MD, PhD; R. Katayama MD; T. Kimura, MD, PhD, Department of Orthopedic Surgery, Toyama Medical and Pharmaceutical University; T. Sawai, MD, PhD; M. Uzuki, MD, PhD, Department of Pathology, Iwate Medical School.

Address reprint requests to Dr. F. Nakazawa, Department of Orthopedic Surgery, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: fujio@ms.toyama-mpu.ac.jp

Submitted May 24, 2000; revision accepted February 21, 2001.

used by these patients before surgery were MTX (15 patients), salazosulfapyridine (16), auranofin (12), and bucillamine (24). In some patients, these drugs had been used in combination. The size of the grafted specimen was adjusted to a block almost 10 mm in diameter before implantation. All surgical procedures were performed under sterile conditions. SCID-HuRAg mice were available for this study at one month after implantation, when survival of the grafted tissue was confirmed.

Induction of apoptosis in grafted synovial tissue by MTX administration. The time course of the induction of apoptosis by MTX was investigated in 60 SCID-HuRAg mice divided into 20 groups, each consisting of 3 mice. MTX (Japan Lederle Co. Ltd., Tokyo, Japan) was dissolved in saline and administered orally to 10 of the 20 groups of SCID-HuRAg mice at 0.3 mg/kg as a single dose (MTX treatment group). The remaining 10 groups were administered an equivalent volume of saline in the same manner (control group). The grafted tissues were examined histologically at 0, 6, 12, 24, and 72 h as well as at 1, 2, 3, 4, and 6 weeks after MTX or saline administration, respectively.

For a comparative study of the histological changes induced by MTX and other drugs, 30 SCID-HuRAg mice were divided into 6 groups, each consisting of 5 mice. For 4 weeks SCID-HuRAg mice in each of 5 groups were orally administered one of the following 5 drugs: MTX (0.3 mg/kg/week), salazosulfapyridine (30 mg/kg/day; Santen Pharmaceutical Co. Ltd., Osaka, Japan), auranofin (0.2 mg/kg/day; SmithKline Beecham Co., Philadelphia, PA, USA), bucillamine (10 mg/kg/day; Santen), and indomethacin (2 mg/kg/day; Sigma Chemical Co., St. Louis, MO, USA). The sixth group was not given a drug (control group). On the third day of treatment, TdT mediated dUTP nick end labeling (TUNEL) positive apoptotic cells were examined in 2 mice from each of the 6 groups. At the end of drug administration, the grafted synovial tissues from the remaining 3 mice in each group were removed for histological observation.

Detection of apoptotic synovial cells. Histological apoptotic changes were examined by TUNEL staining and electron microscopy. The grafted synovial tissue was prepared for histological observation as described[11,14,15].

For TUNEL staining, the excised RA tissue grafts were fixed in 4% buffered formaldehyde and embedded in paraffin to prepare sections of 6 μm thickness. After dewaxing and hydration, proteins were stripped with 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature. Endogenous peroxidase was inactivated with 2% H_2O_2, and then the specimens were labeled in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1.0 mM cobalt chloride), and reacted with biotinylated dUTP dissolved in TdT buffer. Finally, the samples were stained with diaminobenzidine, and sequential tissue sections were stained with hematoxylin and eosin.

For electron microscopy, specimens were also prepared from RA grafted tissues that had been removed 72 h after MTX administration. The samples were fixed in 0.1 M cacodylated buffer (pH 7.4) containing 3% glutaraldehyde at 4°C for 1 h. After subsequent incubation in 1% OsO_4 for 1 h, the sample was dehydrated and sliced to obtain ultrathin sections of 1000 Å thickness. The sections were stained with uranyl acetate and lead citrate for electron microscopic observation.

Tissue samples, except those for electron microscopy, were evaluated by our collaborators blindly selecting 5 regions from each tissue section. The number of targeted cells detectable in each region was counted on computer image analysis software (Mac-Scope; Mitani, Fukui, Japan), and the mean value was calculated for each sample, as described[14].

Induction of apoptosis in cultured synovial cells by MTX. Induction of apoptosis in cultured synovial cells by MTX was examined by agarose gel electrophoresis and flow cytometry. Synovial cells were cultured as described[16]. Synovial tissues were removed from patients with RA (4 women, one man). Part of each synovial sample was engrafted into a SCID-HuRAg mouse, and the remaining portion of the sample was used for cell culture. These synovial tissues were minced and incubated with 0.5 μg/ml collagenase in serum-free DMEM for 3 h at 37°C, filtered through sterile nylon mesh, and washed, then cultured in DMEM supplemented with 10% heat inactivated fetal calf serum, penicillin (100 U/ml), and gentamicin (60 mg/ml) in a humidified 5% CO_2 incubator in culture dishes overnight. Culture supernatants were removed and the resultant adherent cells (1×10^6 cells/ml) were cultured in the presence or absence of the indicated dose of MTX (0-10^{-4} M). Apoptotic cells in the cultured synovial cells were examined by TUNEL staining as described[11,15]. The percentage of TUNEL positive cells was calculated as follows: TUNEL positive cells/total number of observed cells × 100. Further, the inhibitory effect of leucovorin on MTX induced apoptosis was evaluated. Synovial cells were cultured with and without leucovorin (150 μg/ml) and the indicated dose of MTX (0–10^{-4} M). Synovial cells were also cultured with increasing doses of leucovorin (10, 150, 500 μg/ml) plus 1×10^{-6} M MTX.

MTX induced apoptotic DNA fragmentation in cultured synovial cells was detected by agarose gel electrophoresis[17]. Cultured synovial cells (1×10^6 cells) were incubated with MTX for 72 h and resuspended with 0.5 μg/ml collagenase for 15 min at 37°C. Then the synovial cells were washed with PBS and collected by centrifugation (1600 × g, 5 min). The cell pellets were treated briefly with lysis buffer. After centrifugation (1600 × g, 5 min), the supernatants were collected and the extraction was repeated with the same amount of lysis buffer. 1% sodium dodecyl sulfate was added to the collected supernatants and treated for 2 h with RNase A (5 μg/μl) at 56°C followed by digestion with proteinase K (2.5 μg/μl) for at least 2 h at 37°C. After the addition of 1/2 volume of 10 M ammonium acetate, DNA was precipitated with 2.5 volumes of ethanol, dissolved in gel loading buffer, and separated by electrophoresis on 1% agarose gels.

MTX induced apoptosis in synovial cells was also examined using flow cytometry. The synovial cells were cultured with or without the indicated dose of MTX. Double staining with FITC-Annexin V for the cell membrane and propidium iodide (PI) for cellular DNA (PharMingen, San Diego, CA, USA) was performed as described[18]. After washing twice with PBS, 1×10^5 cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). FITC-Annexin V was added to a final concentration of 1 μg/ml Annexin V. Then 0.1 volume of PI (10 μg/ml in binding buffer) was added, resulting in a final concentration of 1 μg PI/ml cell suspension. The mixture was incubated in the dark for 10 min and then measured by FACScan (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis. All data were expressed as mean ± standard error of mean (SEM). Welch's correction test was used for statistical comparison between groups. The statistical significance of the relationship between the doses of leucovorin and the TUNEL positive cultured synovial cells was tested using Spearman's correlation analysis. A p value < 0.05 was considered statistically significant.

RESULTS

Induction of apoptosis in synovial tissue by MTX. The number of TUNEL positive cells in synovial tissues from SCID-HuRAg mice was significantly increased at 24 and 72 h after MTX administration compared with synovial tissues from the controls. The number of TUNEL positive cells increased and peaked at 72 h, followed by a gradual decrease, as shown in Figures 1 and 2. Moreover, apoptotic cells were also detected in similar locations by electron microscopy. Changes that are characteristic of apoptotic cells, including condensed nuclear chromatin and tightly packed cytoplasmic organelles, were observed after MTX treatment (Figure 3). However, the induction of apoptosis in the synovium was not observed with the other antirheumatic drugs used in this study (Figure 4).

Effects of DMARD on synovial tissue. In the MTX treated

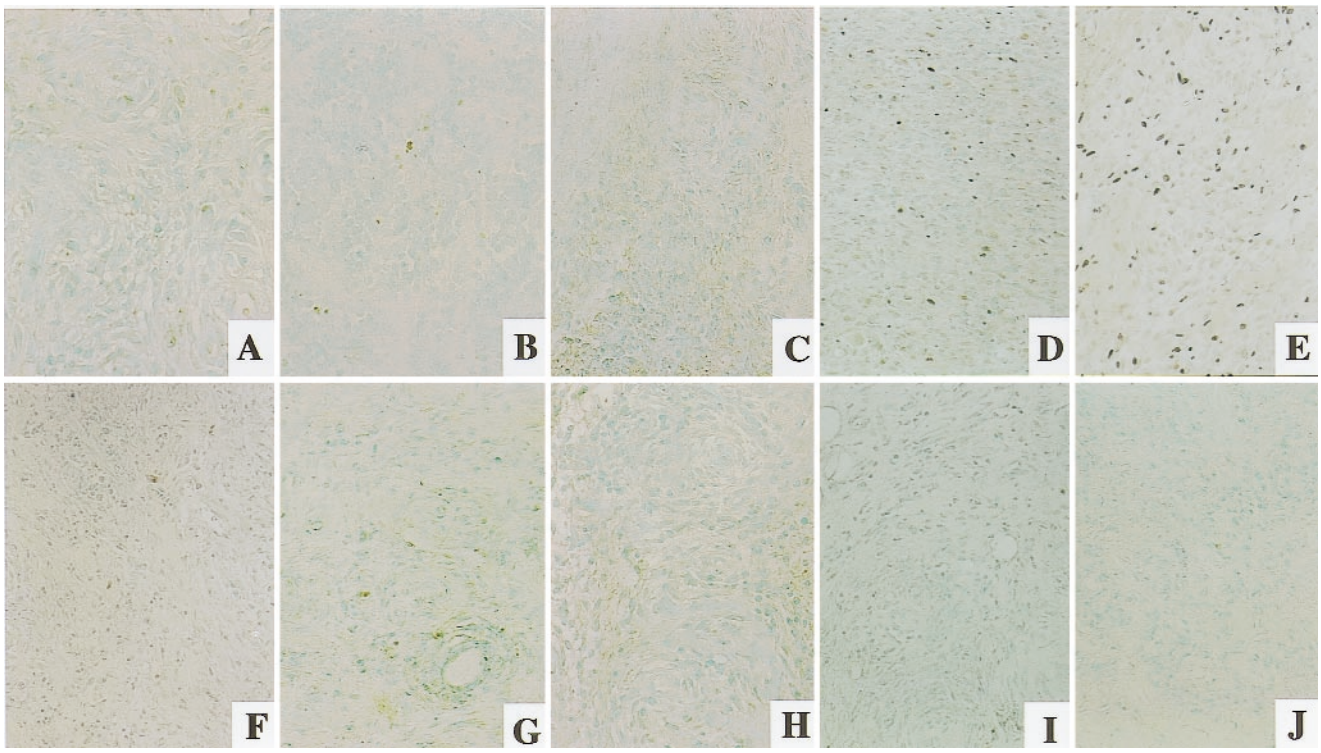

Figure 1. TUNEL positive apoptotic cells were observed in grafted synovial tissues from SCID-HuRAg mice after MTX administration. (A) 0 h, (B) 6 h, (C) 12 h, (D) 24 h, (E) 72 h, (F) 1 week, (G) 2 weeks, (H) 3 weeks, (I) 4 weeks, (J) 6 weeks after MTX administration (original magnification ×200).

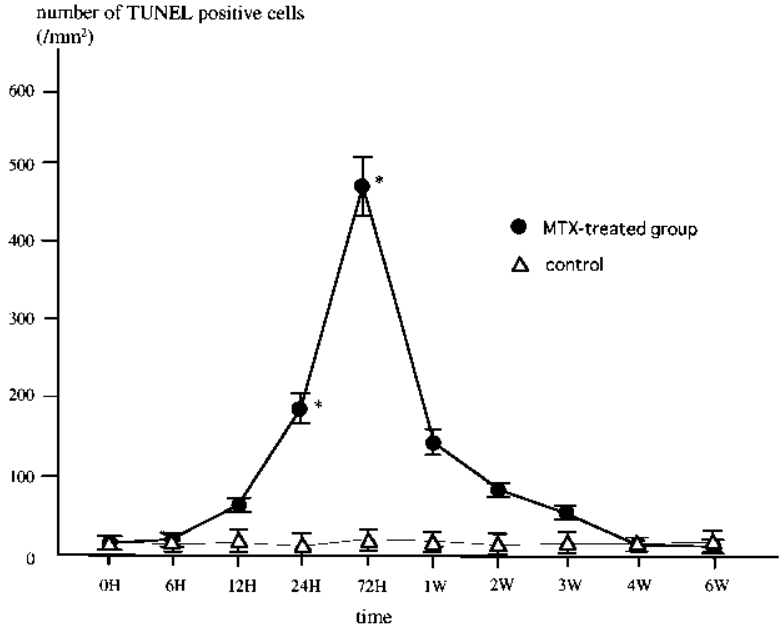

Figure 2. Time course of changes in TUNEL positive apoptotic cells after MTX administration. TUNEL positive apoptotic cells appeared 12 h after MTX administration. The number of TUNEL positive cells peaked at 72 h, and then gradually decreased. At 24 and 72 h after MTX treatment, the ratio of TUNEL positive cells was statistically significantly higher than in controls (*$p < 0.05$).

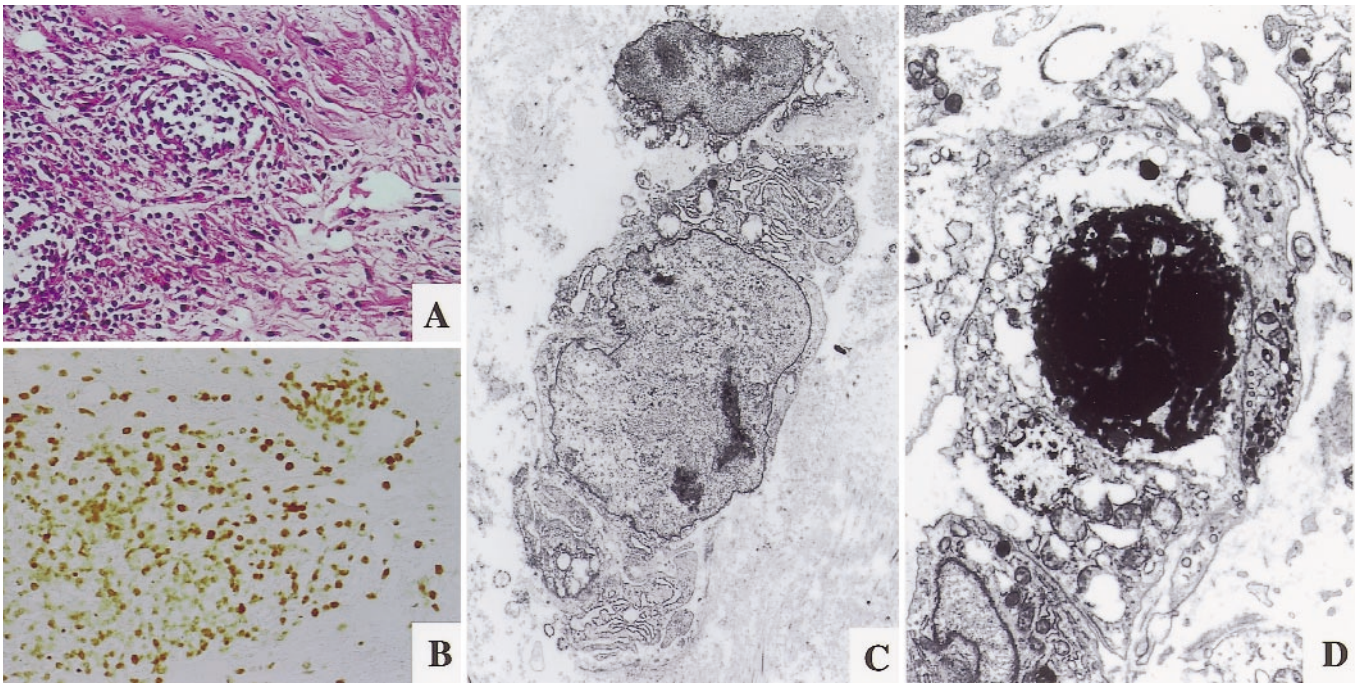

Figure 3. Apoptotic cells were detected by electron microscopy. Electron microscopic analysis was performed at the same location as the TUNEL positive cells 72 h after MTX administration. (A) H&E staining, (B) TUNEL staining (original magnification ×200). Changes that are characteristic of apoptotic cells, including condensed chromatin and tightly packed cytoplasmic organelles, were observed in the electron micrograph (D). Such changes were not observed at the same location as the TUNEL negative cells in the control group (C) (original magnification ×10,000).

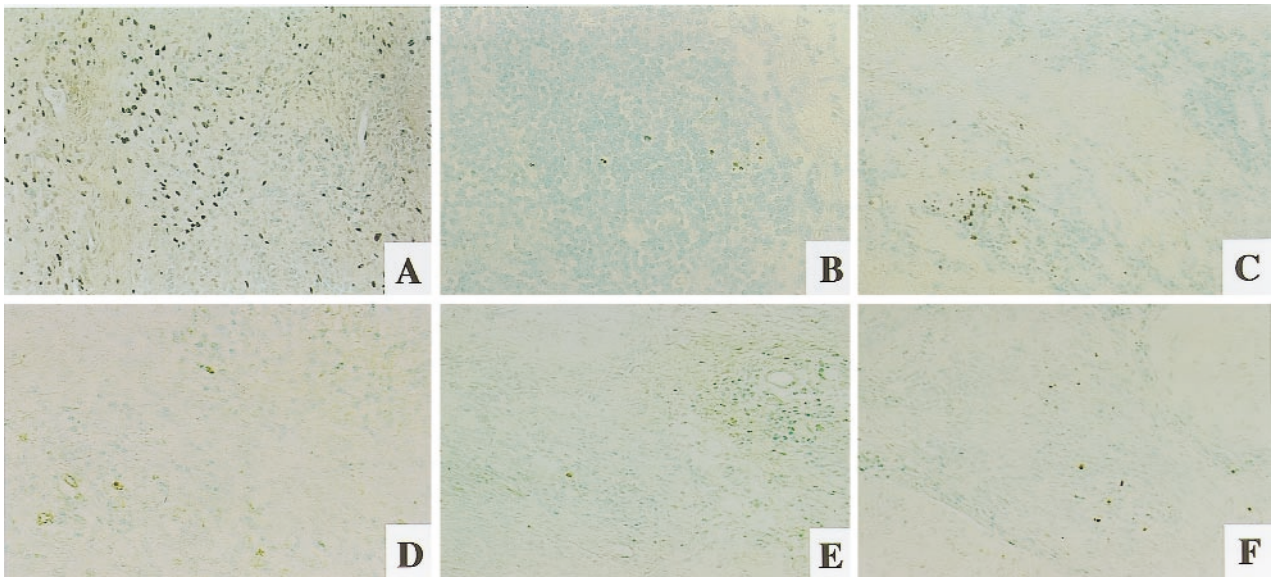

Figure 4. TUNEL positive apoptotic cells were examined in synovial tissue from SCID-HuRAg mice on the third day of treatment with DMARD and an NSAID. Many TUNEL positive cells were observed in the MTX treated group compared with the control group, but no change was seen in the other DMARD and NSAID treated groups. (A) MTX, (B) auranofin, (C) salazosulfapyridine, (D) bucillamine, (E) indomethacin, (F) saline (original magnification ×100).

group, a statistically significant decrease in the number of inflammatory cells was observed compared with the control group that received saline (380.5 ± 38.7 vs 873.6 ± 51.2 cells/mm^2) (Figure 5, Table 1). The inflammatory region was replaced with fibrotic tissue after MTX treatment. In contrast, the number of inflammatory cells detected in the auranofin treated group (824.1 ± 61.5 cells/mm^2) was almost comparable with that in the control group. In the groups that received another DMARD or indomethacin, the mean number of inflammatory cells was reduced compared with the control group, but without statistical significance (salazosulfapyridine treated group 600.3 ± 71.2 cells/mm^2; bucillamine treated group 638.7 ± 81.9 cells/mm^2; indomethacin treated group 688.7 ± 62.6 cells/mm^2). The drug induced histological changes in the synovial tissue grafted into SCID mice were not significantly affected by the DMARD that had been administered to the donor patients prior to the removal of tissue for grafting (data not shown).

Induction of apoptosis in cultured synovial cells by MTX. Many TUNEL positive cells were detected in cultured synovial cells (13.5 ± 5.2%) that had been incubated with even a low concentration of MTX (1×10^{-7} M). The percentage of TUNEL positive cells increased as the concentration of MTX was increased in a dose dependent manner (Figure 6). TUNEL positive cells were not significantly decreased with leucovorin. And as the dose of leucovorin was increased, TUNEL positive cells did not undergo a statistically significant decrease or a trend in this direction ($\sigma = -1.99$, $p = 0.48$) (Figure 7). These data are shown in Tables 2 and 3.

Agarose gel electrophoresis revealed nucleosome ladders

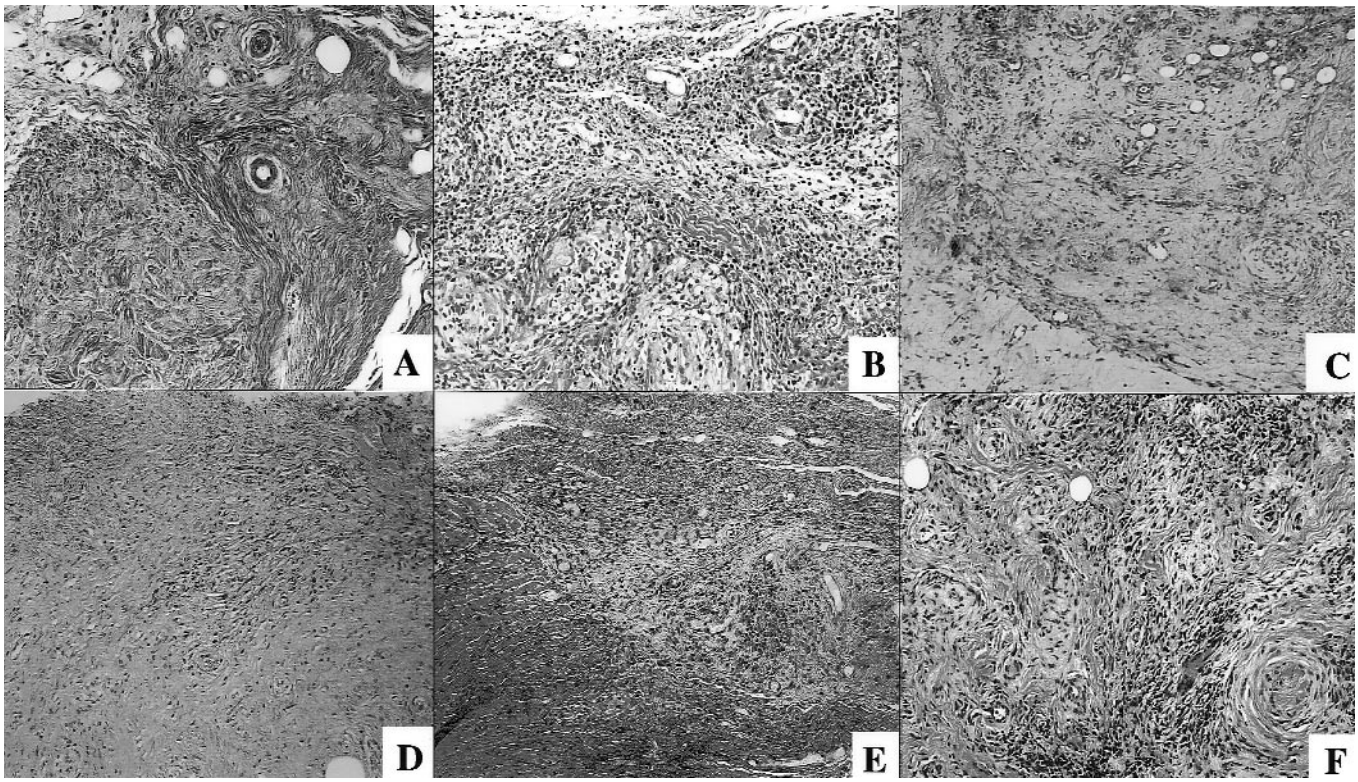

Figure 5. Histological findings of synovial tissue after treatment with DMARD and an NSAID. A decrease in inflammatory cell numbers was observed in the MTX treated group compared with controls, but no change was seen in the other DMARD and NSAID treated groups. (A) MTX, (B) auranofin, (C) salazosulfapyridine, (D) bucillamine, (E) indomethacin, (F) saline (original magnification ×100).

Table 1. Number of inflammatory cells in synovial tissue from SCID-HuRAg mice after administration of DMARD and an NSAID treatment.

	MTX	AF	SASP	Bc	Ind	Saline
Cell count, per mm^2	380.5 ± 38.7*	824.1 ± 61.5	600.3 ± 71.2	638.7 ± 81.9	688.7 ± 62.6	873.6 ± 51.2

*$p < 0.05$ compared to nondrug treatment (saline) group. Values are the mean ± SEM. MTX: methotrexate, AF: auranofin, SASP: salazosulfapyridine, Bc: bucillamine, Ind: indomethacin.

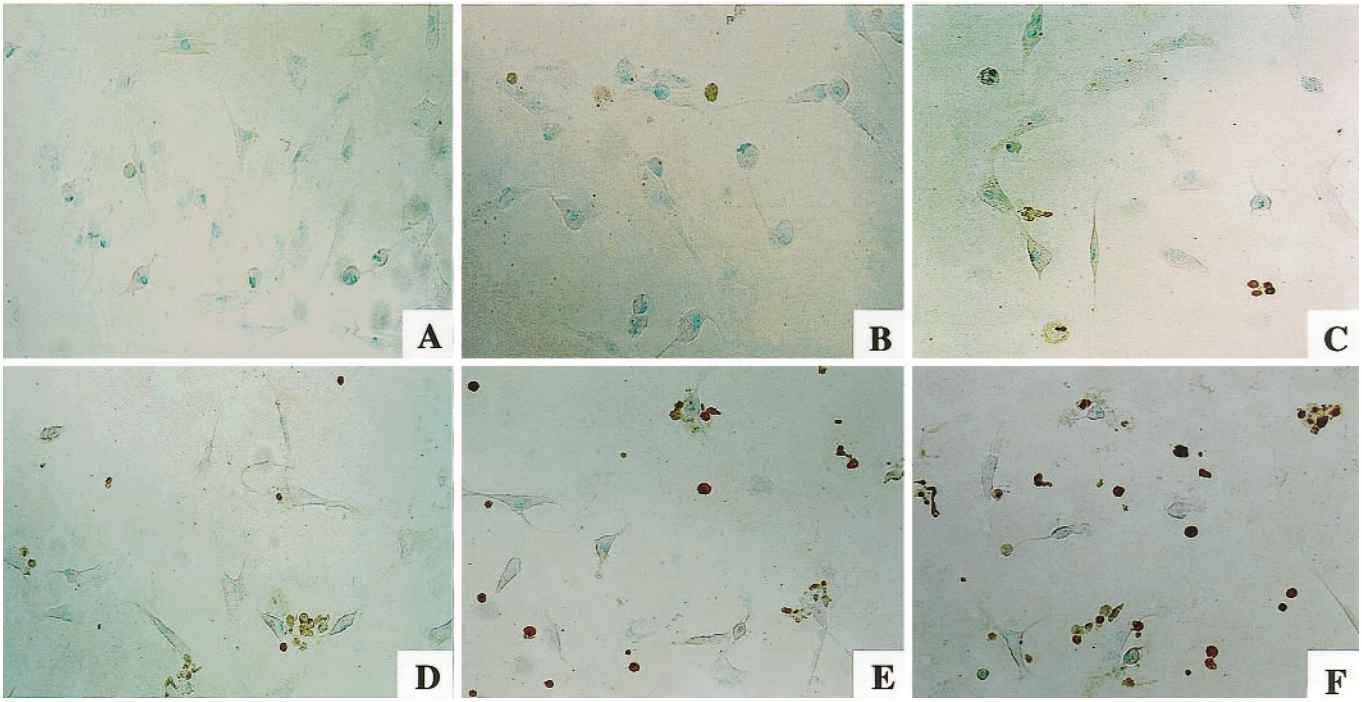

Figure 6. TUNEL positive apoptotic cells in cultured RA synovial cells. The percentage of TUNEL positive cells increased as the concentration of MTX was increased in a dose dependent manner. (A) No MTX administration (control), (B) MTX (1×10^{-8} M), (C) 1×10^{-7} M, (D) 1×10^{-6} M, (E) 1×10^{-5} M, (F) 1×10^{-4} M (original magnification ×400).

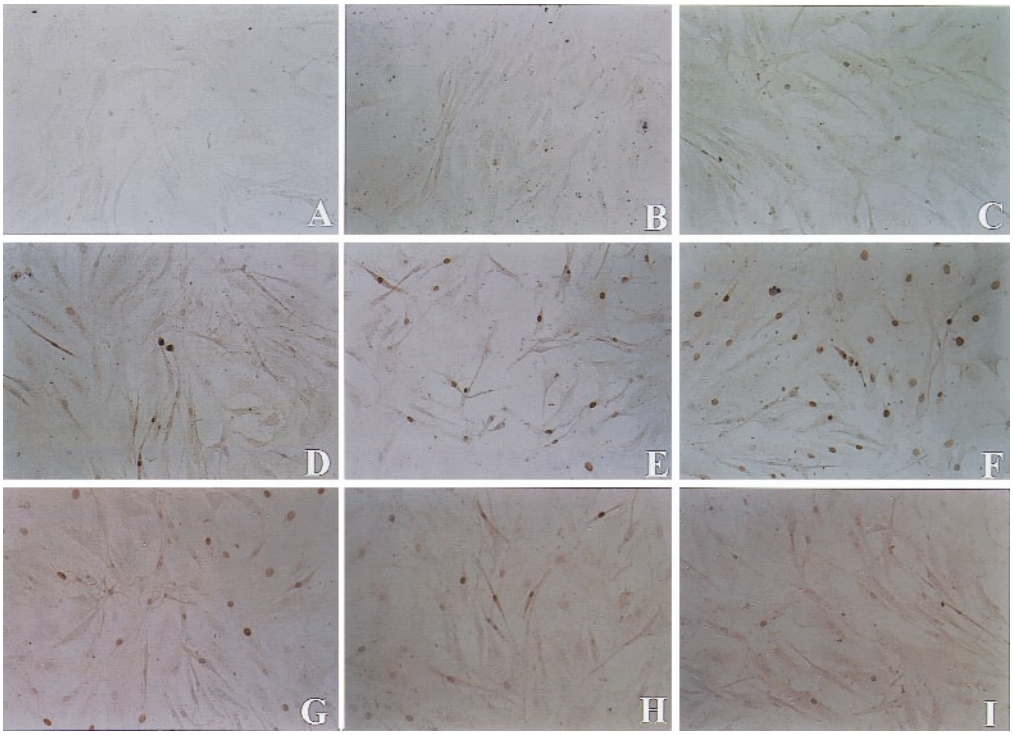

Figure 7. Synovial cells were cultured with MTX and leucovorin. A significant inhibitory effect on MTX induced apoptosis by leucovorin was not seen (A–F). TUNEL positive cells decreased in a nonsignificant manner as leucovorin doses increased (G–I). (A) Control, leucovorin 150 μg/ml. (B) MTX 10^{-8} M, leucovorin 150 μg/ml. (C) MTX 10^{-7} M, leucovorin 150 μg/ml. (D) MTX 10^{-6} M, leucovorin 150 μg/ml. (E) MTX 10^{-5} M, leucovorin 150 μg/ml. (F) MTX 10^{-4} M, leucovorin 150 μg/ml. (G) MTX 10^{-6} M, leucovorin 10 μg/ml. (H) MTX 10^{-6} M, leucovorin 150 μg/ml. (I) MTX 10^{-6} M, leucovorin 500 μg/ml (original magnification ×100).

Table 2. Percentage of TUNEL positive cells in the cultured synovium with and without leucovorin (150 μg/ml) and the indicated dose of MTX.

	Concentration of MTX, M					
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
TUNEL positive cells, %						
without leucovorin	1.5 ± 0.5	1.9 ± 0.6	13.5 ± 5.2	17.1 ± 4.8	39.9 ± 7.9	52.7 ± 15.1
with leucovorin 150 μg/ml	2.0 ± 0.2	1.7 ± 0.3	8.5 ± 6.0	12.6 ± 4.5	30.1 ± 6.7	60.6 ± 14.8

Values are mean ± SEM.

Table 3. Percentage of TUNEL positive cells in the cultured synovium with leucovorin and MTX (10^{-6} M).

	Concentration of Leucovorin (μg/ml) with MTX (10^{-6} M)		
	10	150	500
TUNEL positive cells, %	14.3 ± 6.2	12.6 ± 4.5	9.0 ± 3.3

Values are mean ± SEM.

in the DNA from MTX treated synovial cell cultures, but not in the DNA from non-MTX treated synovial cultures (Figure 8).

Flow cytometric analysis showed that the FITC+/PI– apoptotic cell population increased in cultured synovial cells 12 h after MTX administration in a dose dependent manner (Figure 9).

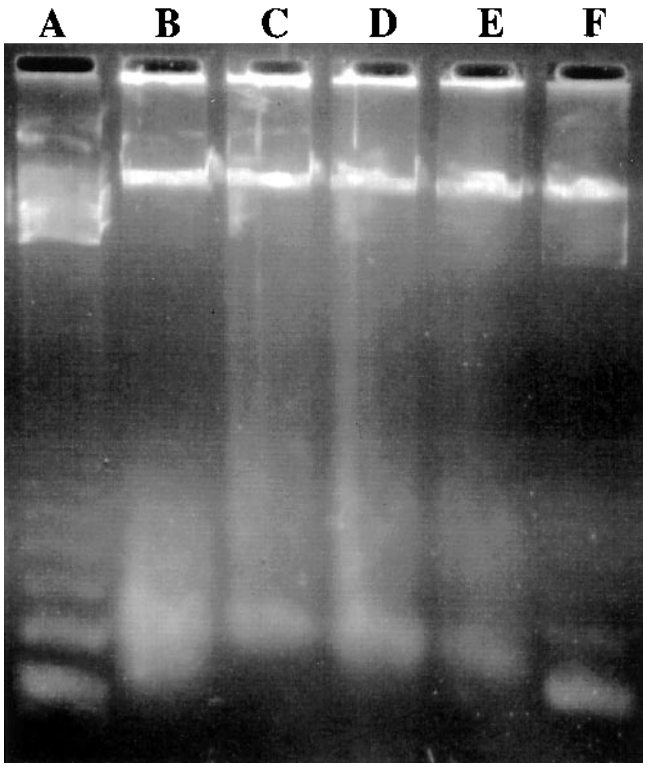

Figure 8. DNA fragmentation in cultured synovial cells. The electrophoresis gel of DNA from MTX treated synovial cells revealed 180 base-pair nucleosome ladders, but these were not seen in the gels of non-MTX treated synovial cells. (A) ladder marker, (B) no MTX administration (control), (C) 1×10^{-7} M, (D) 1×10^{-6} M, (E) 1×10^{-5} M, (F) 1×10^{-4} M.

DISCUSSION

Since MTX was first introduced as a treatment for RA in 1951[19], it has been at the forefront of DMARD for RA because of its excellent antirheumatic effects[20]. MTX, a metabolic antagonist of folinic acid, is an immunosuppressant that binds to dihydrofolate reductase to inhibit nucleic acid synthesis. Recently, it was elucidated that MTX has an antiinflammatory action by inducing an increase in adenosine release at inflamed sites[7]. This adenosine mediated antiinflammatory effect is also observed in NSAID and may partly explain the fast-acting effect of MTX[21]. However, such an antiinflammatory role seems to be insufficient to wholly explain the fast-acting and immunomodulatory effect of MTX. MTX must have other pharmacological actions against RA that are unlike those of NSAID.

It has been shown that some anticancer agents, such as cisplatin, 5-fluorouracil, and etoposide, have the effect of inducing apoptosis in cancer cells[22]. Huschtscha, *et al* recently reported that MTX also induces apoptotic changes in leukemic cells[13]. If MTX could induce apoptosis in RA synovial tissue as well as in leukemic cells, this may represent a new antirheumatic mechanism for MTX, because the induction of apoptosis in RA synovium has provoked antirheumatic effects[11,23,24].

Based on these reasons, we investigated the induction of apoptosis in RA synovium by MTX using SCID-HuRAg mice. MTX induced apoptotic changes in RA synovial tissue were observed as early as 24 h after MTX administration. Such prompt apoptotic changes seemed to be compatible with the faster therapeutic effect of MTX compared with other DMARD[25]. Moreover, the induction of apoptosis seemed to be a specific effect of MTX treatment because many such apoptotic changes were not observed after the administration of other DMARD (salazosulfapyridine, auranofin, and bucillamine) or an NSAID (indomethacin). In addition, histological antirheumatic effects were observed only with MTX treatment in the SCID-HuRAg mice. These results suggest that MTX exerts its effects on RA synovial tissue through the induction of apoptosis.

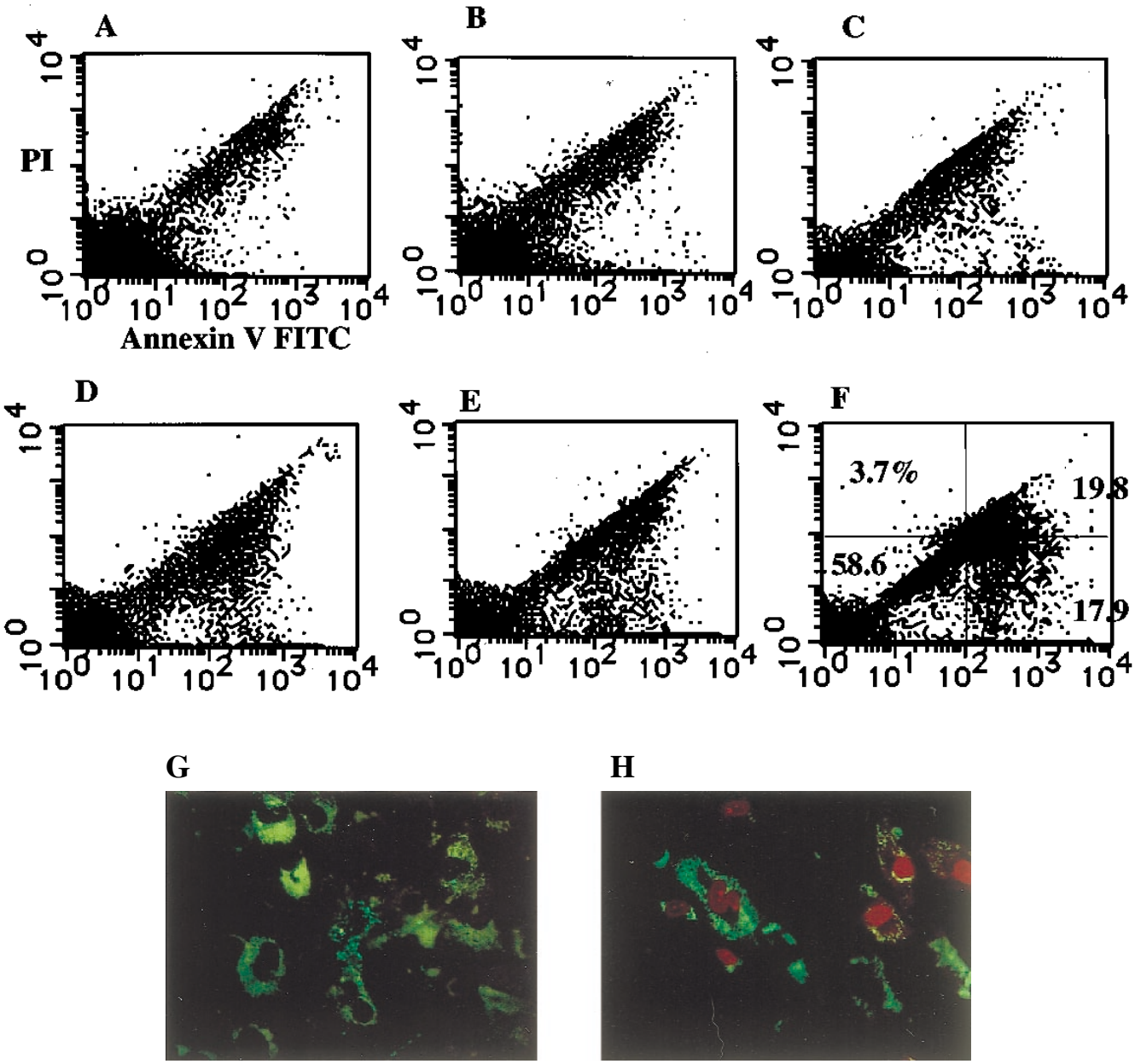

Figure 9. Flow cytometric analysis of apoptotic cells. In the control group, few FITC+/PI– cells were observed. (A) 12 h after MTX administration the FITC+/PI– apoptotic cell population was increased. (B) MTX (1×10^{-8} M), (C) 1×10^{-7} M, (D) 1×10^{-6} M, (E) 1×10^{-5} M, (F) 1×10^{-4} M. Apoptotic cells were observed by fluorescent microscopy with 1×10^{-6} M MTX. (G) Annexin V FITC+/PI– apoptotic cell, (H) Annexin V FITC+/PI+ apoptotic or necrotic cell.

Weekly low dose pulse MTX therapy was performed routinely in patients with RA, resulting in serum concentrations of MTX up to 5.8×10^{-7} M[26]. In our experiment, after oral administration of MTX to mice (0.3 mg/kg/week), the concentration of MTX in the serum was 1.22×10^{-6} M at 2 h (data not shown). Although this concentration is not equivalent to that reached in human subjects, we considered the difference acceptable to examine MTX induced apoptosis.

In MTX therapy for RA, important findings involving the active metabolites and MTX polyglutamate have been reported[7]. However, in this study, such findings were not evaluated and only histological changes were examined. Certainly, MTX suppressed RA synovitis, but the mechanism of MTX induced apoptosis in RA synovial cells still remains unclear. Genestier, *et al*[27] reported that MTX could selectively delete activated peripheral blood T cells by a Fas independent pathway, and MTX induced apoptosis was completely abrogated by addition of folinic acid or thymidine. In our study, leucovorin could not prevent MTX induced apoptosis in the cultured synovial cells. We

consider the difference in findings between these 2 studies a result of differences in cell type. Further studies are needed to clarify the mechanisms of MTX induced apoptosis in RA synovial cells.

MTX has a therapeutic effect in RA by inducing apoptosis of RA synovial cells. Interestingly, other DMARD with lower antiinflammatory potency in RA synovium show little ability to induce synovial cell apoptosis. Thus, it is probable that the different pharmacological action of MTX leads to the different histological changes observed in patients with RA undergoing MTX therapy.

REFERENCES

1. Furst DE, Kremer JM. Methotrexate in rheumatoid arthritis. Arthritis Rheum 1988;31:305-14.
2. Nakajima A, Hakoda M, Yamanaka H, Kamatani N, Kashiwazaki S. Divergent effects of methotrexate on the clonal growth of T and B lymphocytes and synovial adherent cells from patients with rheumatoid arthritis. Ann Rheum Dis 1996;55:237-42.
3. Andersen PA, West SG, O'Dell JR, Via CS, Claypool RG, Kotzin BL. Weekly pulse methotrexate in rheumatoid arthritis. Ann Intern Med 1985;103:489-96.
4. Segal R, Mozes E, Yaron M, Tartakovsky B. The effects of methotrexate on the production and activity of interleukin-1. Arthritis Rheum 1989;32:370-7.
5. Suarez CR, Pickett WC, Bell DH, McClintock DK, Oronsky AL, Kerwar SS. Effect of low dose methotrexate on neutrophil chemotaxis induced by leukotriene B4 and complement C5a. J Rheumatol 1987;14:9-11.
6. Hirata S, Matsubara T, Saura R, Tateishi H, Hirohata K. Inhibition of in vitro vascular endothelial cell proliferation and in vivo neovascularization by low-dose methotrexate. Arthritis Rheum 1989;32:1065-73.
7. Cronstein BN, Naime D, Ostad E. The antiinflammatory mechanism of methotrexate: Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. J Clin Invest 1993;92:2675-82.
8. Danning CL, Boumpas DT. Commonly used disease-modifying antirheumatic drugs in the treatment of inflammatory arthritis: An update on mechanisms of action. Clin Exp Rheumatol 1998;16:595-604.
9. Weinblatt ME, Coblyn JS, Fox DA, et al. Efficacy of low-dose methotrexate in rheumatoid arthritis. N Engl J Med 1985;312:818-22.
10. Okamato K, Asahara H, Kobayashi T, et al. Induction of apoptosis in the rheumatoid synovium by Fas ligand gene transfer. Gene Therapy 1998;5:331-8.
11. Sakai K, Matsuno H, Morita I, et al. Potential withdrawal of rheumatoid synovium by the induction of apoptosis using a novel in vivo model of rheumatoid arthritis. Arthrits Rheum 1998;41:1251-7.
12. Fujisawa K, Asahara H, Okamato K, et al. Therapeutic effect of the anti-Fas antibody on arthritis in HTLV-1 tax transgenic mice. J Clin Invest 1996;98:271-8.
13. Huschtscha LI, Bartier WA, Ross CEA, Tattersall MHN. Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. Br J Cancer 1996;73:54-60.
14. Matsuno H, Sawai T, Nezuka T, et al. Treatment of rheumatoid synovitis with anti-reshaping human interleukin-6 receptor monoclonal antibody. Arthritis Rheum 1998;41:2014-21.
15. Morita I, Matsuno H, Sakai K, et al. Time course of apoptosis in collagen-induced arthritis. Tissue Reactions 1998;20:37-43.
16. Matsuno H, Sugiyama E, Muraguchi A, et al. Pharmacological effects of SA96 (bucillamine) and its metabolites as immunomodulating drugs — the disulfide structure of SA96 metabolites plays a critical role in the pharmacological action of the drug. Int J Immunopharmacol 1998;20:295-304.
17. Herrmann M, Lorenz HM, Voll R, Grünke M, Woith W, Kalden JR. A rapid and simple method for the isolation of apoptotic DNA fragments. Nucl Acids Res 1994;22:5506-7.
18. Engeland M, Ramaekers FCS, Schutte B, Reutelingsperger CPM. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996;24:131-9.
19. Cronstein BN. Molecular therapeutics: Methotrexate and its mechanism of action. Arthritis Rheum 1996;39:1951-60.
20. Guidelines for the management of rheumatoid arthritis. Arthritis Rheum 1996;39:713-22.
21. Cronstein BN, Stouwe MV, Druska L, Levin RI, Weissmann G. Nonsteroidal antiinflammatory agents inhibit stimulated neutrophil adhesion to endothelium: Adenosine dependent and independent mechanisms. Inflammation 1994;18:323-35.
22. Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 1990;40:2353-62.
23. Firestein GS, Yeo M, Zvaifler NJ. Apoptosis in rheumatoid arthritis synovium. J Clin Invest 1995;96:1631-8.
24. Nishioka K, Hasunuma T, Kato T, Sumida T, Kobata T. Apoptosis in rheumatoid arthritis. Arthritis Rheum 1998;41:1-9.
25. Olsen NJ, Callahan LF, Pincus T. Immunologic studies of rheumatoid arthritis patients treated with methotrexate. Arthritis Rheum 1987;30:481-8.
26. Klemer JM, Lee JK. The safety and efficacy of the use of methotrexate in long-term therapy for rheumatoid arthritis. Arthritis Rheum 1986;29:822-31.
27. Genestier L, Paillot R, Fournel S, Ferraro C, Miossec P, Revillard JP. Immunosuppressive properties of methotrexate: Apoptosis and clonal deletion activated peripheral T cells. J Clin Invest 1998;102:322-8.