

Synoviocyte apoptosis may differentiate responder and non-responder patients to methotrexate treatment in rheumatoid arthritis

Sang Yeob Lee · Sun-Hee Park · Sung Won Lee ·
Sang Hwa Lee · Min Kyeong Son · Yung Hyun Choi ·
Won Tae Chung · Young Hyun Yoo

Received: 1 May 2013 / Accepted: 3 March 2014 / Published online: 24 March 2014
© The Pharmaceutical Society of Korea 2014

Abstract We aimed to evaluate whether methotrexate (MTX) *in vitro* induces apoptosis in synoviocytes obtained from rheumatoid arthritis patients and whether the apoptosis inducing effect of MTX to synoviocytes is correlated with the clinical responsiveness to MTX in patients with rheumatoid arthritis (RA). We evaluated 18 patients with RA taking MTX 15–20 mg/week as the subject group (nine responders and nine non-responders) and ten patients with osteoarthritis (OA) and nine patients with ankylosing spondylitis (AS) as the control group. Synoviocytes, cultured from the synovial fluid of the knee joint of each subject, were used for experiments between passages 4 and 6, and were treated with MTX. The induction of apoptosis was determined by the quantification of DNA hypodiploidy by flow cytometry, nuclear morphology, caspases activation, DNA electrophoresis, and mitochondrial membrane potential measurements. The viability of synoviocytes

treated with MTX was different between the MTX responders and nonresponders. MTX induced apoptosis in cultured synoviocytes by mitochondria- and caspase-dependent manners in the MTX responders but did not in the MTX non-responder, OA, and AS patients. The apoptotic responsiveness of the synoviocytes to MTX predicts the sensitivity to MTX treatment and provides a method to determine the early application of an anti-tumor necrosis factor- α agent in RA treatment.

Keywords Apoptosis · Methotrexate · Rheumatoid arthritis · Synoviocyte

Introduction

The synovial membrane in patients with rheumatoid arthritis (RA) is characterized by synovial hyperplasia and a sub-intimal infiltration of inflammatory cells, along with increased vascularity. Macrophage-derived cytokines, such as tumor necrosis factor- α (TNF- α) and interleukins, which are abundant in the synovial fluid and tissues from patients with RA, are known to play the critical role of mitogens for synovial fibroblasts.

In RA, the joint destructive process is suggested to be mediated, at least in part, by fibroblast-like synoviocytes (FLSs) from the synovium. A SCID mouse co-implantation model, showed that FLS from RA patients attached to and invaded normal cartilage (Müller-Ladner et al. 1996). Moreover, other researchers have observed that FLSs present characteristics of transformed cells, such as anchorage-independent growth, insensitivity to apoptosis, and increased proliferation in RA (Lafyatis et al. 1989). The resistance of RA synovial fibroblasts to apoptosis promotes synovial hyperplasia and is closely linked to the

S. Y. Lee · S. W. Lee · W. T. Chung
Division of Rheumatology Department of Internal Medicine,
Dong-A University, Pusan, Republic of Korea
e-mail: harrison1007@hanmail.net

S.-H. Park · S. H. Lee
Department of Microbiology, Dong-A University, Pusan,
Republic of Korea

M. K. Son · Y. H. Yoo (✉)
Department of Anatomy and Cell Biology and Mitochondria
Hub Regulation Center, College of Medicine, Dong-A
University, 3-1 Dongdaesin-dong, Seo-gu, Pusan 602-714,
Republic of Korea
e-mail: yhyoo@dau.ac.kr

Y. H. Choi
Department of Biochemistry and Research Institute of Oriental
Medicine, Dongeui University College of Oriental Medicine,
Pusan, Republic of Korea

invasive phenotype of these cells. Therefore, the suppression of the production of inflammatory molecules, hyperplastic synovium, and increased apoptosis of synovium are considered to be important targets of therapy for RA (Wessels et al. 2008).

Methotrexate (MTX) is one of the most commonly used drugs in the management of rheumatic diseases. It is the anchor drug in the treatment of RA and the basic drug of the disease-modifying anti-rheumatic drug (DMARD) combination therapies used in RA. MTX appears to act as a folate antagonist, although its exact mechanism of action remains unclear despite its widespread use. The effects of MTX *in vivo* may be mediated by reducing cell proliferation, increasing the rate of T cell apoptosis, increasing endogenous adenosine release, altering the expression of cellular adhesion molecules and influencing cytokine production. Previous studies showed that MTX exerted apoptosis-inducing activity in the active lymphocytes of RA patients (Huang et al. 2005; Furst and Kremer 1988; Nakajima et al. 1996). These studies showed that MTX induced reactive oxygen species (ROS) in a time- and concentration-dependent manner, indicating that MTX induces apoptosis in those cells by the alteration of the intracellular ROS levels (Huang et al. 2005; Furst and Kremer 1988; Nakajima et al. 1996).

To date, there is no study reporting whether MTX inhibits rheumatoid synovitis by inducing apoptosis of synoviocytes, depending on the clinical sensitivity of MTX. In this study, we demonstrated that MTX treatment induces apoptosis in synoviocytes obtained from MTX responding RA patients *in vitro*. Importantly, the MTX treatment did not induce apoptosis in synoviocytes obtained from MTX non-responding RA patients *in vitro*.

Materials and methods

Human samples and ethics statement

We enrolled ten patients who fulfilled the 1986 revised American College of Rheumatology criteria for osteoarthritis (OA) (Altman et al. 1986), nine patients who fulfilled the 1984 revised American College of Rheumatology criteria for ankylosing spondylitis (AS) (Van der Linden et al. 1984) and 18 patients who fulfilled the 1987 revised American College of Rheumatology criteria for RA classification (Arnett et al. 1988). At the beginning of our study, the OA and AS patients received non-steroidal anti-inflammatory drug. The RA patients received MTX 15–20 mg/week with prednisolone 5 mg/day. The subjects were classified as MTX responders or non-responders according to the European League Against Rheumatism criteria (Van Gestel et al. 1998), with nine patients

classified as MTX responders and nine as MTX non-responders. The MTX responders had been receiving only MTX with low dose steroids before or during the study and the MTX non-responders had also received anti-TNF therapy during the study because of their insufficient response to MTX. The dose of the treatment drugs was stable before the start of the study and was maintained until the end of the study in the MTX responders and the, OA and AS patients.

MTX non-responders additionally received etanercept subcutaneously at a dose of 25 mg/kg twice a week. No patient had received biological treatment before etanercept therapy. The activity of the disease was measured using the DAS28 (DAS, including a 28-joint count). Patients were excluded if they presented other autoimmune or rheumatic diseases, infectious disorders, or positive serology for hepatitis B or C, or human immunodeficiency virus infection. The protocol used in the cell culture was reviewed and approved by the Dong-A University-Institutional Care and Use Committee under their ethical procedures and scientific care (DIACUC-07-8). The study using human samples was reviewed and approved by the Dong-A University Hospital Institutional Review Board (DUHIRB-10-10-23). Written informed consent was obtained from all participants.

Reagents

The following reagents were obtained commercially: DPL methotrexate injection solution from Hospira (Melbourne, Australia) polyclonal rabbit pro-anti-human caspase-3 and caspase-7 from Santa Cruz Biotechnology (Santa Cruz, CA, USA) HRP-conjugated goat anti-rabbit IgGs from Cell Signaling Technology (Beverly, MA, USA) minimum essential medium (MEM) and fetal bovine calf serum from Hyclone (S. Logan, UT, USA) Hoechst 33342, RNase A, proteinase K, and propidium iodide (PI) from Sigma (St. Louis, MO, USA) and Super Signal West Pico enhanced chemiluminescence western blotting detection reagent from Pierce (Rockford, IL, USA).

Cell culture of fibroblast-like synoviocytes and MTX treatment

Synovial fluid samples were obtained from the knees of OA patients, AS patients and ten RA patients who fulfilled the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) at the time of the therapeutic arthrocentesis (Altman et al. 1986; Van der Linden et al. 1984; Arnett et al. 1988). The patients provided informed consent, and approval was obtained from a local ethics committee. All patients attended the Rheumatology Clinic at Dong-A University Hospital (Busan, Korea). Synovial cells were obtained as

described by Neidhart et al. (2003). Briefly, synovial fluid samples were centrifuged at 450 g for 30 min, and then the cell pellets were resuspended in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and incubated for 24 h at 37 °C in a plastic culture flask. Non-adherent cells were washed out, and medium was changed daily for the next 3 days. The remaining adherent round-shaped monocytes and spindle-shaped fibroblast-like cells were cultured for two additional weeks in a flask. The round-shaped monocytes that attached to the flask were resistant to trypsinization. In contrast, spindle-shaped FLSs were easily collected by trypsinization, relocated to a new culture flask, and used for experiments between passages 4 and 6. A total of 7×10^4 cells were plated in each well of a six well plate, and the cells were treated with 100–500 μM MTX in 2 ml of MEM medium.

Viability assay

An automated trypan blue exclusion assay were undertaken. Total cells and trypan blue-stained (i.e., nonviable) cells were counted, and the percentage of nonviable cells was calculated using the Vi-Cell cell counter (Beckman Coulter, Miami, FL, USA).

Nuclear morphology study for apoptosis

Synoviocytes were harvested and then washed with PBS. They were fixed in 4 % paraformaldehyde for 20 min at room temperature. The cells were washed with PBS twice, and stained in 4 $\mu\text{g}/\text{ml}$ Hoechst 33,342 for 1 h at 37 °C. Stained cells were coated onto clean, lipid-free glass slides and mounted with a cover glass. The samples were observed and photographed under an epifluorescence microscope (Axiophot, Zeiss, Germany).

Western blot analysis

Synoviocytes (2×10^6) were washed twice with ice-cold PBS. Cells were resuspended in lysis buffer [200 μl of ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris–Cl (pH 7.6), 0.5 % Triton X-100, protease inhibitor cocktail)] and incubated at 4 °C for 30 min. The lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. The protein concentrations of the cell lysates were measured with the Bradford protein assay reagent (Bio-Rad). Then, 50 mg of proteins was loaded onto 15 % SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and probed with each antibody. Immunostaining with the antibodies was carried out using the Super Signal West Pico enhanced chemiluminescence substrate and detected with LAS-3000PLUS.

Quantification of DNA hypoploidy and cell cycle phase analysis by flow cytometry

Cells were washed twice with PBS, and fixed with cold 70 % ethanol at 4 °C overnight. The fixed cells were pelleted and ethanol was removed by washing twice with PBS containing 1 % bovine serum albumin (BSA). The cells were resuspended in 1 ml of PBS containing 11 Kunitz U/ml RNase A, incubated at 4 °C for 30 min and washed once with BSA–PBS. Cells were resuspended in PI solution (50 $\mu\text{g}/\text{ml}$) and incubated at 37 °C for 30 min in dark. Cells were washed with PBS, the DNA content of 10,000 cells was used for the generation of simultaneous estimation of the cell cycle parameters and apoptosis using an Epics XL (Beckman Coulter, FL).

DNA electrophoresis

Synoviocytes (1×10^6) were lysed in 1.5 ml of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5 % SDS] including proteinase K (200 $\mu\text{g}/\text{ml}$). After the samples were incubated overnight at 48 °C, 200 μl of ice cold 5 M NaCl was added. The supernatant containing extracted DNA was collected after centrifugation. Samples were added 20 μl of 5 mol/l NaCl and 120 μl of isopropanol and stocked at -20 °C overnight. Before minigel electrophoresis, samples were centrifuged at 16,000 rpm for 15 min to remove isopropanol and RNase A-treated for 1 h at 37 °C. A loading buffer containing 100 mM EDTA, 0.5 % SDS, 40 % sucrose, and 0.05 % bromophenol blue was added at 1:5 (v/v). 12 μl of samples was loaded to each well along with the DNA molecular marker and separation was achieved in 2 % agarose gels in 0.5 X Tris–acetic acid/EDTA buffer containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide using 50 mA for 40 min. The DNA in the gel was detected with LAS-3000PLUS.

Mitochondrial membrane potential (MMP) assay

Dissipation of the mitochondrial membrane potential (MMP) was measured using a mitochondrion specific probe, JC-1. JC-1 was added directly to the cell culture medium (5 $\mu\text{g}/\text{ml}$ final concentration) and incubated for 30 min at 37 °C. Flow cytometry was conducted using an Epics XL flow cytometer (Beckman Coulter, FL, USA). To acquire and analyse the data, the EXPO32 ADC XL 4 color software program was used.

Statistical analysis

Four independent experiments were performed in vitro. The results are expressed as the means \pm SD from three experiments. The results of the experimental and control groups were tested for statistical significance by Student's

Table 1 Clinical and laboratory features of subjects

	Osteoarthritis (n = 10)	Ankylosing spondylitis (n = 9)	Rheumatoid arthritis (n = 18)		p value
			MTX responder (n = 9)	MTX non-responder (n = 9)	
Age (years, mean)	67 ±3.66	25 ±1.25	55.7 ±2.61	52 ±2.22	p > 0.05
Sex, female (male)	9(1)	0(9)	6(3)	8(1)	
Disease duration (years)	17 ±5.61	6 ±0.22	6.7 ±1.54	8.4 ±1.67	p > 0.05
RF/ACPA	nega/nega	nega/nega	pos/pos	pos/pos	
DAS 28 before study	NR	NR	1.72	3.96	p > 0.05
DAS 28 after study	NR	NR	0.74	3.7	p > 0.05
MTX, mg/wk, range	Not used	Not used	15–20	15–20	p > 0.05
Prednisone, mg/day	Not used	Not used	5	5	p > 0.05
Other drugs	Aceclofenac (200 mg/day)	Aceclofenac (200 mg/day)	Not used	Etanercept (50 mg/week)	

MTX methotrexate, DMARDs disease modifying anti rheumatic drugs, TNF tumor necrosis factor, RF rheumatoid factor, ACPA anti-citrullinated protein antibodies, DAS28 28-joint count disease activity score, NR no research

t test. In all cases, *p* value of <0.05 was considered to be significant.

Results

The clinical and laboratory features of the patients are shown in Table 1. The comparisons of the clinical and laboratory variables between the MTX responders and non-responders revealed no significant differences (*p* > 0.05). In this study, we enrolled 18 RA patients (male: 4, female: 14). Among them, nine RA patients were MTX responders (male: 3, female: 6), and nine were MTX non-responders (male: 1, female: 8).

MTX reduced the viability of synoviocytes obtained from MTX responders

Treatment with 100–500 μM of MTX for 24 and 48 h did not reduce the viability of synoviocytes from the OA and AS patients and MTX-non-responders. The treatment with 300 and 500 μM MTX for 24 and 48 h significantly reduced the viability of synoviocytes from the MTX- responders (*p* < 0.05). Although the treatment with 100 μM MTX for 48 h significantly reduced the viability of synoviocytes from the MTX- responders, the treatment of MTX for 24 h at the dose did not reduce their viability (*p* > 0.05) (Fig. 1).

MTX reduced the viability of synoviocytes from MTX responders in a time-dependent manner

The mechanism underlying the reduction of viability by MTX treatment was examined using synoviocytes from nine MTX responders. Synoviocytes from nine MTX non-responders were used as the experimental control. Because the viability of synoviocytes from the MTX responders after

treatment with 500 μM MTX for 48 h was approximately 50 % that of the control values, this concentration was utilized solely in further studies. We first tested the viability of synoviocytes treated with 500 μM MTX for 48 h at various time points. Treatment with 500 μM MTX reduced the viability of synoviocytes from a MTX responder in a time-dependent manner (*p* < 0.05) (Fig. 2a). However, treatment with 500 μM MTX did not reduce the viability of synoviocytes from a MTX non-responder (*p* > 0.05) (Fig. 2b).

MTX reduced the viability of synoviocytes obtained from MTX responders by inducing apoptosis

We next examined whether the reduction of the viability by MTX treatment is mediated via apoptosis. Importantly, MTX induced apoptosis in the synoviocytes obtained from a MTX responder (Fig. 3a). Flow cytometry indicated that the MTX treatment induced the accumulation of apoptotic cells with a subdiploid DNA content. Confocal microscopy of Hoechst staining showed that the MTX treatment induced nuclear condensation, and DNA electrophoresis demonstrated that the MTX treatment induced a DNA ladder in these cells. In contrast, MTX did not induce apoptosis in the synoviocytes obtained from a MTX non-responder (Fig. 3b). The accumulation of apoptotic cells with a subdiploid DNA content, nuclear condensation and DNA ladder was not evident in the synoviocytes obtained from a MTX non-responder after treatment with MTX.

MTX treatment induced the apoptosis of synoviocytes obtained from MTX responders via mitochondria and caspases

We further examined whether the MTX treatment induces apoptosis via mitochondria and caspases. The flow

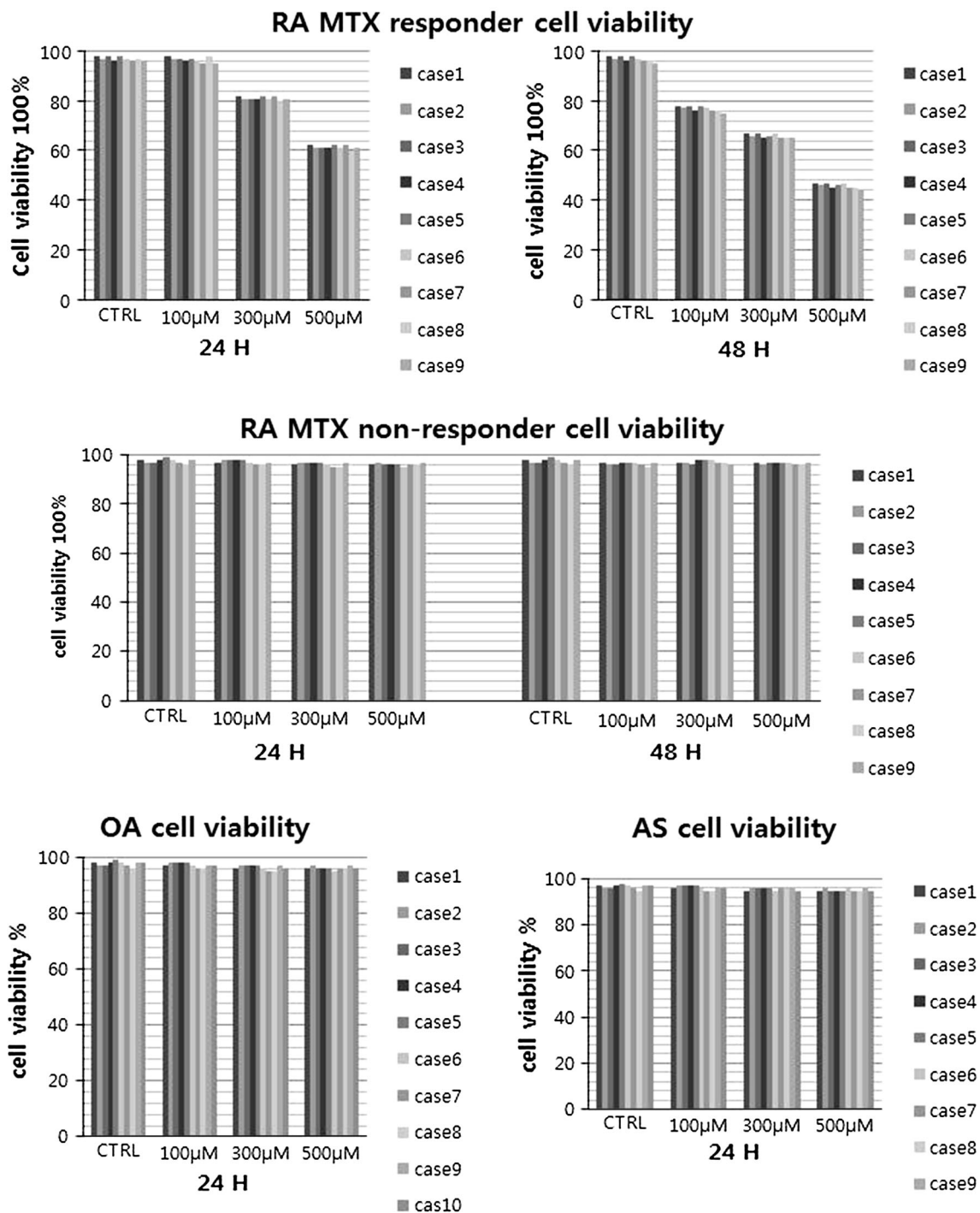


Fig. 1 MTX reduced the viability of synoviocytes obtained from MTX responders. Cells were treated with 100–500 μM MTX. Viability was determined by trypan blue exclusion. MTX treatment significantly reduced the viability of the synoviocytes obtained from

MTX responders dose-dependently ($p < 0.05$). In contrast to the synoviocytes obtained from MTX responders, MTX treatment did not reduce the viability of synoviocytes from MTX non-responders ($p > 0.05$)

cytometric analysis showed that the MTX treatment induced the reduction of MMPs, and the western blot assay showed that the MTX treatment induced the degradation of procaspase-3 and -7 in the synoviocytes obtained from

MTX responders (Fig. 4a). However, the MTX treatment induced neither the reduction of MMP nor the degradation of procaspase-3 and -7 in the synoviocytes obtained from MTX non-responders (Fig. 4b).

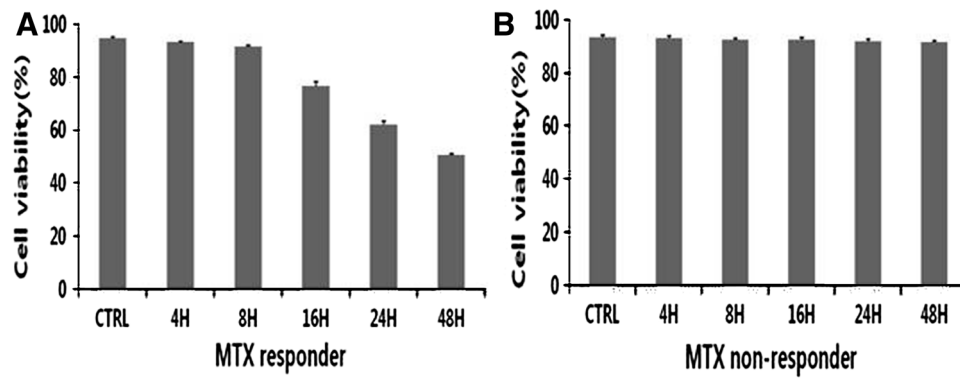


Fig. 2 MTX reduced the viability of synoviocytes from a MTX responder patient in a time-dependent manner. Cells were treated with 500 μ M MTX for the indicated times. Viability was determined by trypan blue exclusion. **a** Viability of synoviocytes from a MTX responder. Representative data from a MTX responder are presented.

MTX treatment significantly reduced the viability of the synoviocytes obtained from MTX responders in a time-dependent manner ($p < 0.05$). **b** Viability of synoviocytes from a MTX non-responder. Representative data from a MTX non-responder are presented

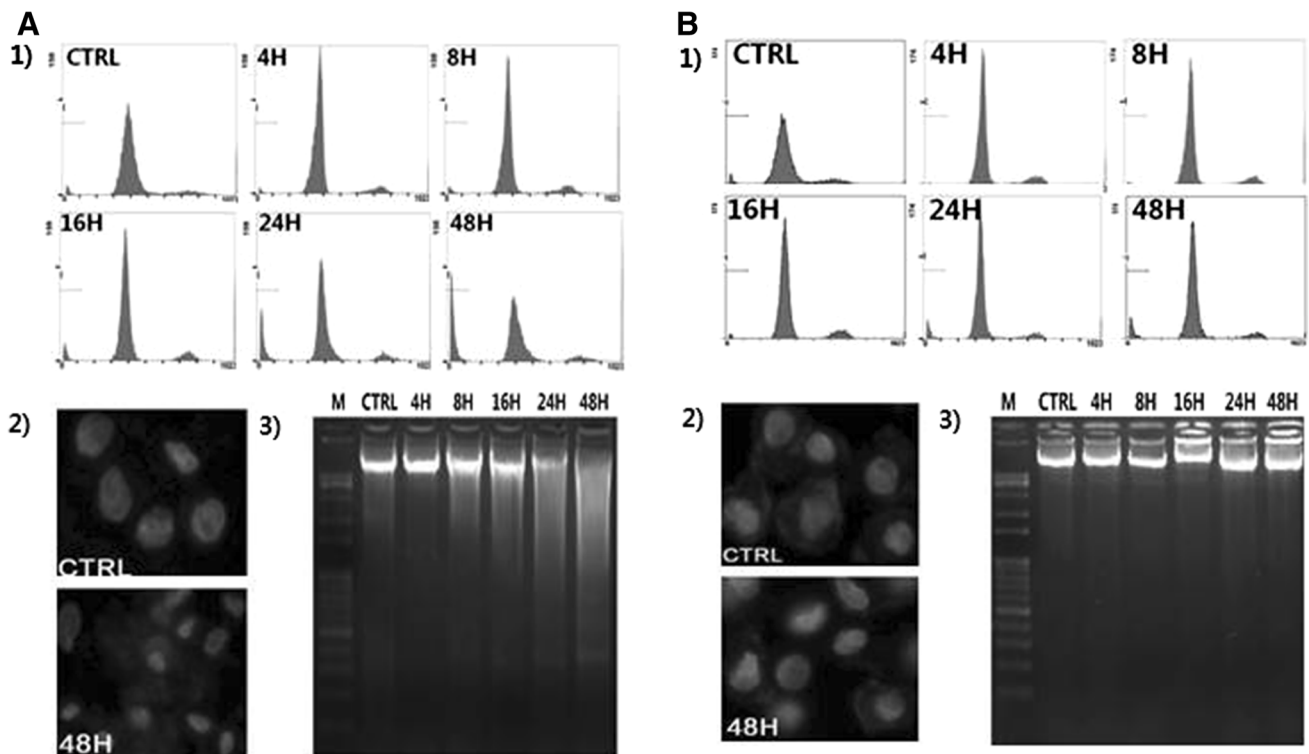


Fig. 3 MTX reduced the viability of synoviocytes obtained from MTX responder by inducing apoptosis. **a** MTX induced apoptosis in synoviocytes obtained from a MTX responder. Cells were treated with 500 μ M MTX for the indicated times. **1** Flow cytometry showing the accumulation of apoptotic cells with subdiploid DNA content in MTX-treated synoviocytes. **2** Confocal microscopy showing nuclear condensation in MTX-treated synoviocytes using Hoechst staining. **3** DNA electrophoresis showing a DNA ladder in MTX-

treated synoviocytes. Representative data from a MTX responder are presented. **b** MTX did not induce apoptosis in synoviocytes obtained from a MTX non-responder. Cells were treated with 500 μ M MTX for the indicated times. **1** Flow cytometry showing a simultaneous estimation of the cell cycle parameters and apoptosis. **2** Confocal microscopy showing the nuclear morphology using Hoechst staining. **3** DNA electrophoresis. Representative data from a MTX non-responder are presented

Discussion

Rheumatoid arthritis is a chronic inflammatory disease that leads to progressive joint destruction and is characterized

by synovial hyperplasia, cell activation, articular inflammation and invasion of the synovium into the adjacent bone and cartilage. In most cases, the inflammatory process initially affects single joints, but the disease usually

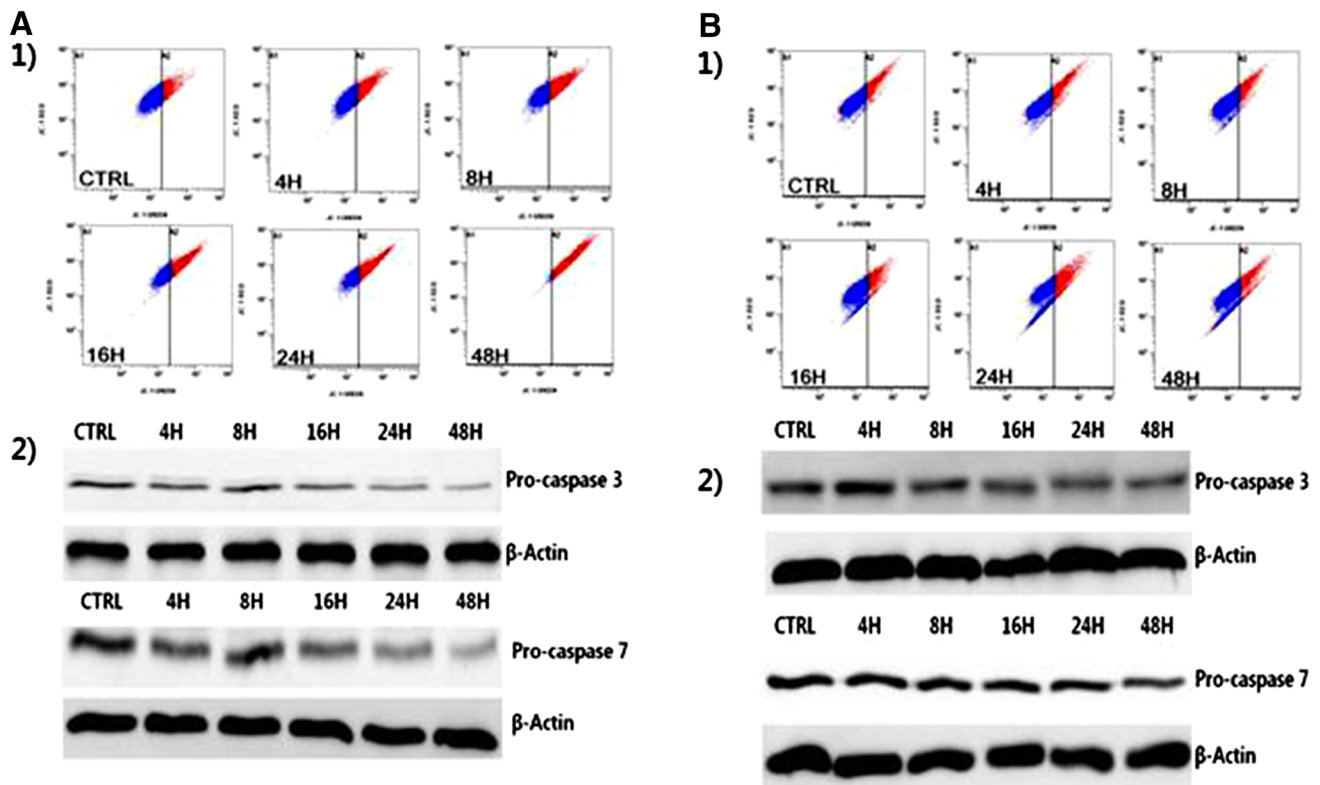


Fig. 4 MTX treatment induced the apoptosis of synoviocytes obtained from a MTX responder via mitochondria and caspases. Cells were treated with 500 μ M MTX for the indicated times. **a** Synoviocytes from a MTX responder. **1** Flow cytometry showing the reduction of MMP in the MTX-treated cells. **2** Western blot assay showing the degradation of procaspase-3 and -7 in the MTX-treated

cells. Representative data from a MTX responder are presented. **b** Synoviocytes from a MTX non-responder. **1** Measurement of MMP by flow cytometry. **2** Western blot assay of procaspase-3 and -7. β -actin, and internal control. Representative data from a MTX non-responder are presented

progresses to affect nearly all joints. The pathophysiology of RA involves many different cell types, including macrophages, B cells, T cells, chondrocytes and osteoclasts, all of which contribute to the destructive process. In these cells, RA synoviocytes, which are present in large numbers in the synovium of RA patients, may be one of the key players in the destructive process of RA.

RA synoviocytes contribute primarily to the progression of the disease by attaching to, invading and degrading cartilage and bone (Firestein 2003; Pap et al. 2005; Rutkauskaitė et al. 2004). To invade and degrade cartilage and bone, the activation of synoviocytes is important. Activated RA synoviocytes are detectable early after RA onset, and erosions are visible at cartilage–bone junctions (Noss and Brenner 2008; Muller-Ladner et al. 2007; Bartok and Firestein 2010; Perlman and Pope. 2010; Lefevre et al. 2009). In this area, various inflammatory factors can enhance the destructive properties of the RA synoviocytes, including tumor necrosis factor (TNF)- α , IL-6 and IL-1 β , which are also therapeutic targets in RA (Gibbons and Hyrich 2009).

DMARDs, the key therapeutic agents, were used for the suppression of systemic inflammation in RA. Among the DMARDs, MTX is internationally the first choice for active RA. MTX has been revealed to be an inhibitor of cytokine production induced by T cell activation in whole-blood cultures of healthy donors and RA patients (Gerards et al. 2003; de lathouder et al. 2004). MTX reduced the production of IL-4, IL-6, IL-13 TNF- α , interferon gamma (IFN- γ) and granulocyte–macrophage colony-stimulating factor (Gerards et al. 2003; de lathouder et al. 2004; Kraan et al. 2004). Several reports show a dose- and time-dependent effect of MTX on the inhibition of cell proliferation and induction of apoptosis (Phillips et al. 2003; Herman et al. 2005; Quemeneur et al. 2003; Möller et al. 2002). One study shows that MTX induces apoptosis in activated leucocytes and resting T cells (Ardoin and Pitsetsky 2008), although apoptosis in resting T cells could not be detected in other studies (Phillips et al. 2003; Herman et al. 2003; Hsu et al. 2006). This observation suggests that MTX induces apoptosis only in highly activated cells, such as activated RA synoviocytes. However, there are few

studies that indicate which apoptotic pathways are involved in the apoptosis of RA synoviocytes following MTX treatment. In this study, we found that MTX induced the apoptosis of RA synoviocytes in MTX responders. In contrast, we did not find that MTX induced apoptosis in MTX non-responders nor in AS and OA patients. In clinical practice, MTX has a therapeutic effect on RA patients but no effect on AS and OA patients. Among the RA patients, the sensitivity to MTX varies, depending on the individual patient. Therefore, we thought the responsiveness of apoptosis in the synoviocytes may determine the responsiveness to MTX.

In the apoptosis of RA, previous research has identified different mechanisms that prevent synovial cells in RA from apoptosis. These mechanisms include changes in the mitochondrial pathway and the altered expression of downstream modulators of death receptors and transcriptional regulators, such as NF kappa B (Korb et al. 2009). In such mechanisms, accumulating evidence has revealed that mitochondria play a pivotal role in the apoptotic process in mammalian cells (Hsu et al. 2006; Korb et al. 2009; Cutolo et al. 2001). The disruption of the MMP (Dcm) is considered to be an indicator of mitochondrial damage and generally characterizes the early stage of apoptosis, preceding the efflux of small molecules from the mitochondria, including cytochrome c, apoptosis-inducing factor, cIAPs, and others, and followed by caspase-3/caspase-7 cascade activation (Nakazawa et al. 2001; Jing et al. 2007).

Caspases are a family of proteases that are the main effectors of apoptosis, and they are activated by receptor- and mitochondria-mediated pathways. In classical apoptosis, caspases 3 and 7 are the effector caspases in the intrinsic mitochondrial pathway, and the caspase proteins are recruited because of the activation of the apoptotic signaling pathway (Kim 2005; Kim et al. 2006; Gu et al. 2003; Da Sylva et al. 2005; Belmokhtar et al. 2003). Therefore, in this study, we investigated the activity of the key caspases, caspases-3 and caspase-7, in MTX-induced apoptosis. Our results revealed the activation of caspase-3 and -7 in the MTX responder group, suggesting that the MTX-induced apoptosis in RA synoviocytes is mediated by a mitochondrion-initiated pathway.

However, this study has important limitations, which are that we simply classified RA patients as MTX responders or non-responders based on the additional treatment with TNF- α and that a small number of subjects were recruited. However, the findings in this study have provided strong in vitro evidence that MTX induced the apoptosis of RA synoviocytes via mitochondria- and caspase-mediated pathways in the MTX responders but not in the MTX non-responders. Therefore, we suggest that the responsiveness of synoviocytes to the apoptosis-inducing efficacy of MTX predicts the sensitivity to MTX treatment and provides a

basis for determining the early clinical application of an anti-tumor necrosis factor (TNF)- α agent in RA.

Acknowledgments This work was supported by the National Research Foundation of Korea grant funded by the Korean government (2011-0001262).

References

- Altman, R., E. Asch, D. Bloch, G. Bole, D. Borenstein, K. Brandt, W. Christy, T.D. Cooke, R. Greenwald, and M. Hochberg. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American rheumatism association. *Arthritis and Rheumatism* 29(8): 1039–1049.
- Ardoin, S.P., and D.S. Pisetsky. 2008. The role of cell death in the pathogenesis of autoimmune disease: HMGB1 and microparticles as intercellular mediators of inflammation. *Modern Rheumatology* 18: 319–326.
- Arnett, F.C., S.M. Edworthy, D.A. Bloch, D.J. McShane, J.F. Fries, N.S. Cooper, L.A. Healey, S.R. Kaplan, M.H. Liang, and H.S. Luthra. 1988. The American rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and Rheumatism* 31: 315–324.
- Bartok, B., and G.S. Firestein. 2010. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunological Reviews* 233: 233–255.
- Belmokhtar, C.A., J. Hillion, C. Dudogon, S. Fiorentino, M. Flexor, M. Lanotte, and E. Ségall-bendirdjian. 2003. Apoptosome-independent pathway for apoptosis. Biochemical analysis of APAF-1 defects and biological outcomes. *Journal of Biological Chemistry* 278: 29571–29580.
- Cutolo, M., A. Sulli, C. Pizzorni, B. Seriola, and R.H. Straub. 2001. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Annals of the Rheumatic Diseases* 60: 729–735.
- Da Sylva, T.R., A. Connor, Y. Mburu, E. Keystone, and G.E. Wu. 2005. Somatic mutations in the mitochondria of rheumatoid arthritis synoviocytes. *Arthritis Research and Therapy* 7: R844–R851.
- de Lathouder, S., A.H. Gerards, B.A. Dijkmans, and L.A. Aarden. 2004. Two inhibitors of DNA syntheses lead to inhibition of cytokine production via a different mechanism. *Nucleosides and Nucleic* 23: 1089–1100.
- Firestein, G.S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423: 356–361.
- Furst, D.E., and J.M. Kremer. 1988. Methotrexate in rheumatoid arthritis. *Arthritis and Rheumatism* 31: 305–314.
- Gerards, A.H., S. de Lathouder, E.R. de Groot, B.A. Dijkmans, and L.A. Aarden. 2003. Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis. *Rheumatology* 42: 1189–1196.
- Gibbons, L.J., and K.L. Hyrich. 2009. Biologic therapy for rheumatoid arthritis: clinical efficacy and predictors of response. *Bio Drugs* 23: 111–124.
- Gu, Y., C. Wang, C.M. Roifman, and A. Cohen. 2003. Role of MHC class I in immune surveillance of mitochondrial DNA integrity. *Journal of Immunology* 170: 3603–3607.
- Herman, S., N. Zurgil, P. Langevitz, M. Ehrenfeld, and M. Deusch. 2003. The induction of apoptosis by methotrexate in activated lymphocytes as indicated by fluorescence hyperpolarization: A possible model for predicting methotrexate therapy for rheumatoid arthritis patients. *Cell Structure and Function* 28: 113–122.

- Herman, S., N. Zurgil, and M. Deutsch. 2005. Low dose methotrexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines. *Inflammation Research* 54: 273–280.
- Hsu, P.C., T.C. Hour, Y.F. Liao, Y.C. Hung, C.C. Liu, W.H. Chang, M.C. Kao, G.J. Tsay, H.C. Hung, and G.Y. Liu. 2006. Increasing ornithine decarboxylase activity is another way of prolactin preventing methotrexate-induced apoptosis: crosstalk between ODC and BCL-2. *Apoptosis* 11: 389–399.
- Huang, C.C., P.C. Hsu, Y.C. Hung, C.C. Liad, C.T. Liu, C.M. Hour, G.J. Kao, G.J. Tsay, G.Y. Hung, and G.Y. Liu. 2005. Ornithine decarboxylase prevents methotrexate-induced apoptosis by reducing intracellular reactive oxygen species production. *Apoptosis* 10: 895–907.
- Jing, X.B., X.B. Cai, H. Hu, S.Z. Chen, B.M. Chen, and J.Y. Cai. 2007. Reactive oxygen species and mitochondrial membrane potential are modulated during CDDP-induced apoptosis in EC-109 cells. *Biochemistry and Cell Biology* 85: 265–271.
- Kim, R. 2005. Recent advances in understanding the cell death pathways activated by anticancer therapy. *Cancer* 103: 1551–1560.
- Kim, R., M. Emi, and K. Tanabe. 2006. Role of mitochondria as the gardens of cell death. *Cancer Chemotherapy and Pharmacology* 57: 545–553.
- Korb, A., H. Pavenstädt, and T. Pap. 2009. Cell death in rheumatoid arthritis. *Apoptosis* 14: 447–454.
- Kraan, M.C., T.J. Smeets, M.J. van Loon, F.C. Breedveld, B.A. Dijkmans, and P.P. Tak. 2004. Differential effects of leflunomide and methotrexate on cytokine production in rheumatoid arthritis. *Annals of the Rheumatic Diseases* 63: 1056–1061.
- Lafyatis, R., E.F. Remmers, A.B. Roberts, D.E. Yocum, M.B. Sporn, and R.L. Wilder. 1989. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. *Journal of Clinical Investigation* 83(4): 1267–1276.
- Lefevre, S., A. Knedla, C. Tennie, A. Kampmann, C. Wunrau, R. Dinsler, A. Korb, E.M. Schnäker, I.H. Tarner, P.D. Robbins, C.H. Evans, H. Stürz, J. Steinmeyer, S. Gay, J. Schölmerich, T. Pap, U. Müller-Ladner, and E. Neumann. 2009. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. *Nature Medicine* 15: 1414–1420.
- Möller, B., N. Kukoc-Zivojnov, S. Okamgba, U. Kessler, E. Puccetti, O.G. Ottmann, J.P. Kaltwasser, D. Hoelzer, and M. Ruthardt. 2002. Folinic acid antagonizes methotrexate-induced differentiation of monocyte progenitors. *Rheumatology International* 22: 60–67.
- Muller-Ladner, U., C. Ospelt, S. Gay, O. Distler, and T. Pap. 2007. Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts. *Arthritis Research and Therapy* 9: 223.
- Müller-Ladner, U., J. Kriegsmann, B.N. Franklin, S. Matsumoto, T. Geiler, R.E. Gay, and S. Gay. 1996. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. *American Journal of Pathology* 149(5): 1607–1615.
- Nakajima, A., M. Hakoda, H. Yamanaka, N. Kamatani, and S. Kashiwazaki. 1996. Divergent effects of methotrexate on the clonal growth of T and B lymphocytes and synovial adherent cells from patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases* 55: 237–242.
- Nakazawa, F., H. Matsuno, K. Yudoh, R. Katayama, T. Sawai, M. Uzuki, and T. Kimura. 2001. Methotrexate inhibits rheumatoid synovitis by inducing apoptosis. *Journal of Rheumatology* 28(8): 1800–1808.
- Neidhart, M., C.A. Seemayer, K.M. Hummel, B.A. Michel, R.E. Gay, and S. Gay. 2003. Functional characterization of adherent synovial fluid cells in rheumatoid arthritis: Destructive potential in vitro and in vivo. *Arthritis and Rheumatism* 48: 1873–1880.
- Noss, E.H., and M.B. Brenner. 2008. The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis. *Immunological Reviews* 223: 252–270.
- Pap, T., I. Meinecke, U. Müller-Ladner, and S. Gay. 2005. Are fibroblasts involved in joint destruction? *Annals of the Rheumatic Diseases* 64(Suppl 4): 52–54.
- Perlman, H., and R.M. Pope. 2010. The synovial lining micromass system: Toward rheumatoid arthritis in a dish? *Arthritis and Rheumatism* 62: 643–646.
- Phillips, D.C., K.J. Woollard, and H.R. Griffiths. 2003. The anti-inflammatory actions of methotrexate are critically dependent upon the production of reactive oxygen species. *British Journal of Pharmacology* 138: 501–511.
- Quemeneur, L., L.M. Gerland, M. Flacher, M. Ffrench, J.P. Revillard, and L. Genestier. 2003. Differential control of cell cycle, proliferation, and survival of primary T lymphocytes by purine and pyrimidine nucleotides. *Journal of Immunology* 170: 4986–4995.
- Rutkauskaite, E., W. Zacharias, J. Schedel, U. Müller-Ladner, C. Mawrin, C.A. Seemayer, D. Alexander, R.E. Gay, W.K. Aicher, B.A. Michel, S. Gay, and T. Pap. 2004. Ribozymes that inhibit the production of matrix metalloproteinase one reduced the invasiveness of rheumatoid arthritis synovial fibroblasts. *Arthritis and Rheumatism* 50: 1448–1456.
- Van der Linden, S., H.A. Valkenburg, and A. Cats. 1984. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis and Rheumatism* 27(4): 361–368.
- Van Gestel, A.M., C.J. Haagsma, and P.L. van Riel. 1998. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. *Arthritis and Rheumatism* 41: 1845–1850.
- Wessels, J.A., T.W. Huizinga, and H.J. Guchelaar. 2008. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. *Rheumatology (Oxford)* 47(3): 249–255.