Arsenic Trioxide Induces Apoptosis of Fibroblast-like Synoviocytes and Represents Antiarthritis Effect in Experimental Model of Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Recent studies have demonstrated that rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) proliferate as fiercely as tumor cells. Induction of apoptosis in RA FLS therefore provides a new approach for the inhibition of joint destruction. Arsenic trioxide (As_2O_3) was reported to be an effective apoptosis inducer in a variety of cell types. We investigated the possible effect of As_2O_3 on apoptosis induction of RA FLS and the mechanisms involved in this process.

Methods. Apoptosis was determined by flow cytometric analysis, terminal deoxynucleotide transferase-mediated dUTP nick end-labeling, and transmission electron microscopy. The activity and messenger RNA (mRNA) expression of nuclear factor-κB (NF-κB) was then detected by ELISA and real-time polymerase chain reaction, respectively. Activities of caspase-3 and caspase-8 were evaluated using luminogenic substrates. The effect of As_2O_3 on the morphology of rats with collagen-induced arthritis was evaluated under a light microscope after H&E staining.

Results. As_2O_3 significantly enhanced the apoptosis of RA FLS. It suppressed the DNA-binding activity and mRNA expression level of NF-κB, probably by inhibiting tumor necrosis factor-α-induced activation of NF-κB. As_2O_3 treatment significantly increased the activity of both caspase-3 and caspase-8. Morphological analysis revealed histological recovery in the synovial membrane. Synovial hyperplasia and inflammation in the joints were effectively inhibited.

Conclusion. As_2O_3 represents an apoptotic effect on RA FLS through NF-κB signaling pathway, and this process is mediated by the activation of caspase cascade. Treatment with As_2O_3 significantly improved the pathologic changes of collagen-induced arthritis and may have potential for treatment of RA. (First Release Oct 1 2010; J Rheumatol 2011;38:36–43; doi:10.3899/jrheum.100299)

Key Indexing Terms:
RHEUMATOID ARTHRITIS ARSENIC TRIOXIDE APOPTOSIS SIGNAL TRANSDUCTION

Arsenic trioxide (As_2O_3), a component of traditional Chinese medicine, is successfully used in the management of acute promyelocytic leukemia (APL). It is an effective therapeutic agent even in patients resistant to retinoic acid and chemotherapy. Various cell types have been reported to be sensitive to As_2O_3-induced apoptosis. As_2O_3 has been reported to be cytotoxic to APL cells and other malignant cells, including myeloma cells, chronic lymphocytic leukemia cells, and a variety of solid tumor cells[1,2,3,4]. Intravenous administration of As_2O_3 to patients with APL leads to a peak plasma level of 5 to 7 μM, with a steady-state concentration of 1 to 2 μM[5]. The 2 main mechanisms involved in the therapeutic effects of As_2O_3 are differentiation and apoptosis. In malignant cells, As_2O_3 induces cell differentiation at low concentrations ($\leq 1\ \mu$M) and promotes apoptosis at high drug levels ($\geq 2\ \mu$M)[6,7]. Recently, As_2O_3 was found to protect animals from developing human lupus-like syndrome. MRL/lpr mice have been widely used to investigate the etiology and treatment of systemic lupus erythematosus. A study involving an MRL/lpr mouse demonstrated that As_2O_3 treatment significantly prolonged survival. As_2O_3 is therefore considered a novel potential agent for autoimmune diseases[8].

Rheumatoid arthritis (RA) is a common human autoimmune disease characterized by chronic inflammation and hyperplasia of the synovial joints and subsequent progressive destruction of articular structures[9]. Although various cell populations may participate in the pathogenesis of RA, fibroblast-like synoviocytes (FLS) are considered crucial in both the initiation and progression of arthritis[10]. Mechanisms of synovial hyperplasia are not fully understood. Recent evidence supports the hypothesis that resistance to apoptosis contributes to the expansion of RA FLS. Apoptosis induction of RA FLS is therefore suggested as a potential therapeutic approach for RA[11,12].

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We investigated whether As_2O_3 had a therapeutic effect on RA. Considering the critical role of FLS in the development of RA, we investigated the possible effect of As_2O_3 on human RA FLS as well as an experimental model of collagen-induced arthritis (CIA) in rats and further explored the molecular mechanisms involved in As_2O_3 treatment.

MATERIALS AND METHODS

Cell culture and reagents. RA FLS derived from inflamed synovial tissues of patients with RA were obtained from Cell Applications (San Diego, CA, USA). Cells were incubated in synoviocyte growth medium (Cell Applications) at 37°C in 5% CO_2. FLS passages 3 to 7 were used for all experiments.

Tumor necrosis factor-α (TNF-α), bovine type II collagen, and Freund's incomplete adjuvant all came from Sigma-Aldrich (St. Louis, MO, USA). As_2O_3 was purchased from Yida pharmaceutical factory (Harbin, Hei Longjiang, China). A stock solution of 1 mM As_2O_3 was obtained by dissolving the drug in phosphate-buffered saline (PBS) and storing at 4°C in the dark. The solution was diluted to working concentration before use.

Apoptosis analysis. Apoptosis was detected by flow cytometry, terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay staining, and transmission electron microscopy (TEM).

For the flow cytometric analysis, RA FLS were treated with As_2O_3 for 24 h and then harvested and washed twice in PBS. Cells (1×10^5) were resuspended in 100 μl annexin V binding buffer, followed by double-staining with FITC-annexin V and propidium iodide using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Samples were analyzed by flow cytometry (BD Biosciences).

TUNEL assay was carried out following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Air-dried cell samples were fixed with freshly prepared fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 1 h at room temperature. Slides were then washed in PBS, and permeabilized with Triton X-100 (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4°C. Next, samples were rinsed with PBS and incubated with TUNEL reaction mixture in a humidified chamber at 37°C for 60 min. After washing 3 times with PBS, samples were analyzed under a fluorescence microscope (Eclipse E800, Nikon, Tokyo, Japan). The apoptosis index was defined as the percentage of positive cells in 1000 RA FLS.

Apoptosis of RA FLS was also determined under TEM directly through observations of morphological changes at the subcellular level. Cells were cultured in the presence or absence of As_2O_3. After 72 h incubation, RA FLS were harvested by centrifugation and fixed with 2.5% cold glutaraldehyde. Fixed samples were dehydrated with a graded series of ethanol and embedded. Ultrathin sections were subsequently stained with uranyl acetate and lead citrate. Ultrastructural features of cells were examined under a TEM (JEM-120, JEOL, Tokyo, Japan).

DNA-binding activity of NF-κBp65. Specific binding of NF-κBp65 subunit was measured using a TransAM NF-κBp65 kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. After treatment with As_2O_3 and/or TNF-α, cells were collected by scraping and centrifugation. Cell nuclear extracts were then added into a 96-well plate immobilized with oligonucleotide containing the NF-κB consensus site (5'-GGG ACT TTC C-3'). Using a primary antibody directed against the NF-κBp65 subunit and a second antibody conjugated to horseradish peroxidase, the optical density was qualified by spectrophotometry using the SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Real-time polymerase chain reaction (PCR). Total RNA was extracted using an RNeasy Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reverse transcription was carried out with AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed with the Premix Ex Taq PCR kit (Takara, Dalian, China) and conducted with an iCycler iQ real-time detection system (Bio-Rad, Hercules, CA, USA). Sequences for the NF-κB gene-specific primers were as follows: 5'-AGA AGT CAC ATC TGG TTT GAT TTC T-3' (forward), 5'-CCA GCC CTC AGC AAA TCC-3' (reverse), and FAM-TGA TCT GTA CCA GAC GCC CTT GCA C-BHQ1 (probe). PCR was performed with the following cycling measurements: 95°C for 5 min followed by 40 cycles, each comprising 95°C for 10 s and 60°C for 30 s. A total of 20 μl PCR mixture contained 10 μl 2× Premix Ex Taq, 1 μl (0.2 μM, final concentration) of each primer, 2 μl of cDNA sample, 0.5 μl TaqMan probe, and 5.5 μl ddH_2O. The housekeeping gene GAPDH was used for internal normalization. Fold-changes of gene induction were calculated relative to untreated sample.

Caspase activity assays. Activities of caspase-3 and caspase-8 were measured using caspase-Glo 3/7 assay kit and caspase-Glo 8 assay kit (Promega), respectively. RA FLS were seeded into a white 96-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of 5000 cells/well in triplicate wells. Cells were cultured in the presence or absence of As_2O_3 for 12 h and subsequently mixed with an equal volume of caspase substrates. Luminescence was measured after 30 min incubation using a plate reader (SpectraMax M5, Molecular Devices).

CIA model in rats. Twelve-week-old male dark agouti (DA) rats were used in our experiments. Animals were obtained from the China Academy of Chinese Medical Sciences. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Committee of Harbin Medical University. Rats were well fed and housed in an animal facility with 12-hour light cycles.

Bovine type II collagen (BII) was dissolved in 0.1 M acetic acid, and the collagen solution was emulsified in an equal volume of incomplete Freund's adjuvant. CIA was induced in rats by intradermal injections at the tail base with 2 mg/kg BII. A booster injection of 100 μg BII was administered on the seventh day. Twenty-one days after collagen injection, CIA was well established in the immunized rats. Animals were randomly divided into the following 6 groups: normal control, CIA control, and As_2O_3-treated CIA groups (1.0 mg/kg, 2.0 mg/kg, 4.0 mg/kg, and 6.0 mg/kg). Each group comprised 12 DA rats. Twenty-two days after the first immunization, DA rats received a treatment of As_2O_3 for 7 days. The drug was injected intraperitoneally with specified doses daily. Control groups received a daily intraperitoneal injection of an equal volume of saline.

Hind limbs of the DA rats were dissected and fixed immediately in 10% formalin, embedded in paraffin and subsequently stained with H&E. Samples were then observed under a light microscope. Sections were examined for the presence of hyperplasia of the synovium, pannus formation, and destruction of the joint.

Statistical analysis. Results are expressed as means ± SD. Statistical analysis was performed with paired t test or ANOVA as appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

As_2O_3-induced RA FLS apoptosis. After RA FLS were cultured in the presence or absence of As_2O_3, apoptosis was first determined using flow cytometry. On the basis of annexin V and propidium iodide binding, we found that As_2O_3 treatment induced an apoptotic process in RA FLS. A lower percentage of binding (1.6% ± 0.3%) was detected in the untreated cells group. Enhanced apoptotic rate of RA FLS was found with the treatment of 2 μM As_2O_3, and even higher when 8 μM As_2O_3 was applied (10.6% ± 2.1%). Thus, As_2O_3 induced RA FLS apoptosis in a dose-dependent manner (Figure 1A).

As_2O_3-associated apoptotic effects were further con-

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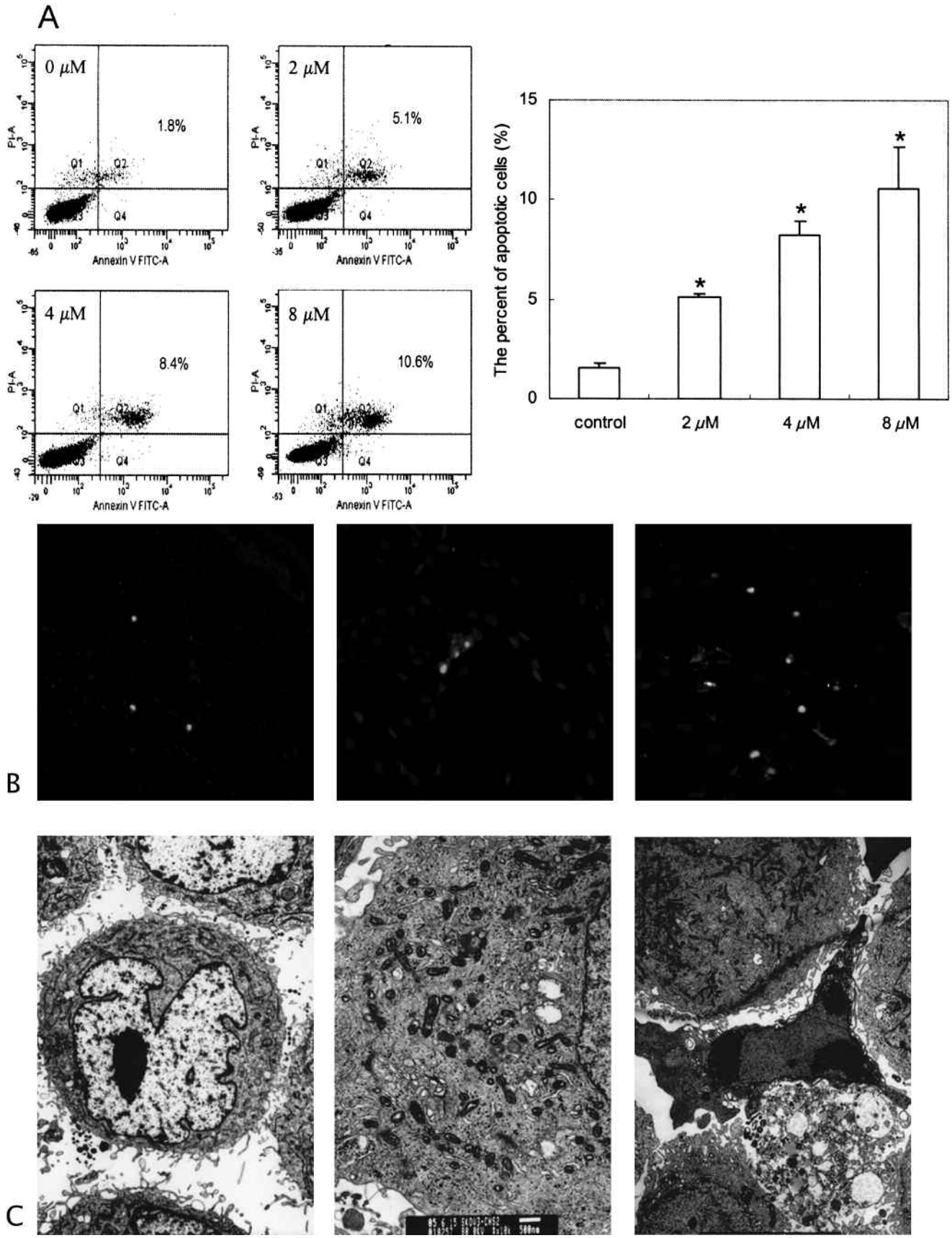

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Figure 1. Arsenic trioxide (As_2O_3) shows apoptotic effect on RA FLS. A. Apoptotic rate was evaluated by flow cytometry. Cells were incubated with indicated concentrations of As_2O_3 for 24 h, followed by double-staining with FITC-annexin V and propidium iodide. Apoptotic percentage of RA FLS increased in accord with concentration of As_2O_3. *Significant differences compared with control untreated cells. B. Apoptosis was examined by TUNEL assay (400×). Light areas are apoptotic cells. Only a few positive cells were detected in the control group, and apoptotic cells were easily observed in As_2O_3-treated groups. C. Ultrastructural changes of RA FLS examined under transmission electron microscopy (8000×). Early apoptosis was observed following treatment with 2 μM arsenic agent. Cells incubated with 8 μM As_2O_3 revealed typical manifestations of apoptosis.

firmed by TUNEL staining. Apoptosis index in the 0.5 μM As_2O_3-treated group showed no difference compared with that of the untreated cells (1.5% ± 0.6%). As illustrated in Figure 1B, quantitative analysis showed a predominant increase of cell death following treatment with 2 μM or 8 μM As_2O_3, with apoptotic indexes of 7.4% ± 1.6% and 9.3% ± 2.6%, respectively ($p < 0.05$ vs control).

Ultrastructural changes of cells were observed under TEM. Untreated RA FLS displayed normal shape with developed rough endoplasmic reticulum. Cells treated with 2 μM As_2O_3 did not display altered morphology. However, early characteristics of apoptosis were observed, including chromatin condensation and marginalization. After a treatment with 8 μM As_2O_3, irregular contour of cells was noted and other typical characteristics of apoptosis appeared including vacuolar formation, mitochondrial swelling, pyknosis and condensation, and fragmentation of nuclear chromatin (Figure 1C). These observations further demonstrated As_2O_3-induced apoptosis of RA FLS.

Effects of As_2O_3 on NF-κB messenger RNA (mRNA) expression and DNA-binding activity. We then studied the possible mechanisms involved in the apoptotic effect of As_2O_3. Inhibition of NF-κB was shown to play an important role in As_2O_3-induced tumor cell apoptosis[1]. We assessed NF-κB mRNA expression and DNA-binding activity of the p65 subunit in cultured RA FLS. The mRNA level of NF-αB was predominantly upregulated with TNF-α stimulation. This action was efficiently suppressed through the addition of As_2O_3. As_2O_3 treatment downregulated NF-κB mRNA expression in a dose-dependent manner (Figure 2A). Indeed, the mRNA level was diminished to control level when treated with 8 μM As_2O_3.

DNA-binding activity of the NF-κBp65 subunit was evaluated using a sensitive multiwell colorimetric assay. Cells were pretreated with 4 μM As_2O_3 for 6 h, followed by a 2-h incubation with TNF-α (20 ng/ml). Pretreatment with TNF-α resulted in 2.5-fold-enhanced DNA-binding capacity of the p65 subunit. Cells treated with As_2O_3 itself showed weaker NF-κBp65 activity and no statistical significance was detected. With TNF-α stimulation, NF-κB activation in RA FLS was markedly decreased (Figure 2B). These observations indicate that the NF-κB pathway plays an essential role in the As_2O_3-induced apoptotic process.

As_2O_3-induced RA FLS apoptosis involves activation of caspases. It has been reported that NF-κB protects cells from apoptosis by restraining the upstream caspase cascade. Moreover, As_2O_3-induced apoptosis could be effectively blocked by the inhibitors of caspase-8 and caspase-3. To further clarify the apoptotic signaling pathway underlying As_2O_3 toxicity, we investigated the effect of As_2O_3 on the activation of the caspase cascade in RA FLS. Caspase-8 and caspase-3 activities were examined using the luminescent caspase activity assay with luminogenic substrates. Enzymatic activities of caspase-8 and caspase-3 were measured by detecting the luminescent signals of the caspases and results were expressed as relative light units compared

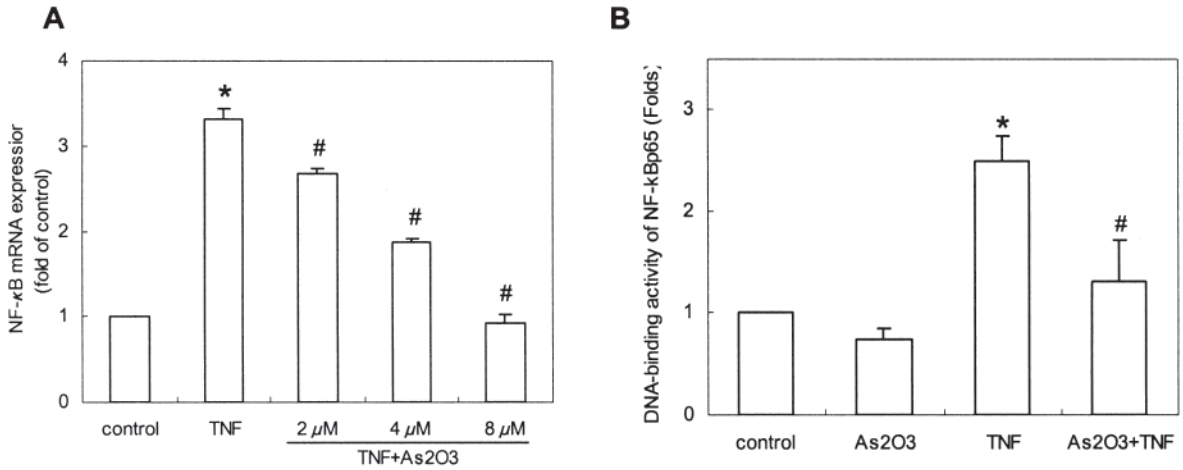

Figure 2. Arsenic trioxide treatment reduced NF-κB activity of RA FLS. Untreated cells were used as negative control. NF-κB mRNA expression (A) and DNA-binding activity of the NF-κBp65 subunit (B) was described as fold-change compared with negative control. Results are representative of 3 independent experiments. *Significant difference ($p < 0.05$) from control and # TNF-α-treated group.

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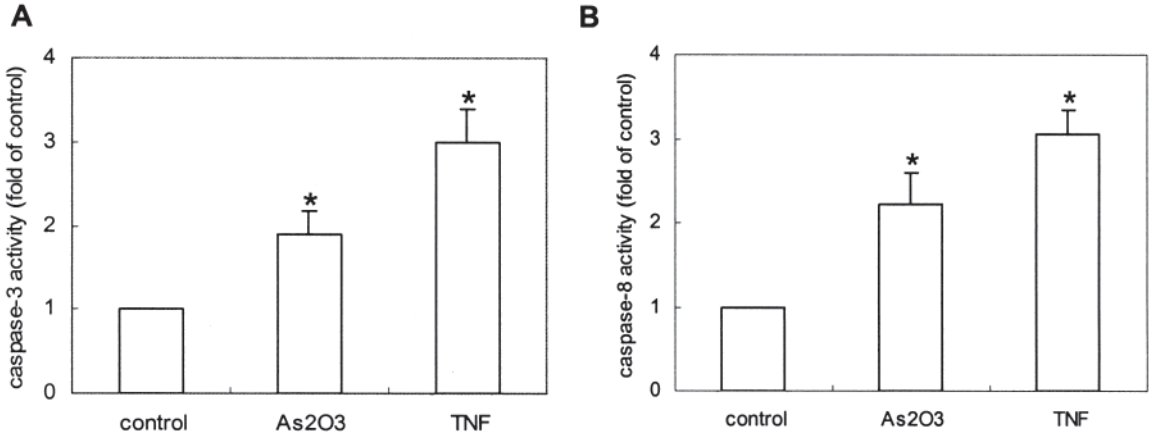

Figure 3. Effect of arsenic trioxide (As_2O_3) on caspase activities of RA FLS. After 12-hour incubation with As_2O_3, enzymatic activities of caspase-3 and caspase-8 were evaluated by detecting luminescent signals. TNF-α was used as a positive control in both experiments. *Significant difference ($p < 0.05$) compared with untreated cells (control).

with the untreated group. Our data demonstrate that As_2O_3 treatment significantly increased the activities of caspase-8 and caspase-3 (2.2-fold and 1.9-fold, respectively; Figure 3). These results indicate that caspase activation plays a potent role in the process of As_2O_3-induced RA FLS apoptosis.

Effect of As_2O_3 on CIA rats. CIA rats displayed limited activity compared with normal rats during the experiment. Joint swelling subsided in As_2O_3-treated rats, and they exhibited no behavioral abnormalities compared with normal controls. Absolute increments in body weight were measured on the first, seventh, and fifteenth days after As_2O_3 treatment, and no significant differences were observed between groups.

With H&E staining and morphology detection of the animal joints, no synovial hyperplasia or pannus formation was observed in the control rats. Arthritis developed in the CIA model, characterized by synovial hyperplasia, pannus formation, erosion of bone and cartilage, inflammatory cell infiltration, and arthrodial cartilage degradation (Figure 4). Animals treated with As_2O_3 revealed less evidence of inflammation or destruction in the joint, and this particularly applied to CIA rats treated with 4.0 mg/kg and 6.0 mg/kg As_2O_3. Indeed, histology of the synovial membrane in these groups was similar to normal synovium, and synovial hyperplasia and inflammation were effectively suppressed by As_2O_3.

DISCUSSION

Proliferation of RA synovial cells is considered to be as fierce as that of tumor cells[13]. The hypertrophied synovium invades and erodes the cartilage and adjacent bone, resulting in progressive destruction of the joint. Although the pathogenesis of RA is only partially understood, it has been demonstrated that FLS are important in arthritis and are involved in the initiation and perpetuation of RA. RA FLS are therefore considered important targets for novel approaches in the management of RA. Synovial hyperplasia appeared to be mediated at least in part by defective apoptosis of RA FLS. Previous studies suggested that induction of apoptosis is a promising therapeutic strategy to eliminate RA synovial pannus[14]. In our study, apoptosis of RA FLS was proved by both direct and indirect observations. Our results indicate that As_2O_3 can effectively induce the programmed cell death in RA FLS in a dose-dependent manner.

NF-κB is widely expressed in almost all animal cell types. This ubiquitous transcription factor is involved in the activation of a multitude of cellular and viral genes and plays a pivotal role in cell death and survival pathways[15]. NF-κB is known to be activated in RA[16]. Activation of NF-κB by inflammation protects synovial cells from apoptosis[17]. Data from studies using experimental animals and human joints have shown obvious increases in the DNA-binding activity of NF-κB in both the CIA model and patients with RA[18,19]. Prior studies suggested that inhibition of NF-κB activity essentially contributes to the cytotoxic action of arsenic. Cells transfected with NF-κBp65 expression structures are resistant to arsenic-mediated induction of apoptosis[20]. We therefore hypothesize that NF-κB plays a role in RA FLS apoptosis induced by As_2O_3. Our data demonstrate for the first time that As_2O_3 has the ability to block TNF-α-induced activation of NF-κB in RA FLS. DNA-binding activity and the mRNA level were both effectively suppressed during As_2O_3 incubation. A variety of drugs (e.g., sulfasalazine, gold compounds, and corticosteroids) have been reported to exhibit their therapeutic properties, at least in part, through the NF-κB pathway[21,22,23]. Besides classical agents, gene therapy and novel agents containing small-molecule inhibitors of NF-κB have exhibited efficacy in animal arthritis models[24,25]. Thus, blockade of

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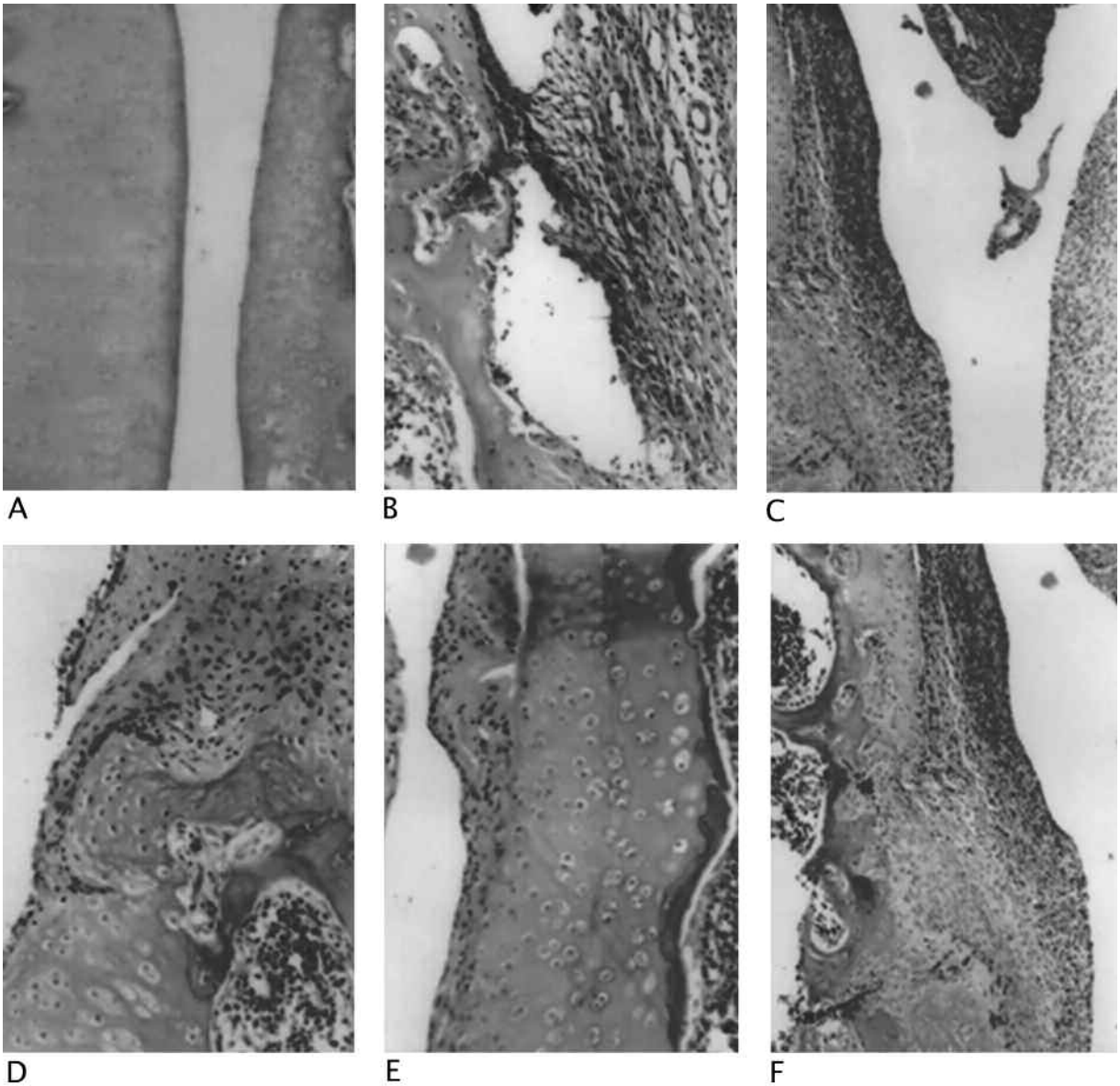

Figure 4. Effect of arsenic trioxide (As_2O_3) treatment on collagen-induced arthritis (CIA) rats. Morphological changes in joints of the 6 groups were evaluated under a light microscope (H&E stain). A. Smooth articular facets were seen in the control group. B. Arrows indicate excessive proliferation of synovium and angiogenesis in the positive control (CIA). These observations diminished gradually with increase of As_2O_3 concentration (C, 1 mg/kg; D, 2 mg/kg; E, 4 mg/kg; F, 6 mg/kg).

the NF-κB signaling pathway has a precise therapeutic effect on RA. In addition, activation of NF-κB enabled the activation of multiple inflammatory molecules. TNF-α, interleukin 1ß, matrix metalloprotease, and various proinflammatory factors are considered to be under the control of NF-κB transcription factors[15]. As_2O_3 may consequently exhibit antiinflammatory effects, which are of great benefit in the control of the disease. In short, the definite inhibitory action against the NF-κB pathway makes it possible to use As_2O_3 as a novel agent for RA. It is also important to note that the drug concentrations we used correlate closely with clinical concentrations and can closely mimic the *in vivo* status.

Previous studies have demonstrated that specific inhibition of NF-κB activity potentiates TNF-α and FasL-related apoptosis[26]. Caspase-8 is known to be the apical caspase that participates in the TNF-α and FasL pathway. Studies on the HT1080 fibrosarcoma cell line validated that NF-κB suppressed the initiation of the caspase cascade through caspase-8 blockade. In our study, As_2O_3 treatment effectively enhanced the activity of caspase-8, suggesting that NF-κB inhibition by As_2O_3 led to the activation of the upstream

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caspase. Meanwhile, caspase-3 was also activated. Thus, NF-κB inactivation brought about the enzymatic activity of both initiated and executive caspases and enabled an extrinsic apoptotic pathway.

Actions of As_2O_3 were further detected *in vivo* in the CIA model. The 7-day treatment with As_2O_3 significantly improved arthritis of the rat model. Pathological changes of the synovium were markedly relieved in a dose-dependent manner. These results further suggest the therapeutic effect of As_2O_3, especially for joint destruction and angiogenesis. Although no toxic side effects were observed during the treatment, the possible toxicity of As_2O_3 should be noted. Thus, the comparative efficacy of different delivery methods (e.g., venous, articular cavity, or intraperitoneal injections) is worth studying in future research. Considering the direct interactions of As_2O_3 with RA FLS, it will be of great interest to investigate the feasibility and effect of As_2O_3 through intraarticular administration.

Notable progress has been made in the treatment of RA, especially for the application of biological agents that specifically suppress the actions of TNF-α or interleukin 1. However, not all patients with RA benefit from those drugs. A certain number of patients exhibit poor responses to the biologics, indicating that new strategies should be applied to the management of RA. High cost is another major obstacle. Its effectiveness and relatively modest cost make As_2O_3 useful. We observed a case with both APL and active RA, in which APL treatment with As_2O_3 relieved the joint symptoms. This finding further confirmed the usefulness of As_2O_3 in clinical application.

A recent case report[27] recorded a relapse of RA in a patient with APL who received combination therapy with all-transretinoic acid and As_2O_3. This seems to conflict with our findings. However, the patient in that report received both all-transretinoic acid and As_2O_3 treatment, while our patient received a treatment with only As_2O_3. In addition, a modified method, known as continuously slow As_2O_3 intravenous infusion, was used in our patient, in accordance with a prior study[28]. This delivery method kept the drug in stable concentrations in blood circulation and effectively decreased the side effects. Thus, the rational method for the use of As_2O_3 should be noted.

Our study demonstrates that As_2O_3 can restrain the proliferation of RA FLS and consequently alter the histomorphological changes in RA synovium. The NF-κB signaling pathway and caspase activation participate in As_2O_3-induced apoptosis of RA FLS. These findings show As_2O_3 to be a novel and economic therapeutic agent for RA treatment. Further studies are required to evaluate the possible toxicity of As_2O_3.

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