The Effect of Apoptosis Signal-Regulating Kinase 1 Gene Transfer on Rat Collagen Induced Arthritis

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ABSTRACT. ***Objective.*** To examine the apoptosis-inducing effect of apoptosis signal-regulating kinase 1 (ASK1) gene transfer into synovial cells *in vitro* and *in vivo*.

Methods. An adenovirus vector was constructed so that a constitutively active form of ASK1 gene (ASK1ΔN) was expressed in the presence of the Cre recombinase. The ASK1ΔN and Cre adenovirus vectors were cotransduced into cultured synoviocytes derived from patients with rheumatoid arthritis (RA), and apoptosis was evaluated by TUNEL and Hoechst staining. Collagen induced arthritis (CIA) was induced in 8-week-old male DA rats, and 10 days later the 2 adenovirus vectors were coadministered into the ankle joints of the animals. As indicators of severity of arthritis, swelling of the ankle and articular index (AI) scores were evaluated, while histopathological observation of articular tissue was also performed.

Results. In the cultured human RA synoviocytes, overexpression of the ASK1ΔN significantly reduced cell viability and induced apoptosis. In the CIA rats transduced with the ASK1ΔN gene, arthritis was significantly promoted in terms of the swelling of the ankle joints and elevation of the AI scores. Histopathological observation also revealed that the constitutively active ASK1 induced massive infiltration of inflammatory cells into the synovial membrane as well as proliferation of synovial fibroblasts. Degeneration of the synovial membrane was not evident.

Conclusion. Adenoviral transduction of ASK1ΔN induced apoptosis in RA synoviocytes *in vitro*, but not in CIA synovium *in vivo*. (J Rheumatol 2005;32:2373–80)

Key Indexing Terms:
ARTHRITIS APOPTOSIS GENE THERAPY
ASK1 APOPTOSIS SIGNAL-REGULATING KINASE 1

Rheumatoid arthritis (RA) is an autoimmune disease characterized by systemic inflammation, which progresses through the involvement of external and genetic factors, causing severe pain and functional impairment of the joints. The synovial membrane is the predominant tissue where pathological changes are seen, including infiltration of lymphocytes as well as proliferation of fibroblasts and vascular endothelial cells[1]. During the progression of RA, bone and cartilage are degraded through osteoclast activation and protease production[2]. Recently, significant therapeutic efficacy was demonstrated in clinical trials with biological agents that suppress inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6)[3]. To achieve longterm therapeutic effects in RA, gene therapy has also been attempted, in which genes effective for treatment are introduced into the joint using some gene delivery vector. For example, *ex vivo* transduction of the IL-1 receptor antagonist (IRAP) gene, which competitively blocks IL-1, has been performed in human clinical trials[4]. In *in vivo* approaches, RA model animals were transduced into the synovium with adenovirus vectors carrying the genes for antiinflammatory cytokines including IL-4[5], IL-10[6], and IL-13[7], while other studies employed the genes encoding Iκß[8] and p16 cyclin-dependent kinase inhibitor (cdk)[9] to suppress nuclear factor-κB (NF-κB) activation and cell cycle progression, respectively.

Apoptotic cell death is involved in the maintenance of body homeostasis, and the induction of apoptosis in the synovial membrane of patients with RA is considered a mechanism by which inflammatory synovial cells that would otherwise continue proliferating are degraded. In synovial tis-

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sues obtained from RA patients, not only proliferation but also apoptosis of synoviocytes has been detected. However, the incidence of apoptotic cells is limited, suggesting that apoptosis induction in RA synovial cells may be suppressed by various apoptosis inhibitory molecules, which are thought to be one of the factors causing the abnormal proliferation of synoviocytes[10-12]. Thus, adequate induction of apoptosis by gene transfer to a RA synovial membrane would modulate the proliferation of synovial cells as an effective genetic synovectomy. Once carried out, the therapeutic effect of synovectomy may persist for a long period. The genetic synovectomy would be more advantageous than other gene therapy strategies in that it does not require longterm expression of the therapeutic genes.

Apoptosis signal-regulating kinase 1 (ASK1) molecule, which is involved in the stress induced signal cascade, is reported to be expressed in RA synovial tissue[13]. If ASK1 expression is controlled experimentally in synovium, the results may greatly contribute to the understanding of the mechanisms of synovial proliferation, while induction of ASK1 gene would provide a novel and effective strategy of genetic synovectomy. Based on these hypotheses, we investigated apoptosis induction in synoviocytes that were transduced with the ASK1 gene.

MATERIALS AND METHODS

This study was approved by the Safety Committee for Recombinant DNA Experiments and the Ethical Review Board on Clinical Research and conducted according to the regulations for animal research of Kyoto Prefectural University of Medicine. All human samples were obtained in accord with the Ethical Review Board on Clinical Research of Kyoto Prefectural University of Medicine.

Adenovirus vectors. Human ASK1HA cDNA was a generous gift from Prof. H. Ichijo (Laboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, University of Tokyo)[14]. AxCALNL-ASK1ΔN was prepared by inserting the N-terminus truncated mutant of ASK1 (ASK1ΔN) gene into the AxCALNL (Takara, Otsu, Japan) at the downstream of the stuffer fragment that was flanked by 2 loxP sites[15]. AxCAN-Cre is an adenovirus vector containing the Cre recombinase gene (Takara). AxCA-LacZ was prepared by inserting the ß-galactosidase gene (LacZ) into the AxCA (Takara) at the downstream of the CAG promoter. Recombinant virus clones were isolated and titrated as described[16].

Synoviocytes. At the time of total knee arthroplasty, synovial tissues were obtained from 8 patients with RA that met the diagnostic criteria of the American College of Rheumatology[17]. Informed consent was obtained from the patients before the surgery. The synovial tissues were washed with phosphate buffered saline (PBS) and homogenized in 1 mg/ml collagenase (Collagenase S-1; Nitta Gelatin, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM) that contained 4.5 g/l glucose and supplemented with 1 μM sodium pyruvate (Nacalai Tesque, Kyoto, Japan). After 2 h agitation at 37°C, cells were washed with PBS and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Trace Scientific, Melbourne, Australia), 100 units/ml penicillin, and 100 μg/ml streptomycin (10% FBS/DMEM; Gibco BRL, Gaithersburg, MD, USA) at 37°C in 5% CO_2/95% humidified air (standard conditions).

Gene transduction to synoviocytes. Synoviocytes were suspended in 10% FBS/DMEM and seeded in collagen type I-coated 8-well chamber slides (Asahi Techno Glass, Tokyo, Japan), 1×10^5 cells/cm^2. After 48 h culture under standard conditions, culture medium was removed and adenovirus vectors suspended in 80 μl of 10% FBS/DMEM were added to the cells. AxCALNL-ASK1ΔN and AxCA-LacZ were infected into the cells at a multiplicity of infection (MOI) of 500 plaque-forming units (pfu)/cell, while AxCAN-Cre infection was performed at a MOI of 50 pfu/cell. After incubation under standard conditions for 1 h, the culture supernatant was replaced with fresh 10% FBS/DMEM.

Colorimetric cell proliferation assay and phase-contrast microscopic observation. Tetrazolium salt based colorimetric assay was performed to evaluate cell proliferation. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) was added to the cells at a concentration of 5 mg/ml, followed by incubation under standard conditions for 4 h. Optical densities (OD) at 570 and 630 nm were measured using a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). Phase-contrast microscopic observation was performed by inverted microscopy (IX-70/DP-50; Olympus, Tokyo, Japan).

Hoechst 33342 staining and terminal dUTP nick end-labeling (TUNEL) assay. Synoviocytes were stained with 2% bisbenzimide H33342 fluorochrome (Hoechst 33342; Nacalai Tesque) in 10% FBS/DMEM followed by incubation under the standard conditions for 30 min. Cells were observed by fluorescence microscopy (IX-FLA/IX-70/DP-50; Olympus). The TUNEL assay was performed using the In Situ Cell Death Detection Fluorescein Kit (Roche Diagnostics, Mannheim, Germany) as described[18]. TUNEL-positive cells were visualized by fluorescence microscopy as described above.

Collagen induced arthritis (CIA) and in vivo gene transfer. Eight-week-old male DA rats (Shimizu Laboratory Supplies, Kyoto, Japan) were subcutaneously injected into the tail base with a mixture of 100 ml of bovine type II collagen (Collagen Research Center, Tokyo, Japan) and 100 ml of Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA). Ten days after the immunization, various doses of AxCALNL-ASK1ΔN and AxCANCre were suspended in 10 μl and 1 μl of PBS, respectively, and the mixture was injected into the ankle joint of the animals. Control rats were administered with 11 μl of PBS.

Evaluation of clinical manifestations. To evaluate the paw swelling, the height of the dorsum of the foot was measured using a micrometer caliper (Digimatic; Mitsutoyo, Kanagawa, Japan). Gross observations of the toes and paws of the rats were assessed using arthritis index (AI) scores according to the methods of Woods, *et al*[19].

Histological examination. Rats were sacrificed with sodium pentobarbital. The ankles were fixed with 4% paraformaldehyde (pH 7.4) and decalcified in 10% formic acid. Embedded in paraffin, the specimens were cut into sections and stained with hematoxylin-eosin (H&E).

Statistical analysis. Comparisons between 2 groups with equal variance were performed using Student's t test, and those with unequal variance with Student's t test with Welch's correction. Statistical significance was defined as a p value < 0.05.

RESULTS

Effect of ASK1ΔN gene transfer on the viability of cultured RA synoviocytes. Adenoviral vectors carrying a constitutively active form of ASK1 (ASK1ΔN) gene were constructed. Since the ASK1 gene impairs host cell function during the process of the adenovirus vector generation, the Cre/loxP expression control system was employed[20] so that the ASK1 was expressed only in the presence of Cre recombinase. Human RA synoviocytes were infected with either AxCALNL-ASK1ΔN alone, a mixture of control adenovirus vector (AxCAN-LacZ) and Cre gene adenovirus (AxCAN-Cre), or a mixture of the AxCALNL-ASK1ΔN and AxCAN-Cre. Figures 1A–1D show RA synoviocytes 60 h after adenovirus vector infection. Compared with other cell groups,

the AxCALNL-ASK1ΔN + AxCAN-Cre group showed an obvious decrease in the cell number. The cells coinfected with the ASK1ΔN and Cre adenovirus vectors were observed over time. There was no change in the cultured human RA synoviocytes until 48 h after the gene transfer, but then the cell number decreased by 60 h, and continued to decrease until 96 h (Figures 1E–1H). MTT assay was performed 60 h after the gene transfer. The OD values were 131.4 ± 5.0 (control), 110.2 ± 20.7 (AxCALNL-ASK1ΔN), 116.8 ± 5.8 (AxCA-LacZ + AxCAN-Cre), and 26.4 ± 1.9

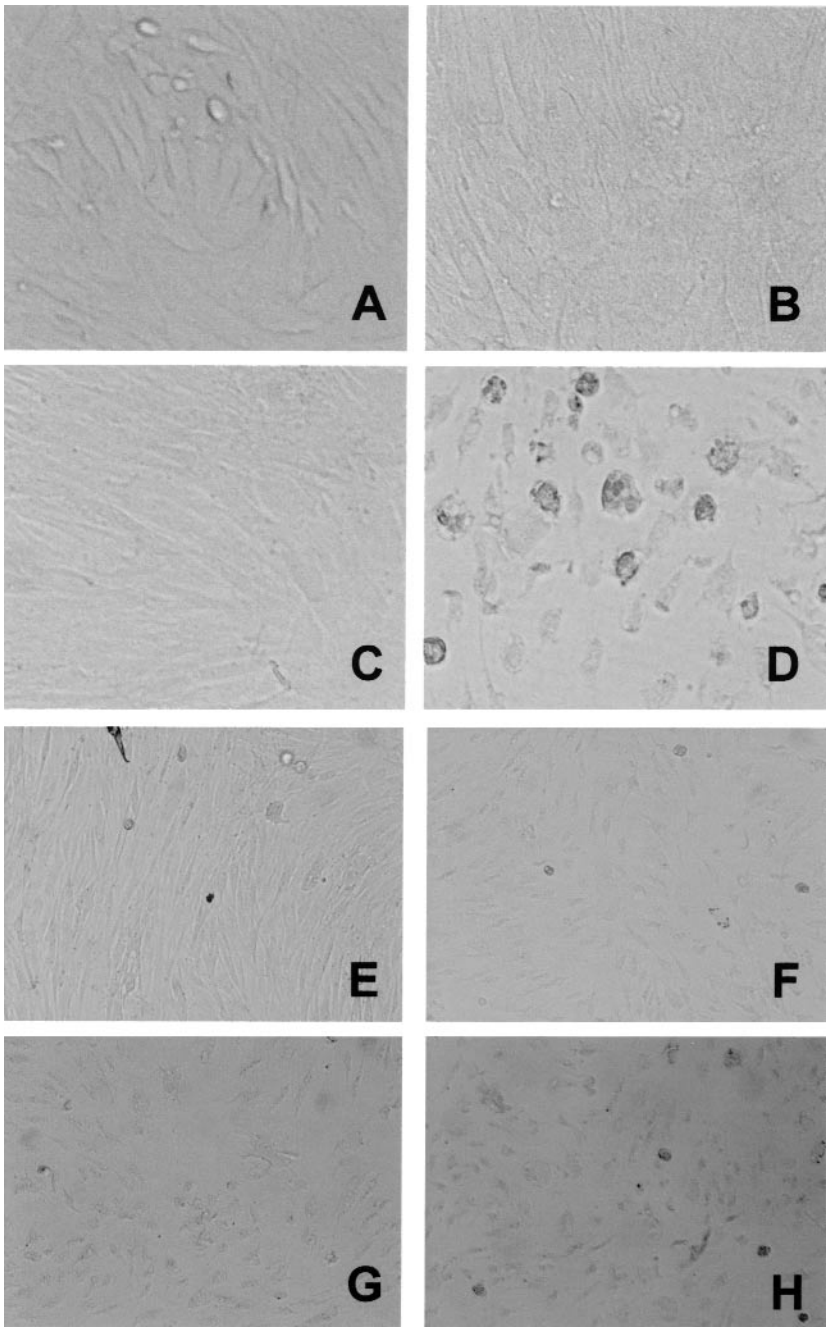

Figure 1. Morphological change of synoviocytes transduced with ASK1 gene. A–D: Human RA synoviocytes were infected with AxCALNL-ASK1ΔN (B), AxCA-LacZ and AxCAN-Cre (C), or AxCALNL-ASK1ΔN + AxCAN-Cre (D). Control cells were left uninfected (A). Cells were observed by phase-contrast microscopy 60 h after gene transfer. Original magnification ×100. E–H: RA synoviocytes were infected with AxCALNL-ASK1ΔN and AxCAN-Cre. Cells were observed by phase-contrast microscopy 48 h (E), 60 h (F), 72 h (G), and 96 h (H) after gene transfer. Original magnification ×40.

(AxCALNL-ASK1ΔN + AxCAN-Cre), indicating that cell viability in the AxCALNL-ASK1ΔN + AxCAN-Cre group was significantly lower than in the other groups (Figure 2).

Apoptosis-inducing effect of ASK1ΔN gene transfer in cultured RA synoviocytes. To examine the apoptosis-inducing effect, each cell group was stained with Hoechst 33342 at 60 h after adenovirus infection. In the AxCALNL-ASK1ΔN + AxCAN-Cre group, the viable cell number was reduced and apoptotic cells with fragmented nuclei were observed (Figures 3A–3D). In the other cell groups, no apoptotic cells were observed. Apoptosis induction was also evaluated using TUNEL staining. TUNEL-positive cells were observed in the AxCALNL-ASK1ΔN + AxCAN-Cre group, but not in the other cell groups (Figures 3E–3H). These results revealed that the Cre/lox system worked accurately, and that the overexpression of ASK1ΔN gene induced apoptosis in cultured human RA synoviocytes.

Changes in paw swelling in CIA rats treated with ASK1ΔN adenovirus vector. Various doses of AxCALNL-ASK1ΔN and AxCAN-Cre were simultaneously administered to the ankles of rats with or without CIA, and paw thickness was measured as an indication of severity of arthritis. Paw swelling was enhanced dose-dependently in all groups in which the ASK1ΔN gene was transduced (Figure 4, groups A–C), whereas normal DA rats showed no significant changes in paw thickness after the ASK1ΔN adenovirus infection (Figure 4, groups E–G). In Group A, in which the ASK1ΔN gene was transduced at the highest dose, the paw thickness was significantly increased to 7.0 ± 0.7 mm on Day 2, but then decreased to 5.4 ± 1.0 mm on Day 3. The swelling deteriorated again from Day 4 and reached a maximum of 10.9 ± 0.6 mm on Day 6. In groups B and C, paw swelling was enhanced over time. Therefore, *in vivo* adenoviral transduction of the active form of ASK1 gene resulted in the deterioration of arthritis.

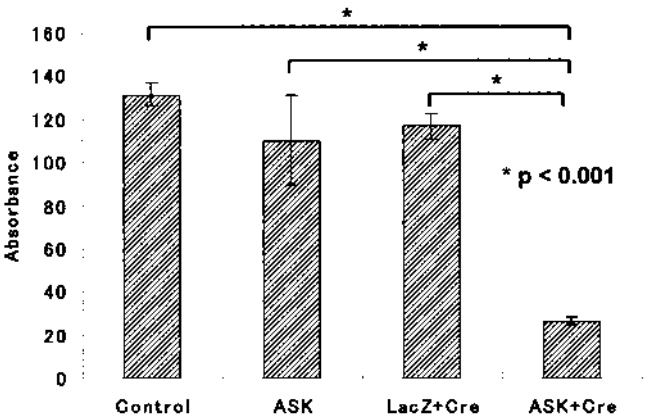

Figure 2. ASK1 gene transduction reduced viability of synoviocytes *in vitro*. RA synoviocytes were infected with AxCALNL-ASK1ΔN (ASK), AxCA-LacZ and AxCAN-Cre (LacZ+Cre), or AxCALNL-ASK1ΔN + AxCAN-Cre (ASK+Cre). Control cells were left uninfected (Control). MTT was added 60 h after gene transfer, and absorbance was measured 4 h later (n = 5).

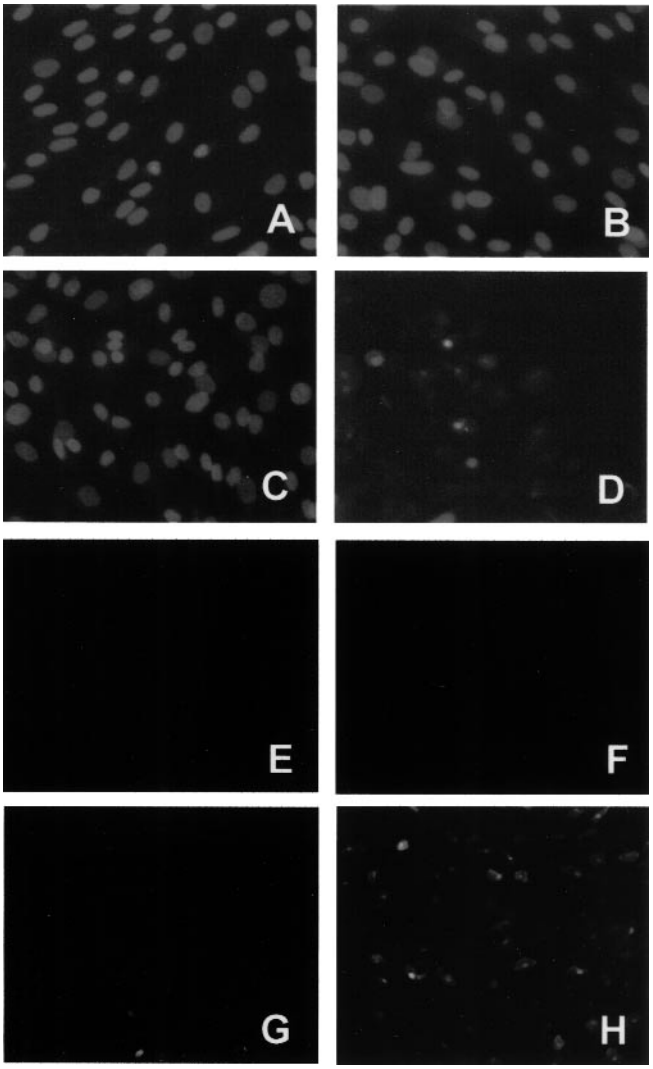

Figure 3. RA synoviocytes underwent significant apoptosis after ASK1 gene transduction *in vitro*. Human RA synoviocytes were infected with AxCALNL-ASK1ΔN (B and F), AxCA-LacZ and AxCAN-Cre (C and G), or AxCALNL-ASK1ΔN + AxCAN-Cre (D and H). Control cells were left uninfected (A and E). Sixty hours later cells were stained with Hoechst 33342 (A–D) or TUNEL staining (E–H) followed by fluorescent microscopic observation. Original magnification ×100.

Changes in AI score after ASK1ΔN gene transfer. The AI score was elevated dose-dependently in all CIA rat groups in which the ASK1ΔN gene had been infected, while normal DA rats showed no significant changes in AI score regardless of the doses of ASK1ΔN adenovirus (Figure 5). In Group A, the AI score increased significantly to 2.8 ± 0.8 on Day 2, followed by reduction to 1.3 ± 1.0 on Day 3. The score increased again from Day 4 and reached a peak of 4.0 ± 0.0 on Day 6 ($n = 4$; $p < 0.05$). In groups B and C, the AI scores were continuously elevated. Therefore, *in vivo* transduction of the ASK1ΔN gene promoted arthritis in terms of the AI score. Normal joints were not affected by *in vivo* administration of the ASK1 gene.

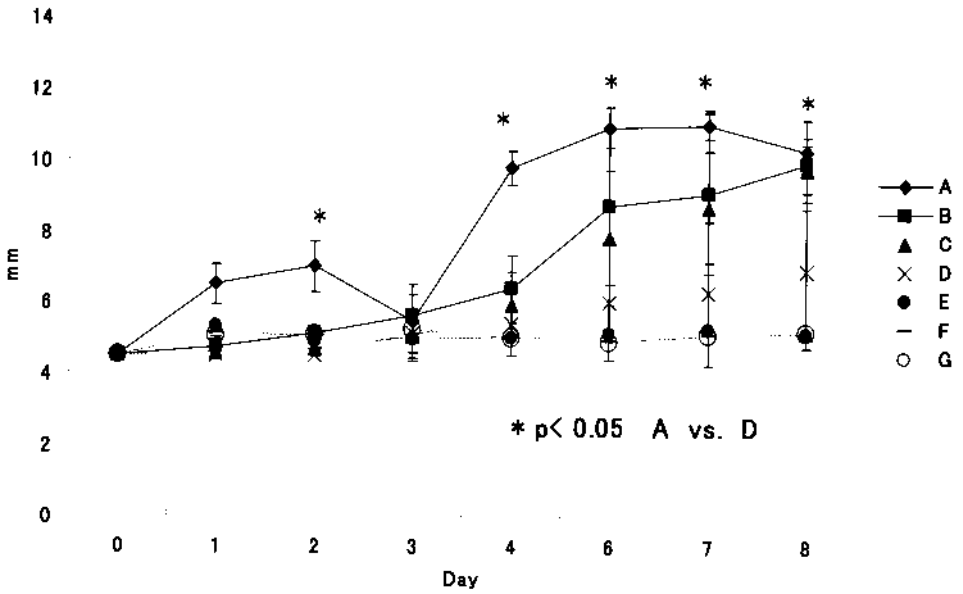

Figure 4. ASK1 gene transduction led to deteriorated paw swelling in CIA rats (groups A–C) and normal DA rats (E–G). Rats were given ankle joint injection of a mixture of AxCALNL-ASK1ΔN (7.6×10^{-5} plaque-forming units, pfu) and AxCANCre (7.6×10^{-4} pfu) (A and E), a mixture of AxCALNL-ASK1ΔN (7.6×10^{-4} pfu) and AxCANCre (7.6×10^{-3} pfu) (B and F), or a mixture of AxCALNL-ASK1ΔN (7.6×10^{-3} pfu) and AxCANCre (7.6×10^{-2} pfu) (C and G). As a control, CIA rats were intraarticularly injected with PBS (D). Means ± SD of paw diameters are plotted here (n = 6 in each group).

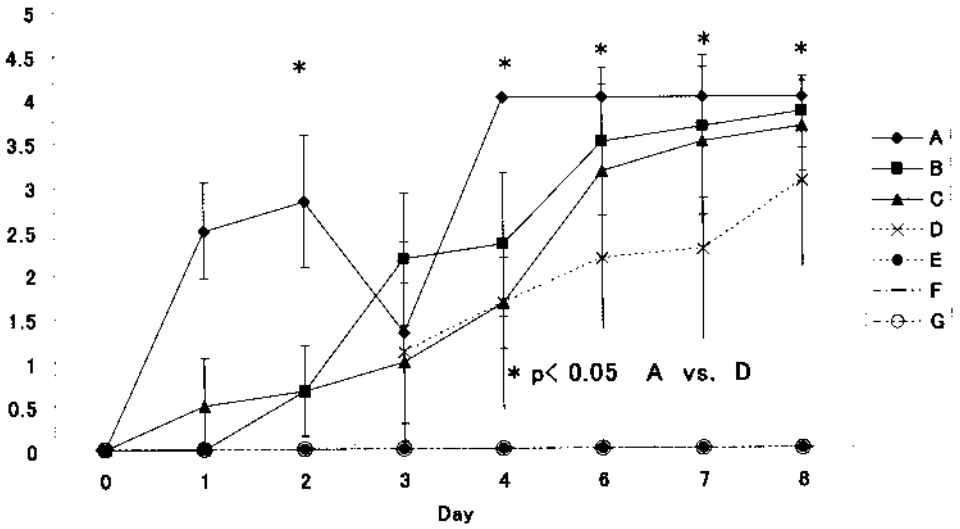

Figure 5. CIA progression was significantly promoted by ASK1 gene transfer. CIA rats (groups A–C) and normal DA rats (E–G) were injected in the ankle joint with a mixture of AxCALNL-ASK1ΔN (7.6×10^{-5} pfu) and AxCANCre as shown in Figure 4. As a control, CIA rats were given PBS intraarticularly (D). Means ± SD of AI scores are plotted here (n = 6 in each group).

Histopathological findings. Histological examination revealed that the ASK1 gene-transduced CIA rats showed remarkable infiltration of lymphocytes and monocyte lineage cells as well as infiltration of inflammatory cells into the synovial membrane, while such morphological changes were not evident in the normal DA rats infected with ASK1ΔN adenovirus, or in the control CIA rats given an administration of PBS (Figure 6). In groups A, B, and C, proliferation of synovial fibroblasts was observed, but degradation of the synovial membrane due to apoptosis was not observed. Pannus formation and destruction of bone and cartilage were also apparent in these groups.

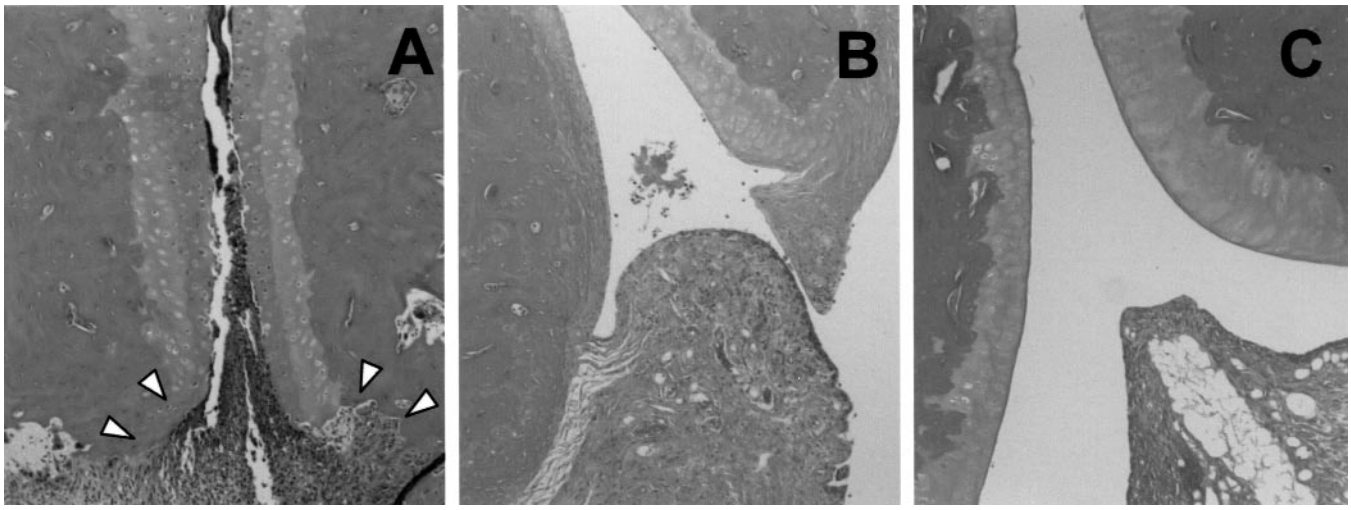

Figure 6. Remarkable synovial inflammation in ASK1 gene-transduced CIA rats (A, white arrowheads). CIA (A and B) or DA (C) rats were injected into the ankle joint with a mixture of AxCALNL-ASK1ΔN (7.6×10^{-5} pfu) and AxCANCre (7.6×10^{-4} pfu) (A and C) or PBS (B). Ten days later articular tissue sections were stained with H&E. Original magnification ×40.

DISCUSSION

In normal tissues, homeostasis is maintained by cell differentiation, proliferation, and apoptosis, while in pathological conditions such as RA, insufficient apoptosis might contribute to the hyperplasia of synovial tissue. This process involves some pathways that inhibit apoptosis[10,21]. There are 2 major apoptotic pathways. In the first, ligand-dependent trimerization of cell-surface death receptors, such as FAS and TNF receptors, induces polymerization of FADD and TRADD, which subsequently activates caspase-8, followed by activation of caspase-3 and apoptosis induction. In the second, various stimuli including stresses induce cytochrome C release from the mitochondria. The cytochrome C binds to Apaf1 and caspase-9 to form a complex, which activates caspase-3 to induce apoptosis[22,23]. Recently, intraarticular gene transfer approaches have been developed[24,25], and several studies have attempted to develop therapeutic methods for RA by inducing apoptosis in the synovial membrane through activation of the FAS/TNF pathways by introducing genes for the Fas ligand, FADD, and TRAIL[26-28]. However, no studies have reported on the application of apoptosis induction through the mitochondrial pathway to RA therapy. In our study, using adenovirus vectors[5-9,29] that enabled the introduction of genes into the synovial membrane, the ASK1ΔN gene was introduced into the synovial membrane, and whether apoptosis could be induced through activation of the mitochondria pathway was examined *in vitro* and *in vivo*.

ASK1 is a serine/threonine kinase with a molecular weight of 160 kDa that belongs to the MAPKKK family. It was originally cloned in 1997 by Ichijo, *et al*[14]. ASK1 has been identified in a wide range of tissues and cells, and ASK1 mRNA expression has also been confirmed in RA synovial tissues. In addition to Fas receptor stimulation through the Fas ligand and cytokine receptor stimulation through TNF-α, IL-1, and other agents, ASK1 is also activated by physicochemical stresses such as reactive oxygen, serum deprivation, and anticancer agents. When activated, ASK1 is overexpressed in the cells, the MKK4/MKK7-JNK and MKK3/6-P38 pathways are activated at the same time, and apoptosis is induced in various cells[30]. On the other hand, when the inactive form of ASK1 is overexpressed in the cells, activation of the JNK and p38 pathways by stimulation and apoptosis is partially suppressed[27]. The mechanism of apoptosis induction by ASK1 is considered to be mainly through the mitochondria-dependent signaling pathway, since overexpression of ASK1 caused the release of cytochrome C from mitochondria and activation of caspase-3 and caspase-9[31]. In this study, the ASK1 gene was initially introduced into cultured human RA synoviocytes *in vitro*. The cells into which the ASK gene was introduced showed a significantly lower absorbance in the MTT assay than the control cells, and the induction of apoptotic cells was clearly observed by Hoechst and TUNEL staining. These results show that apoptosis can be induced by introduction of the ASK1 gene into synoviocytes.

However, in the *in vivo* study, introduction of ASK1ΔN gene into the ankle joint of CIA rats enhanced the joint swelling and AI scores dose-dependently. Histologically, inflammatory cells, including lymphocytes and monocyte-lineage cells, and synovial fibroblasts were increased in the synovial membrane. Apoptosis was not detected in the synovial membrane. Pannus formation was observed along with destruction of bone and cartilage as the inflammation progressed. In contrast, when the ASK1ΔN gene was similarly introduced into the ankle joints of control rats, inflammation

or joint swelling was not caused. Direct intraarticular administration of adenovirus vectors can lead to inflammation or hyperplasia of the synovial membrane depending on the concentration or dose of the vectors and the animal species[32,33]. However, since the control rats did not develop inflammatory reactions in this study, the cause of the enhanced inflammation in the CIA rats is considered to be the effect of ASK1 on the synovial membrane, rather than the administration of the adenovirus vectors.

It was recently reported that ASK1, having the ability to induce apoptosis, also plays an important role in cell differentiation and survival. In human epithelial primary cultures, strong expression of ASK1 induced apoptosis, while weak expression of ASK1 induced differentiation through p38 activation[34]. Overexpression of ASK1 induced apoptosis mainly through the JNK pathway under strong stress stimulations, such as serum deprivation, in primary cultures of nerve cells and a differentiated pheochromocytoma cell line (PC12), while it induced differentiation and survival of nerve cells, such as enhancing neurite elongation, mainly through the p38 pathway in undifferentiated PC12 cells[35]. In addition, ASK1 enhanced hyperplasia of the vascular endothelium and proliferation of cultured vascular smooth cells in rat damaged vascular models through the p38 or JNK pathways[36]. It is considered that ASK1 plays a role in the maintenance of the homeostatic balance in the body by finely adapting to the strength and quality of various stress stimulations as well as to the type of cell, and by regulating signals in the JNK and p38 pathways.

JNK and p38, which are located downstream of the ASK1 transduction pathway, play contradictory roles in both survival signaling and apoptosis signaling. Miyazawa, *et al*[37] and Han, *et al*[38] reported that JNK and p38 are activated in the RA synovial membrane and promote the production of inflammatory cytokines and matrix metalloproteinase (MMP), leading to induction of proliferative synovitis. However, Okamoto, *et al*[39] reported that JNK is activated in the FAS mediated apoptosis induction pathway, and contributes to the induction of apoptosis in the synovial membrane. JNK and p38 also have contradictory functions in RA depending on the situation. In our study, *in vivo* introduction of the ASK1ΔN gene induced apoptosis in cultured synoviocytes. However, although *in vivo* introduction of the ASK1ΔN gene into normal joints did not cause inflammation, it induced cell proliferation and inflammation in CIA rats, in contrast to the effect expected from the results of the *in vitro* study. In the CIA model, in which the immune system is activated by adjuvant arthritis, ASK1 may activate JNK and p38 in synovial fibroblasts, lymphocytes, and monocytes, and work as a potent molecule for inducing inflammation, but not apoptosis.

Future analyses of the involvement of ASK1 in the switching of JNK and p38 may lead to elucidation of the mechanism of RA.

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