椎弓切除纖維化之實驗研究 - 探討纖維化引起神經傳導之變化即以承載脂肪細胞之植入物防止纖維化之效果

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-006-030
執行期間：92年08月01日至93年07月31日
執行單位：國立成功大學醫學系骨科

計畫主持人：周一鳴

報告類型：精簡報告
處理方式：本計畫可公開查詢

中華民國93年6月4日
Thrombospondin-1 as arthritis therapy

Thrombospondin-1 as an Effective Gene Therapeutic Strategy
in Collagen-Induced Arthritis

I-Ming Jou, MD, PhD,1* Ai-Li Shiau, PhD,2* Sheu-Yao Chen, MS,3 Ching-Shan Tsai, MS,2
Chrong-Reen Wang, MD,4 Dar-Bin Shieh, DDS,5 and Chao-Liang Wu, PhD3

Departments of 1Orthopedics; 2Microbiology and Immunology; 3Biochemistry, Section of Rheumatology; 4Internal Medicine; and 5Dentistry; Medical College, National Cheng Kung University, Tainan, Taiwan

*Both authors contributed equally to this work.

Address correspondence and reprint requests to: Chao-Liang Wu, Department of Biochemistry, Medical College, National Cheng Kung University, 1 Dahsueh Road, Tainan 701, Taiwan. E-mail: wumolbio@mail.ncku.edu.tw

Tel: 886-6-235-3535 ext. 5536; Fax: 886-6-274-1694; E-mail: wumolbio@mail.ncku.edu.tw

Financial support: This work was supported by grants NSC 91-2320-B-006-085 and NSC 92-2320-B-006-095 to C.L.W. from the National Science Council of Taiwan, and by the Jieh Chen Scholarship, The Dr Chen Foundation, Tainan, Taiwan.
Abstract

Objective. Angiogenesis is a key process in the pathogenesis of rheumatoid arthritis, a chronic inflammatory joint disease. Because thrombospondin (TSP)-1 inhibits angiogenesis and activates transforming growth factor (TGF)-β, a potent immunosuppressive and anti-inflammatory cytokine, we investigated the therapeutic effects of TSP-1 mediated by adenoviral gene transfer in the collagen-induced arthritis (CIA) model in rats.

Methods. Adenoviral vectors encoding mouse TSP-1 (Ad-TSP-1) or β-galactosidase (Ad-LacZ) were administered by intraarticular injection into CIA rats using various treatment regimes. Clinical parameters, including articular index (AI) score, hindpaw swelling, and ankle width, were assessed in the treated rats. The treated ankles were also removed for radiographic and histological evaluations. Expressions of TSP-1, TGF-β, vascular endothelial growth factor (VEGF), and interleukin-1β (IL-1β) in the synovial tissues were also examined.

Results. Intraarticular TSP-1 gene therapy reduced the severity of CIA in clinical, radiological, and histological aspects. The synovial tissues from Ad-TSP-1-treated ankles revealed considerably fewer blood vessels than those from Ad-LacZ-treated ankles. Expression of TSP-1 and TGF-β, concomitant with decreased lymphocyte infiltration, were increased, whereas production of VEGF and IL-1β were reduced in the synovial tissues from Ad-TSP-1-treated ankles.

Conclusion. Direct intraarticular administration of adenoviral vectors encoding TSP-1...
significantly suppressed clinical inflammatory presentations in CIA, accompanied by reduction of synovial hypertrophy and blood vessels. The antiangiogenic and anti-inflammatory activities of TSP-1 may account for its therapeutic effect on CIA. Taken together, these results suggest that TSP-1 gene therapy may have therapeutic potential for the treatment of rheumatoid arthritis.
Introduction

Rheumatoid arthritis (RA) is not consistently treated with success despite extensive therapeutic regimens now available (1). This suggests that fundamental questions about the pathomechanism of RA remain unanswered. Although there is no generally accepted mechanism of RA, many investigators report that neovascularization is one of the earliest histopathologic findings in RA and that it appears to be necessary for pannus development (2,3). It is consequently hypothesized that suppression of blood vessel growth may ameliorate arthritis by diminishing the delivery of required blood-borne elements to the pannus, and by endothelial-cell production of chemokines and cytokines, thereby suppressing chemokine- and cytokine-induced inflammation. This hypothesis is supported by experimental data showing that vascular endothelial growth factor (VEGF) is expressed during the development of collagen-induced arthritis (CIA) and that the synovitis of CIA can be attenuated by passive immunization with a neutralizing anti-VEGF antibody (4); this result supports the hypothesis that the expression of specific angiogenic factors during the development of CIA is a key pathologic component mediating neovascularization in the pannus and the surrounding joint tissue. Based on these findings, numerous therapeutic approaches targeting angiogenesis, including conventional drugs or local gene-delivery systems that inhibit neovascularization, have been introduced and have shown their beneficial effects in treating or preventing CIA (5-11).
Thrombospondin-1 (TSP-1) is an endogenous inhibitor of angiogenesis and tumor growth. This large multimodular protein influences cellular phenotypes and the structure of the extracellular matrix, both of which affect tissue remodeling associated with angiogenesis and tumorigenesis. In addition to its well-documented antiangiogenic effect and related physiological effects, TSP-1 may have an anti-inflammatory property because TSP-1 upregulates members of the TGF-β, which plays an important role in the progression of RA (12,13). These characteristics prompted us to investigate the preventive and protective effects of TSP-1 in a rat CIA model.

In the present study, we generated the replication-defective adenoviral vector Ad-TSP-1 by encoding mouse TSP-1 under the transcriptional control of the CMV promoter, and we tested its effect on CIA in rats. Our results show that transfer of TSP-1 to the synovial lining of the ankle joints of CIA rats by intraarticular injection of Ad-TSP-1 resulted in the efficient local expression of TSP-1 in synovial tissue and the efficacious inhibition of arthritic deterioration in the joints.
Materials and methods

Cell lines, mice, and rats

The MBT-2 murine transitional cell carcinoma cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 µg/ml gentamicin. Inbred six- to eight-week-old female C3H/HeN mice and adult male Sprague-Dawley rats, 400-550 g at eight weeks of age, were obtained from the laboratory-animal center of our medical college. Both local and international guidelines for the humane treatment of laboratory animals were followed.

Construction of adenoviral vectors

Replication-defective adenoviral vectors based on E1/E3-deleted adenovirus type 5 were generated. The 3.7-kb HindIII-NotI fragment containing the coding region of the murine TSP-1 gene was cloned into the adenovirus shuttle vector pAd5L at the HindIII/NotI sites. The resulting plasmid was co-transfected with pJM17 into 293 cells to generate Ad-TSP-1 (14). Ad-K1-5 and Ad-LacZ serving as the control vector, that encoded kringles 1-5 of mouse plasminogen and β-galactosidase, respectively, have been described previously (15).

Treatment of tumor-bearing mice with adenoviral vectors

Groups of 6-7 C3H/HeN mice were inoculated subcutaneously with MBT-2 tumor cells (10^6) on day 0, and treated intratumorally with 4 × 10^6 and 8 × 10^6 plaque-forming units (PFU) of Ad-TSP-1, Ad-K1-5, or Ad-LacZ on days 13 and 14, respectively. Tumor growth
and survival of the mice were documented by 60-day follow-ups. Tumor measurements were performed in two perpendicular axes with calipers every 3-4 days, and volumes were calculated as $\text{length} \times \text{width}^2 \times 0.45$. The mean tumor volumes were calculated only when mice within the same treatment group were all alive. All mice were sacrificed by intraperitoneal overdose of sodium pentobarbital (Nembutal; 50 mg/kg; intraperitoneally; Abbott, North Chicago, IL) at the end of the experiments. Tumor tissues were fixed in phosphate-buffered 4% formaldehyde solution and embedded in paraffin. Five-µ thick sections were prepared for histological staining with hematoxylin and eosin (H & E).

**Induction and treatment of CIA**

To induce experimental arthritis, rats were immunized, by two dorsal intradermal injections (400 µg and 100 µg, respectively) one week apart, of bovine type II collagen (Elastin Products, Owensville, MO) emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI). Different treatment regimes with Ad-TSP-1 or Ad-LacZ serving as the control were used for the treatment of CIA. Rats were injected intraarticulary with $5 \times 10^7$ PFU of viral vectors in the right and left ankle joints one or three times on different days after immunization with collagen.

**Clinical assessments**

Clinical parameters measured three times per week included body weight, hindpaw
swelling, and ankle width (mediolateral diameter) by an investigator blinded to the treatment of the ankles. Hindpaw swelling was graded from 0 to 4 (0 = no edema, 1 = slight edema of the digit joints and foot pad, 3 = gross edema of the entire foot pad below the ankle, and 4 = gross edema of the entire foot pad including the ankle) as described (16). The width of the ankles was measured with a micrometer (Presto, USA).

**Radiographic evaluation**

The ankles and feet of the animals were anesthetized and then X-rayed after treatment with adenoviral vectors. Radiographs were scored from 0 to 4 using Clark's method (17): 0 = no involvement, and 4 = extensive involvement based on bone mineralization, erosion, periostitis, cartilage space, soft tissues, alignment, and associated degenerative changes. An orthopedic surgeon blind to the treatment evaluated the radiographs.

**Histological assessment**

After final radiological examination, the hind paws were removed from the rats after a lethal dose of anesthetic. The ankles (joint specimens) designated for sectioning were skinned, decalcified in decalcifying solution, embedded in OCT, and then frozen at –80°C. A blade suitable for bone cutting was used to generate 5-µm sections, which were then stained with H & E. Two sections of each specimen were read for pannus formation and cartilage depletion, and scored arbitrarily as 0 when no pannus formed or there was normal cartilage in the joint space, or 1-3 according to the degree (1 = mild, 2 = moderate, and 3 = severe) of pannus
formation or depletion of cartilage (18).

**Immunohistochemical analysis**

Cryostatic sections (5 µm) of the synovial tissues taken from the ankle joints were prepared and incubated with polyclonal goat anti-human TSP-1 peptide (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-human TGF-β1 peptide (sc-146-G; Santa Cruz), known to be reactive with rat counterparts, or rabbit anti-factor VIII (von Willebrand's factor; DAKO Corporation, Carpinteria, CA). After sequential incubation with peroxidase-labeled donkey anti-goat IgG (Santa Cruz) or goat anti-rabbit IgG (Santa Cruz), and aminoethyl carbazole (AEC) as substrate chromogen, the slides were counterstained with hematoxylin. Stained blood vessels within the synovial tissues were counted in three blindly chosen random fields at ×100 magnification.

**ELISA**

Synovial tissues taken from ankle joints were snap frozen in liquid nitrogen, and then homogenized. One milliliter of PBS containing protease inhibitor (Halt Protease Inhibitor Cocktail; Pierce Biotechnology, Inc., Rockford, IL) was added to the homogenate, and the tissue solution was centrifuged at 2000 × g for 10 min at 4°C to remove insoluble debris. After filtration through a 0.45-µm membrane, the protein content of the lysate was measured by BCA assay (Pierce). The levels of VEGF and interleukin-1β (IL-1β) in the lysate were quantified by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's
Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Rat ankles were treated independently for statistical purposes, and comparisons were made between Ad-TSP-1- and Ad-LacZ-treated groups. Differences between Ad-TSP-1 and Ad-LacZ groups were compared by Student's t-test. $P$ values less than 0.05 were considered significant.
Results

Characterization of Ad-TSP-1 in vitro and its effect on tumor growth in vivo

The expression of TSP-1 protein was verified in Ad-TSP-1-transduced A549 lung cancer cells by immunohistochemical staining (data not shown). The expressed TSP-1 mediated by Ad-TSP-1 gene transfer displayed antiangiogenic activity, as determined by inhibition of the proliferation and migration of HMEC-1 endothelial cells as well as inhibition of angiogenesis in the chorioallantoic membrane assay. As expected, adenovirus-mediated TSP-1 expression did not alter the in vitro growth of MBT-2 bladder tumor cells (data not shown). We have shown previously that in vivo Ad-K1-5 exerts an anti-tumor effect that is mediated by its antiangiogenic activity (15). As Ad-TSP-1, like Ad-K1-5, exhibited an antiangiogenic effect in vitro, we next tested whether Ad-TSP-1 also possessed anti-tumor activity on established MBT-2 bladder tumors in mice. Bladder tumor-bearing mice treated with Ad-K1-5 exhibited smaller tumor size and longer survival time than those treated with Ad-LacZ, but no therapeutic effect was observed in those treated with Ad-TSP-1 (Figures 1A and B). On day 31, when all the mice were still alive, the mean tumor volumes of mice treated with Ad-K1-5, Ad-TSP-1, and Ad-LacZ were 308 ± 179 mm³, 869 ± 856 mm³, and 989 ± 732 mm³, respectively. Furthermore, on day 33, when Ad-LacZ-treated mice began to die of a large tumor burden, all the Ad-K1-5-treated mice were alive. In contrast, tumors grew progressively in Ad-TSP-1-treated mice, and their survival was even worse than those treated
with the control Ad-LacZ vector (Figure 1B). By microscopic examination, MBT-2 tumors injected with Ad-LacZ (Figures 1C and D) appeared much more vascularized than those injected with Ad-K1-5 (Figures 1E and F) or Ad-TSP-1 (Figures 1G and H). In addition, TGF-β was detected by immunohistochemical analysis in tumors treated with Ad-TSP-1, but not in those treated with Ad-K1-5 (data not shown). Taken together, these results indicate that, under our treatment regime, although both Ad-TSP-1 and Ad-K1-5 inhibited tumor angiogenesis in vivo, Ad-TSP-1 treatment had no therapeutic efficacy in bladder-tumor-bearing mice and may even have worsened the disease. Because TSP-1 is a major activator of TGF-β in vivo, and TGF-β has pleiotropic effects on cell growth, differentiation, and immune modulation, TGF-β detected in the tumors from Ad-TSP-1-treated mice may be attributable, in part, to the failure of Ad-TSP-1 to inhibit tumor growth.

**TSP-1 gene transfer reduced the incidence and severity of CIA**

Although TGF-β induced in the tumors by Ad-TSP-1 treatment may result in immunosuppression in the tumor microenvironment and counteract the antiangiogenic effect of TSP-1, thereby enhancing tumor growth, TGF-β has an anti-inflammatory effect that may be beneficial for the treatment of arthritis. Therefore, we next examined whether Ad-TSP-1 gene therapy was effective in the treatment of CIA through its direct antiangiogenic activity and indirect anti-inflammatory effects mediated by TGF-β.
Direct intraarticular injection of adenoviral vectors led to transgene expression in lining synovial cells, as judged by X-gal staining 4 days following injection with Ad-LacZ ($5 \times 10^7$ PFU) (data not shown). Detection of TSP-1 protein in the synovial tissues following administration of Ad-TSP-1 confirmed the transduction of cells associated with synovial tissues. Trace expression of TSP-1 was also present in the Ad-LacZ injection side because of the endogenous TSP-1 of the normal tissue. These data, therefore, suggest no systemic leakage of adenoviral vectors following intraarticular injection. To obtain more direct evidence that TSP-1-induced antiangiogenic or anti-inflammatory effects, or both, could prevent the deterioration of CIA, Ad-TSP-1 ($5 \times 10^7$ PFU) was injected into the right ankle joints of rats two weeks following successful induction of CIA. An equal dose of Ad-LacZ was injected into the left ankle joints as a control. The hindpaw swelling resolved 5 days after local treatment with Ad-TSP-1, but not with Ad-LacZ (Figures 2A and B). Higher levels of TSP-1 expression were detected in the synovial tissue from Ad-TSP-1-treated ankle joints (Figure 2C) than in those from Ad-LacZ-treated counterparts (Figure 2D). To monitor the histopathologic response of TSP-1 gene therapy on CIA, ankle joint sections stained with H & E were analyzed. The joint sections from CIA rats exhibited severe hyperplastic synovial tissues with multi-foci pannus formation, and destruction of cartilage compared to normal rats (data not shown). Reductions of hyperplastic synovial tissues, pannus formation, and mononuclear cell infiltration were seen in Ad-TSP-1-treated ankles (Figure 2E) compared to
Ad-LacZ-treated ankles (Figure 2F). These results demonstrate the therapeutic effect of Ad-TSP-1 on CIA rats.

We next conducted another experiment involving a larger number of animals and 2 doses of adenoviral vectors to address the ameliorative effect of Ad-TSP-1 gene transfer on CIA rats. Rats were immunized and boosted with Freund's incomplete adjuvant on days 0 and 7, respectively, followed by intraarticular treatment with $5 \times 10^7$ PFU of Ad-TSP-1 or Ad-LacZ into the ankle joints on days 7 and 10, respectively.

(i) Clinical response. The mean and the difference in paw swelling (Figure 3A) and ankle circumference (Figure 3B) of Ad-TSP-1-treated (right) and Ad-LacZ-treated (left) ankles 15 days after the induction of CIA are shown. There was a trend in the data showing that Ad-TSP-1 was capable of reducing the width and the swelling of the paw compared with the control vector. TSP-1 gene transfer resulted in a statistically significant reduction of joint inflammation (ankle width and swelling) 8 days following treatment.

(ii) Radiological response. Eight days after gene transfer, plain X-rays of the ankle joint injected with Ad-TSP-1 revealed fewer radiological features of severe joint destruction (Figure 4A) than the control side (Figure 4B). The Ad-TSP-1-injected side had a significantly smaller mean global score than did the Ad-LacZ-treated control side ($p < 0.001$; Figure 4C) according to the global radiological scores (maximum 8).
(iii) Immunohistopathological response of angiogenesis. Because microvascular density is a hallmark for angiogenesis, we investigated the vascularity of the synovial tissues by immunohistochemical staining to evaluate the effect of Ad-TSP-1 on angiogenesis. Synovial tissues from the experimental ankles revealed considerably fewer blood vessels than those from the control ankles (Figure 4C). To evaluate the biochemical changes in the synovial tissues, the levels of VEGF and IL-1β were measured by ELISA. Expressions of VEGF and IL-1β in synovial tissues were significantly decreased in the Ad-TSP-1-treated tissues compared to the Ad-LacZ-treated tissues (Figure 5A). Moreover, immunohistochemical staining demonstrated reduced arthritis-associated vascularity in Ad-TSP-1-treated synovium (Figure 5B) compared to its Ad-LacZ-treated counterpart (Figure 5C). In contrast to the synovial tissue from Ad-LacZ-treated ankles (Figure 5D), TGF-β expression, concomitant with decreased lymphocyte infiltration, was seen in the synovial tissue from Ad-TSP-1-treated ankles (Figure 5E), which may be attributed to the anti-inflammatory activity of TGF-β induced by TSP-1 expression. Consistent with clinical, radiological, and histopathological scores, a significant decrease in vascular numbers was observed in the experimental ankle joints treated with Ad-TSP-1, compared to the control ankle joints treated with Ad-LacZ. Histomorphometric and
quantitative analysis confirmed the hypothesis that TSP-1 gene transfer inhibits CIA-induced angiogenesis and inflammation, preventing the deterioration of CIA.

To estimate the therapeutic effects of late administration of Ad-TSP-1 on CIA, another treatment regime that began after the onset of CIA was performed. Similarly, rats were immunized twice within a 7-day interval and were administered intraarticularly with $5 \times 10^7$ PFU of Ad-TSP1 three times 14, 17, and 20 days after the first immunization in swollen ankle joints shown in the early stage of RA. The widths of ankle joints treated with Ad-TSP-1 and the control joints treated with Ad-LacZ of four CIA rats were compared. Eight days after the first treatment with adenoviral vectors, there was a significant difference between the width of the ankle joints treated with Ad-TSP-1 and those treated with Ad-LacZ ($p < 0.01$). Plain radiographs showed that joint-space reduction, soft-tissue swelling, and bony erosion were serious in Ad-LacZ-treated joints but absent in Ad-TSP-1-treated joints (data not shown). Notably, recovery of its gait pattern following TSP-1 gene therapy allowed the CIA rat to stand on its right hind limb (Figure 6A). Radiography shows the difference between the two hind limbs (Figure 6B), which confirmed symptoms displayed in the left hind limb (Figure 6C).
Discussion

RA is a syndrome characterized by severe inflammatory hyperplasia in the synovium with subintimal infiltration of T and B lymphocytes and other inflammatory cells of the multiple joints, that leads ultimately to the destruction of the affected joint (19,20). The effect of treatment is always disappointing when the eventual joint destruction contributes to a complex cellular interaction and the production of a variety of humoral factors occurs (1). The occurrence of these phenomena has been confirmed in murine models in which autoimmune models of inflammatory synovitis were induced. Because angiogenesis is one of the earliest histopathologic findings and appears to be necessary for pannus formation that irreversibly destroys cartilage and bone in the affected joint (2,3,21,22), development of effective interventions to suppress neoangiogenesis, either through conventional drugs or a local gene delivery system, is assumed to be a promising alternative to prevent the deterioration caused by RA. In the present study, we demonstrated the effectiveness of intraarticular administration of adenoviral vector expressing TSP-1 in reducing the severity of CIA, thus suggesting that TSP-1 gene transfer may be a novel and feasible means for the treatment of RA.

First, our \textit{in vitro} study demonstrates that the proliferation and migration of endothelial cells were inhibited by Ad-TSP-1. Furthermore, neovascularization in chick embryos was inhibited and tumor vascularization was reduced by Ad-TSP-1 treatment. Although
Ad-TSP-1 had no therapeutic effect in our bladder tumor model, which may have been due to TGF-β induction by TSP-1 in the tumor microenvironment, these findings suggest that TSP-1 may suppress neovascularization and that TGF-β induced by TSP-1 may suppress inflammation in RA, which can be exploited in the CIA model. In the work described here, successful transfection of the TSP-1 gene to the synovial tissue was verified immunohistochemically, and the expression of the gene was effective in controlling the progression of CIA manifestations. This was demonstrated by significantly lower clinical, radiological, and histological grades that reflected less inflammation and destruction of the CIA joints. Reductions in vascularity and immunoreactivity to factor VIII also indicate that neovascularization in CIA was suppressed by Ad-TSP-1 treatment.

Gene therapy for RA has been widely investigated during the past few years. Several strategies for disrupting the immuno-related abnormality network of joint inflammation and destruction in RA animal models have been reported (5,6,23-30,31-36). There are two main feasible strategies for inhibiting the development of RA. One strategy uses different techniques for modulating the balance between proinflammatory and anti-inflammatory cytokines to inhibit the induction of immune responses in RA. Proinflammatory cytokines, including IL-1, IL-2, IL-6, IL-12, IL-16, IL-17, tumor necrosis factor-α, and interferon-γ, are the reported mediators for the development of human RA and experimental RA models, and considerable interest has been shown in possible utilization of gene transfer to neutralize
Thrombospondin-1 as arthritis therapy

those cytokines for the amelioration of CIA and other models of RA (26,32,36). On the other hand, increasing the levels of anti-inflammatory cytokines by administration of recombinant IL-4, IL-10, and IL-13 genes is also effective in preventing the progression of CIA and other experimental autoimmune arthritis models (24,27,29-31,33-35). Another type of immunotherapy for RA utilizes various gene transfers to inhibit neoangiogenesis and block synovial hypertrophy in RA. Many factors or molecules related to angiogenesis in RA, such as IL-8 (a potent anti-angiogenesis cytokine), basic fibroblast growth factor (bFGF), VEGF, vascular cell adhesion molecules, E-selectin, and soluble adhesion molecules, are enriched in both the synovial tissue and synovial fluid of RA patients (4,37). Furthermore, antagonists or blockers of the aforementioned molecules also prevent angiogenesis after CIA in rats and mice (7-11). Invasive pumping devices, however, were needed to maintain adequate concentration of the molecules within the joints. Antiangiogenic genes, in contrast, may be able to maintain a relatively long-term effect with only one administration, which is assumed to be effective to prevent the development of RA. Recently, administration of the angiostatin gene or urokinase plasminogen inhibitor gene directly inhibited plasminogen activation on the cell surface and restrained endothelial migration with resultant protection of the local degradation of stroma and suppression of neovascularization (5,6). TSP-1 is one of the first endogenous inhibitors of angiogenesis discovered. This large matricellular glycoprotein is produced by stromal fibroblasts, endothelial cells, and immune cells. TSP-1 is effective in
Thrombospondin-1 as arthritis therapy

inhibiting angiogenesis through direct effects on endothelial cell proliferation, migration, and morphogenic organization to capillary tubes, and through indirect effects on growth factor mobilization (12,13). The antiangiogenic effect of TSP-1 is not the only mechanism with the potential to prevent RA; TSP-1 also acts to suppress inflammation through activation of TGF-β (13). TGF-β effectively lowers joint inflammation in already-established arthritis and inhibits the spread of the disease to other joints (38). Cells engineered to express TGF-β can prevent or ameliorate autoimmune disease process (24,39). These antiangiogenic, antimetastatic, and anti-inflammatory properties of TSP-1 merit additional investigation of its possible therapeutic potential in RA. Our use of Ad-TSP-1 is a novel approach to treating RA in an animal model. After the intraarticular injection of Ad-TSP-1, TSP-1 was detected in the synovial tissue of the treated joint and the progression of CIA was inhibited. Furthermore, significantly increased TGF-β production and decreased IL-1β expression were also observed in the synovial tissue from Ad-TSP-1 treated ankle joints, which suggests that anti-inflammatory effect was induced by TSP-1. Our data indicated that Ad-TSP-1 not only controlled pronounced inflammation and vascularization and suppressed synovial hypertrophy in the involved joint, but that it also had a strong protective effect on the cartilage and bone within the joint following the induction of CIA. It is likely that the effects of intraarticular injection of Ad-TSP-1 can inhibit the neoangiogenesis of developing inflammation and, furthermore, can suppress the established pannus. These positive findings
contributed to the efficacy of the local expression of the TSP-1 gene's inhibiting CIA by persistent secretion of TSP-1, which exerted antiangiogenic and anti-inflammatory effects. It is noteworthy that the antiangiogenic effect of transfected Ad-TSP-1 was also sufficiently potent to suppress angiogenesis in established pannus. In antitumor experiments (40), exogenous TSP-1 inhibited angiogenesis in well-vascularized, rapidly growing primary tumors via the same mechanism that prevented neovascularization of dormant micrometastases, i.e., inducing apoptosis in microvascular endothelial cells.

The issues of the efficacy and safety of a viral-vector delivery system are of major concern in the development of a clinically useful gene therapy for RA. Two recent reports showed that retroviral vector transfections of angiostatin (5) and IL-4 (24) increased the endogenous responding cytokine and ameliorated the disease; many other reports seem to have established proof of the effectiveness of adenoviral transfection: they used the adeno-associated viral vectors encoded with IL-1 receptor antagonist (26), urokinase plasminogen inhibitor (6), CTLA4IgG (28), IL-4 (5,29,30), IL-10 (31), and interleukin-13 (27) to inhibit the development of RA in animal models. The use of adenoviral vector is assumed safe in human populations, because the overwhelming majority of individuals have been exposed to adenovirus without any known pathological effects (41). The inflammation and immune reactivity caused by viral-vector transfection pose another potential risk. This reactivity was not investigated in the present study; another study (10) however, showed that
Thrombospondin-1 as arthritis therapy

Adeno-associated virus (AAV) vector-induced immunity was modest and indicated that a prechallenge did not affect AAV vector persistence in vivo. It also showed that persistent transgene expression was detectable in the synovial tissue after two weeks, which coincided with subsidence of the disease. Furthermore, local gene expression in injected joints has inhibited arthritis in the joint adjacent to it (28,31,32,35). Our study results were compatible with these.

Sustained transgene expression by cells associated with synovial tissue can be obtained by intraarticular or systemic injection of adenoviral or retroviral vectors. Although no adverse effect has been reported by using systemic gene therapy for RA, intravenous administration of viral vectors leading to substantial destructive inflammation and activation of innate immunity in the early elimination of the vectors has been observed (28). Local intraarticular injections, however, ensure gene delivery to the joint cavity and possibly eliminate unnecessary systemic effects. Another advantage of intraarticular injection of viral vectors is direct transfection of the synovial tissue. Targeting synovial tissue is assumed to be more efficient than routing the vector through other tissues because synovial tissue has a large area, high cellularity, and close contact with the cartilage and joint space (28,33,35). We selected a local gene therapy strategy to avoid systemic complications and to treat inflamed joints of RA rats for comparison with untreated inflamed joints from the same or different animals.

Although intraarticular injection of adenovirus vector induces inflammatory reactions in
murine joints (6), we did not observe any such side effect, which may have been due to TGF-β induction by TSP-1. In this study, intraarticular injection of adenoviral vectors led to transgene expression by cells associated with synovial tissue, and the possible appearance of secretable transgene products within the joints. Our data do not discern whether circulating TSP-1 or TSP-1 produced locally within the joint was more important in preventing CIA.

This is the first report to exploit TSP-1 gene therapy as an effective anti-RA strategy. In the work described here, direct intraarticular administration of adenoviral vectors carrying the mouse TSP-1 gene significantly suppressed clinical inflammatory presentations, accompanied by a reduction of synovial hypertrophy and blood vessels, and by a lower grade of bone and cartilage destruction. The antiangiogenic and anti-inflammatory activities of TSP-1 may constitute a contributory mechanism by which TSP-1 reduced joint inflammation caused by CIA. The detailed mechanism-of-action of TSP-1 gene therapy for the treatment of RA, however, warrants further investigation.

In conclusion, the results of the present study demonstrate that TSP-1 effectively ameliorated the course of CIA in rats. Furthermore, these findings suggest that TSP-1 gene therapy may have therapeutic potential for the treatment of RA.
References


14. Hitt M, Bett AJ, Prevec L, Graham FL. Construction and propagation of human...
Thrombospondin-1 as arthritis therapy


Figure Legends

Figure 1. Effects of Ad-TSP-1 and Ad-K1-5 gene transfer on tumor growth. Groups of 6-7 C3H/HeN mice were inoculated subcutaneously with MBT-2 cells (10^6) at day 0, and treated intratumorally with 4 × 10^6 and 8 × 10^6 PFU of Ad-TSP-1 or Ad-LacZ at days 13 and 14, respectively. A, The tumor volume in each mouse is shown. The lines denote the mean tumor volume in each group and are stopped when any mice were expired. B, Kaplan-Meier survival curves at day 60 are shown. Paraffin-embedded tumor sections from mice treated with Ad-LacZ (C, D), Ad-K1-5 (E,F), and Ad-TSP1 (G, H) were stained with hematoxylin and eosin.

Figure 2. Amelioration of CIA and TSP-1 expression in Ad-TSP-1-injected ankle. A, Ankle swelling is shown in both hind limbs of a CIA rat before treatment. B, The swelling progressed in the left ankle treated with Ad-LacZ (shown on the right of the photograph), but not in the right ankle treated with Ad-TSP-1 (shown on the left of the photograph) 5 days post-treatment. C, Low level of endogenous TSP-1 expression was found in the synovial tissue of the left ankle treated with Ad-LacZ as determined by immunohistochemical staining. D, TSP-1 expression was more evident in the synovial tissue of the right ankle treated with Ad-TSP-1. E, Histological appearance of the synovial tissue from the left ankle treated with Ad-LacZ. F, The synovial tissue from the right ankle treated with Ad-TSP-1 showing reduction of pannus formation and mononuclear cell infiltration compared with that treated.
with Ad-LacZ.

**Figure 3.** Suppression of ankle swelling in CIA rats by Ad-TSP-1 gene transfer. Seven days after the first immunization with collagen, rats without any RA syndrome on their both hind limbs were intraarticularly injected with $5 \times 10^7$ PFU of Ad-TSP-1 or Ad-LacZ into their ankle joints. Repeated treatment was performed again after 3 days. The therapeutic group treated with Ad-TSP-1 showed reduced RA syndrome on their both hind limbs by (A) articular index and (B) measuring ankle circumference, compared with the control group treated with Ad-LacZ (*, p<0.05; **, p<0.01)

**Figure 4.** TSP-1 gene transfer prevented the swelling of ankle joint and angiogenesis in the early stage of RA. The width of ankle joints treated with Ad-TSP-1 and the control joints treated with Ad-LacZ of four rats were compared. Joint space narrowing down, soft tissue swelling, bony erosion were serious in the group treated with Ad-LacZ (A), but prevented in those treated with Ad-TSP-1 (B) by X-ray radiography. C, Comparison of ankle joints treated with Ad-TSP-1 or Ad-LacZ by radiography score and vessel density. (***, p < 0.001).

**Figure 5.** VEGF, IL-1β, and TGF-β expressions as well as vascularity in the synovial tissues of CIA rats treated with Ad-TSP-1. A, Reduced VEGF and IL-1β expressions were detected in synovial tissues of CIA rats treated with Ad-TSP-1 compared with those treated with Ad-LacZ, as determined by ELISA. Immunohistochemical staining of synovial tissues with anti-factor VIII antibody reveals decreased vascularity in (C), the ankle joint treated with
Ad-TSP-1, as compared with (B), the ankle joint treated with Ad-LacZ. TGF-β expression in the synovial tissue from Ad-TSP-1-treated ankle joint, which was localized in the area of more lymphocyte infiltration (E) in contrast to that treated with Ad-LacZ (D).

**Figure 6.** Recovery of gait pattern following TSP-1 gene therapy. After administration with Ad-TSP-1 into the ankle joint of the right hind limb, the rat could stand on its right hind limb (B) but not with its left hind limb (A) treated with Ad-LacZ as the control. This difference between two hind limbs is also demonstrated in a radiogram (C).
Thrombospondin-1 as arthritis therapy

A

Articular index

- Ad-LacZ (n=10)
- Ad-TSP-1 (n=10)

0 6 8 10 12 14 16

Days after first immunization

B

Ankle circumference

0 45 50 55 60 65 70 75 80 85 90

0 6 8 10 12 14 16