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Hiroshi Takayanagi, ... , Kozo Nakamura, Sakae Tanaka

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Article

Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovial joints resulting from hyperplasia of synovial fibroblasts and infiltration of lymphocytes, macrophages, and plasma cells, all of which manifest signs of activation. Recent studies have revealed the essential role of osteoclasts in joint destruction in RA. Src family tyrosine kinases are implicated in various intracellular signaling pathways, including mitogenic response to growth factors in fibroblasts, activation of lymphocytes, and osteoclastic bone resorption. Therefore, inhibiting Src activity can be a good therapeutic strategy to prevent joint inflammation and destruction in RA. We constructed an adenovirus vector carrying the *csk* gene, which negatively regulates Src family tyrosine kinases. Csk overexpression in cultured rheumatoid synoviocytes remarkably suppressed Src kinase activity and reduced their proliferation rate and IL-6 production. Bone-resorbing activity of osteoclasts was strongly inhibited by Csk overexpression. Furthermore, local injection of the virus into rat ankle joints with adjuvant arthritis not only ameliorated inflammation but suppressed bone destruction. In conclusion, adenovirus-mediated direct transfer of the *csk* gene is useful in repressing bone destruction and inflammatory reactions, suggesting the involvement of Src family tyrosine kinases in arthritic joint breakdown and demonstrating the feasibility of intervention in the kinases for gene therapy in RA.

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Suppression of arthritic bone destruction by adenovirus-mediated *csk* gene transfer to synoviocytes and osteoclasts

Hiroshi Takayanagi,¹ Takuo Juji,¹ Tsuyoshi Miyazaki,¹ Hideharu Iizuka,¹ Tokiharu Takahashi,² Masashi Isshiki,³ Masato Okada,⁴ Yoshiya Tanaka,⁵ Yasuko Koshihara,⁶ Hiromi Oda,¹ Takahide Kurokawa,¹ Kozo Nakamura,¹ and Sakae Tanaka¹

¹Department of Orthopaedic Surgery,

²Third Department of Internal Medicine, and

³Fourth Department of Internal Medicine, Faculty of Medicine, the University of Tokyo, Tokyo 113-0033, Japan

⁴Division of Protein Metabolism, Institute for Protein Research, Osaka University, Osaka 565-0871, Japan

⁵First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan

⁶Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Tokyo, 173-0015, Japan

Address correspondence to: Sakae Tanaka, Department of Orthopaedic Surgery, Faculty of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81-3-3815-5411 ext. 3375; Fax: 81-3-3818-4082; E-mail: TANAKAS-ORT@h.u-tokyo.ac.jp.

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Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovial joints resulting from hyperplasia of synovial fibroblasts and infiltration of lymphocytes, macrophages, and plasma cells, all of which manifest signs of activation. Recent studies have revealed the essential role of osteoclasts in joint destruction in RA. Src family tyrosine kinases are implicated in various intracellular signaling pathways, including mitogenic response to growth factors in fibroblasts, activation of lymphocytes, and osteoclastic bone resorption. Therefore, inhibiting Src activity can be a good therapeutic strategy to prevent joint inflammation and destruction in RA. We constructed an adenovirus vector carrying the *csk* gene, which negatively regulates Src family tyrosine kinases. Csk overexpression in cultured rheumatoid synoviocytes remarkably suppressed Src kinase activity and reduced their proliferation rate and IL-6 production. Bone-resorbing activity of osteoclasts was strongly inhibited by Csk overexpression. Furthermore, local injection of the virus into rat ankle joints with adjuvant arthritis not only ameliorated inflammation but suppressed bone destruction. In conclusion, adenovirus-mediated direct transfer of the *csk* gene is useful in repressing bone destruction and inflammatory reactions, suggesting the involvement of Src family tyrosine kinases in arthritic joint breakdown and demonstrating the feasibility of intervention in the kinases for gene therapy in RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by invasive synovial hyperplasia leading to progressive joint destruction. Investigations into the pathogenesis of joint destruction in RA have revealed the transformed phenotype of rheumatoid synovial cells (1, 2), although the etiology of RA remains unknown. Rheumatoid synovial cells are not only morphologically characterized by their transformed appearance (3) but also are phenotypically transformed to proliferate abnormally (4, 5); they invade bone and cartilage by producing an elevated amount of proinflammatory cytokines (6) and metalloproteinases (7) and by inducing osteoclast formation and activation (8, 9). Their activated phenotype is marked by an upregulation of proto-oncogenes (10), which are involved in important cellular events such as intracellular signaling and gene transcription. Osteoclasts, the multinucleated cells exclusively responsible for bone resorption, have been observed to resorb bone actively at the site of invasion of the proliferated synovial membrane into the adjacent bone (11). The cell types

responsible for bone resorption in RA have been characterized as authentic osteoclasts (9), and we reported previously that rheumatoid synovial fibroblasts are involved in bone destruction by inducing osteoclastogenesis (8). Thus, rheumatoid bone destruction, attributed to activated synoviocytes and bone-resorbing osteoclasts, cannot be easily controlled by inhibiting only 1 of the factors involved in these multiple pathological processes.

Cytoplasmic tyrosine kinases of the Src family are involved in the regulation of a number of cellular processes, including signal transduction of various growth factor receptors and cytokine receptors (12, 13), immunorecognition of T cells and B cells (13), and activation of osteoclasts (14). Consequently, Src family kinases are implicated in a variety of pathological events in RA, characterized by the proliferation of activated synoviocytes, abnormal immune responses, and elevated production of proinflammatory cytokines. Regulation of the activity of Src family kinases can therefore be a good therapeutic target in the treatment of RA.

The kinase activity of Src family kinases is negatively

regulated by another cytoplasmic tyrosine kinase, Csk (C-terminal Src kinase), by the phosphorylation of tyrosine residue at the carboxyl-terminus (Tyr 527) (15, 16). In the present study, we constructed a replication-deficient adenovirus vector carrying the *csk* gene to investigate the effect of introducing the *csk* gene into synoviocytes and osteoclasts *in vitro* and *in vivo*. Adenovirus-mediated overexpression of Csk, by repressing Src kinase activity, dramatically decreased the proliferation rate and IL-6 production of human rheumatoid synovial cells in culture; also, the bone-resorbing activity of human osteoclasts formed in coculture of rheumatoid synoviocytes and PBMCs was remarkably suppressed by Csk expression. Importantly, direct injection of Csk virus into rat ankles with adjuvant arthritis not only ameliorated the inflammatory reactions but also suppressed bone destruction.

Methods

Antibodies. Anti-Csk rabbit polyclonal antibody (C-20; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), anti-v-Src mouse mAb (clone 327; Oncogene Research Products, Cambridge, Massachusetts, USA), anti-Fyn mouse mAb (clone 15; Santa Cruz Biotechnology Inc.), anti-c-Yes rabbit polyclonal antibody (clone 3; Santa Cruz Biotechnology Inc.), and anti- β -actin polyclonal antibody (Sigma Chemical Co., St. Louis, Missouri, USA) were used. For Western blotting, horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) were used as secondary antibodies.

Synovial cell cultures. With the use of enzymatic digestion methods described previously (8, 18), primary rheumatoid synovial cells were obtained from synovial tissues dissected from 10 female patients (age range: 49–72 years) who fulfilled the American Rheumatism Association criteria for RA (17). In brief, the minced synovial tissue was digested in 1 mg/mL collagenase (Wako Pure Chemicals Industries, Osaka, Japan), and the cell suspension was purified using Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). The cells were suspended

in DMEM (GIBCO BRL, Rockville, Maryland, USA) plus 10% FBS (Cell Culture Laboratory, Cleveland, Ohio, USA) and used for experiments after 3–6 passages. Subcultured synovial cells were composed of synovial fibroblasts that were free of macrophages positively stained for CD11b or nonspecific esterase (data not shown). Informed consent to subsequent experiments was given by each patient.

Adenovirus vector construction and gene transduction *in vitro*. The replication-deficient recombinant adenovirus vector carrying the rat *csk* gene (AxCATcsk; Csk virus) under the control of the CAG [cytomegalovirus IE enhancer plus chicken β -actin promoter plus rabbit β -globin poly(A) signal] promoter was constructed using established methods (19). The control virus (Ax1w1; WT virus) and adenovirus vector carrying the β -galactosidase (β -gal) gene (AxCASLacZ; LacZ virus) were kindly provided by Izumu Saito (University of Tokyo). The titer of each virus was 1×10^{10} plaque-forming units (PFU) per milliliter. Infection of adenovirus vectors to cultured synoviocytes and osteoclasts was carried out following the method described previously (20). The cells in culture were incubated with adenovirus (1×10^{10} PFU/mL) diluted 1:10 in DMEM (the final titer of the virus was 1×10^9 PFU/mL) for 1 hour at 37°C, after which 10 times more medium containing 10% FBS was added. The number of virus particles (measured in PFU) per cell was expressed as moi.

X-gal staining for β -gal expression *in vitro*. Rheumatoid synovial cells were infected with LacZ virus (moi = 10, 30, 100). After 24 hours, the cells were fixed with 3.7% formaldehyde in PBS for 15 minutes, and incubated overnight at 37°C in X-gal solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 2 mM 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) in PBS] as described (21).

Immunofluorescence staining and confocal microscopy. WT or Csk virus-infected (moi = 100) synoviocytes plated on glass coverslips were washed with PBS, fixed in 3.7% formaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Cells were sequentially incubated in PBS plus 1% BSA for 30 minutes, 2.0 μ g/mL rabbit anti-Csk polyclonal antibody in PBS for 1 hour, and then with 10 μ g/mL goat Cy5-labeled anti-rabbit IgG antibody (FluoroLink; Amersham Pharmacia Biotech) in PBS for 1 hour. Microscopy was performed using a confocal microscope (MRC-1000UV; Bio-Rad Laboratories Inc., Hercules, California, USA) as described previously (22).

Protein extraction and Western blot analysis. Twenty-four hours after inoculation with Csk virus (moi = 10, 30, 100) or WT or LacZ virus (moi = 100), synovial cells were lysed in TNE buffer (10 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2 mM Na_3VO_4 , 10 mM NaF, and 10 μ g/mL aprotinin). The supernatants were separated by SDS-PAGE under a reducing condition, transferred electrophoretically onto a nitrocellulose membrane, and probed sequentially with 0.2 μ g/mL anti-Csk antibody followed by 0.1 μ g/mL horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were viewed by ECL (enhanced chemiluminescence) Western blotting detec-

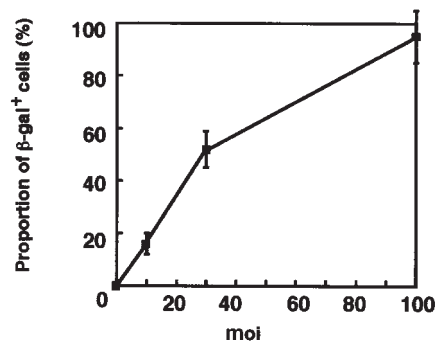


Figure 1

Efficiency of adenovirus-mediated gene transduction into human rheumatoid synovial cells in culture. The percentage of synoviocytes positive for β -gal expression increased in an moi-dependent manner. Data are expressed as mean \pm SD of quintuple measurements.

tion reagents (Amersham Pharmacia Biotech), following the manufacturer's protocol.

Immunoprecipitation and in vitro kinase assay. Equal amounts of protein (100 µg) extracted from LacZ or Csk virus-infected synoviocytes (moi = 100) were incubated with 1 µg of anti-v-Src, anti-Fyn, or anti-Yes antibody for 1 hour at 4°C, and the immune complexes were recovered with protein G-Sepharose (GIBCO BRL). One half of the immunoprecipitate was used for in vitro kinase assay and the other half for Western blotting analysis, to ensure that an equal amount of c-Src protein was recovered. In vitro kinase assay was performed as reported previously (23). Briefly, the samples were resuspended in 60 µL of kinase buffer (20 mM HEPES-NaOH [pH 7.4] and 10 mM MgCl₂) with 1 µCi (37 kBq) of [γ -³²P]ATP (Amersham Pharmacia Biotech) in the presence of 1 µg of acid-treated enolase. They were then incubated for 15 minutes at 30°C and subjected to 8% SDS-PAGE under a reducing condition followed by autoradiography.

Cell proliferation assay. Rheumatoid synovial cells (5 × 10⁴ cells per well) seeded in culture plates were infected with WT, LacZ, or Csk virus (moi = 100). On days 0, 2, 5, and 8, cells were recovered by trypsin-EDTA treatment, and their number was counted. Cell growth rate was also determined by a cell proliferation kit (Amersham Pharmacia Biotech) using immunostaining for a thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), incorporated into replicating DNA according to the manufacturer's protocol.

ELISA for IL-6. Rheumatoid synovial cells (1 × 10⁵ cells per well) were infected with Csk virus (moi = 10, 30, 100) or WT or LacZ virus (moi = 100). FBS was removed from the medium 24 hours after inoculation. After an additional 24 hours, conditioned medium was recovered, and IL-6 concentration in the medium was determined using a human IL-6 ELISA kit (Fujirebio Inc., Tokyo, Japan).

Northern blot analysis. Total RNA was extracted from rheumatoid synovial cells infected with Csk virus (moi = 10, 30, 100) or WT or LacZ virus (moi = 100) 24 hours after inoculation, using acid-guanidinium isothiocyanate-phenol-chloroform (Isogen; Nippon Gene, Toyama, Japan) following the established method (24). Equal amounts (8 µg) of RNA were denatured in formaldehyde, separated on 1% agarose gel, and transferred to a nitrocellulose membrane (Hybond-N; Amersham Pharmacia Biotech) followed by ultraviolet cross-linking. ExpressHyb hybridization solution (CLONTECH Laboratories Inc., Palo Alto, California, USA) was used according to the manufacturer's protocol. The blots were hybridized with a cDNA probe labeled with [α -³²P]dCTP (NEM Life Science Products, Boston, Massachusetts, USA) by a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA). Human IL-6 probe was the PCR product of synoviocytes using the primers described previously (25), and human β -actin probe was obtained from Wako Pure Chemicals Industries.

Resorption assay. Pit-forming assay by human osteoclast-like cells (OCLs) was performed as described previously (8). PBMCs isolated using Ficoll-Paque were cocultured with rheumatoid synovial fibroblasts in α -MEM containing 20% horse serum (GIBCO BRL) in the

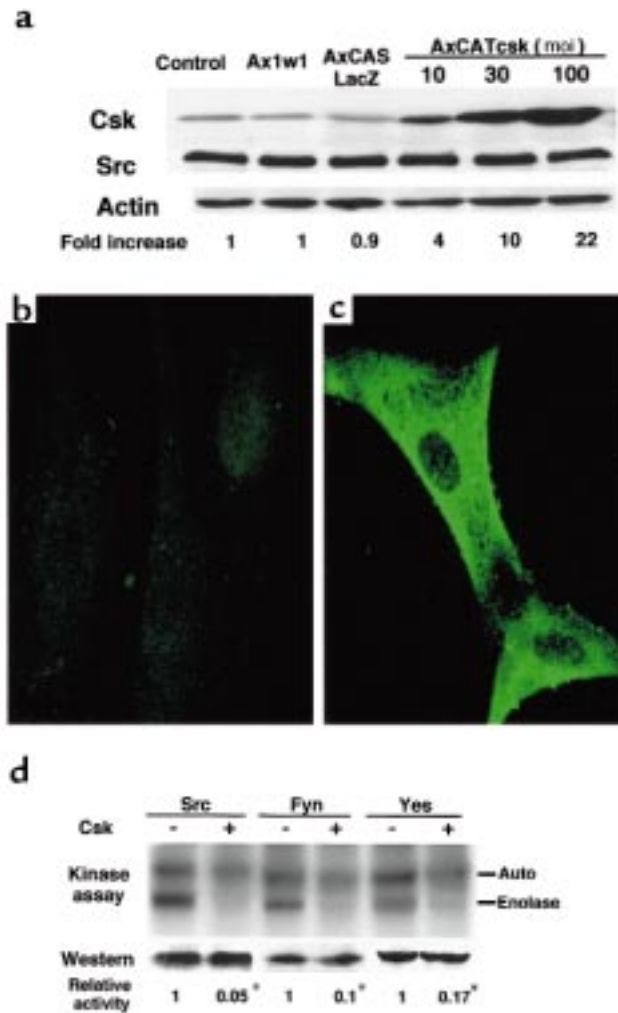
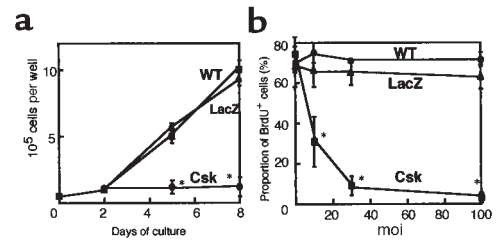


Figure 2 Csk expression and the kinase activity of synoviocytes. (a) Western blot analysis shows that Csk virus induced Csk expression in human synovial cells in an moi-dependent manner without significant changes in expression of c-Src protein. Fold increase in Csk expression normalized by β -actin is shown below each blot. The results shown are from a single experiment typical of 3 experiments yielding identical results. (b) Immunofluorescence staining using anti-Csk antibody shows that WT virus-infected synoviocytes expressed a very small amount of Csk protein. (c) Diffuse cytoplasmic staining in Csk virus-infected (moi = 100) synoviocytes. (d) In vitro kinase assay, using enolase as a substrate demonstrated that kinase activity of c-Src, Fyn, and c-Yes was remarkably repressed in Csk-overexpressing synoviocytes, but the expression of each protein was unaffected by Csk expression. Relative kinase activity of each kinase is shown below. The results were derived from 3 separate experiments. Suppression on each kinase was statistically significant (* $P < 0.05$ Csk⁺ vs. Csk⁻).

presence of 10⁻⁷ M 1,25(OH)₂ vitamin D₃ (Wako Pure Chemicals Industries) and 300 U/mL recombinant human M-CSF (Austral Biologicals, San Ramon, California, USA). After 3 weeks, multinucleated OCLs formed in coculture were isolated by pronase E treatment. OCLs (2 × 10⁴ cells per well) were seeded on calcium phosphate-coated discs (Millenium Biologix Inc., Kingston, Ontario, Canada) (day 0) and infected with WT or LacZ virus (moi = 100) or Csk virus (moi = 10,

Figure 3

Inhibitory effects of adenovirus-mediated Csk overexpression on cell growth of rheumatoid synoviocytes. (a) Cell growth curves of rheumatoid synoviocytes inoculated with WT, LacZ, or Csk virus (moi = 100). Proliferation of Csk virus-infected cells was strongly suppressed. Data represent mean \pm SD of triplicate measurements (* P < 0.05 Csk vs. WT, Csk vs. LacZ). (b) Proportion of BrdU-positive cells in synoviocytes inoculated with WT, LacZ, or Csk virus. Percentage of BrdU-positive cells inoculated with Csk virus decreased in an moi-dependent manner, but WT or LacZ virus had no effect on cell growth rate. Data are expressed as mean \pm SD of quintuple measurements (* P < 0.01 Csk vs. WT, Csk vs. LacZ).



30, 100). On day 5, the discs were washed in 6% sodium hypochloride to remove the adherent cells. The resorbed area was measured by scanning densitometry and analyzed by NIH Image software (National Institutes of Health, Bethesda, Maryland, USA).

Animals and induction of adjuvant arthritis. Inbred male Sprague-Dawley rats were purchased from Sankyo Laboratory Services (Tokyo, Japan). The animals, 6–7 weeks old, were injected subcutaneously into the right hind paw (day 0) with 100 μ L of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA) containing 10 μ g/ μ L *Mycobacterium butyricum* (Difco Laboratories). Arthritis of the bilateral ankle joints developed in 100% of animals after day 7. The effect of Csk injection was evaluated in right ankle joints, which showed severe bone destruction, and in left ankle joints, which exhibited significant inflammatory reactions.

β -gal expression in situ and Csk expression in vivo. Fifteen rats with adjuvant arthritis (immunized on day 0) were injected with 30 μ L of LacZ virus into the inflamed right ankle joint on day 7. On days 14, 21, 28, 35, and 42 (3 rats each day), the right ankle joint was dissected out en bloc and cut longitudinally. After fixation in 3.7% formaldehyde for 12 hours, the tissues were decalcified in 10% EDTA and embedded in paraffin to be sectioned. Serial sections were stained for β -gal and tartrate-resistant acid phosphatase (TRAP) as described previously (21, 26). Counterstaining was performed by

nuclear fast red for β -gal, and by hematoxylin for TRAP. To determine in vivo expression of Csk by the adenovirus, 6 rats were injected with 30 μ L Csk virus, and 2 rats with 30 μ L WT virus, into the right ankle joint (day 0). Csk virus-injected rats were sacrificed on days 7, 21, and 42 (2 rats each day), and WT virus-injected rats (n = 2) were sacrificed on day 7. The protein was extracted from the synovial and periarticular tissues and applied for Western blot analysis using anti-Csk antibody.

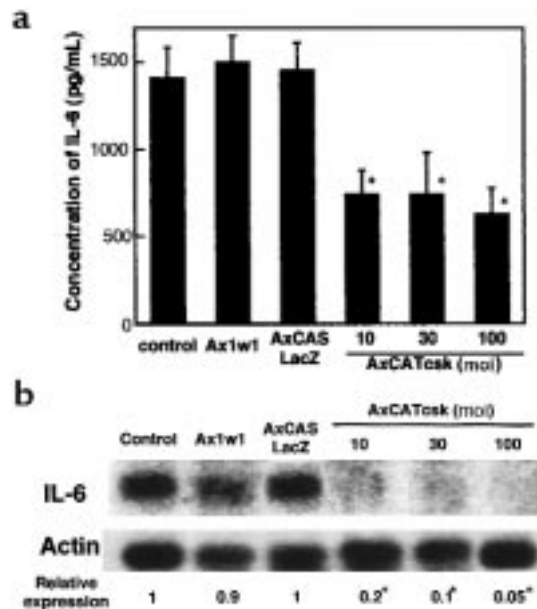
Therapeutic protocol. For virus injection into right ankles, 30 rats were immunized with adjuvant in the right foot (day 0). On day 7, the WT group (n = 10; body weight 151 \pm 2.9 g) was intra-articularly injected with 30 μ L of WT virus; the LacZ group (n = 10; body weight 150 \pm 4.0 g), with 30 μ L of LacZ virus; and the Csk group (n = 10; body weight 147 \pm 6.7 g), with 30 μ L of Csk virus. The viruses (3.0×10^8 virus particles per rat) were directly injected into the inflamed right ankle joint space.

For virus injection into left ankles, 30 rats immunized in the right foot (day 0) were intra-articularly injected with 30 μ L of WT virus (n = 10; body weight 148 \pm 4.0 g), LacZ virus (n = 10; body weight 150 \pm 5.1 g), and Csk virus (n = 10; body weight 150 \pm 4.5 g) on day 7.

Assessment of therapeutic effects by Csk virus on adjuvant arthritis. Therapeutic effects of virus injection were examined by arthritis score and paw volume on days 7, 14, 21, 28, and 35 under inhalation anesthesia with diethyl ether. The rats were sacrificed for radiological and his-

Figure 4

Effect of adenovirus-mediated Csk overexpression on IL-6 production. (a) IL-6 concentration in conditioned medium of rheumatoid synoviocytes inoculated with WT, LacZ, or Csk virus detected by ELISA. IL-6 production was reduced to about 50% below the normal level in rheumatoid synoviocytes inoculated with Csk virus at an moi of 10 or greater. Data are expressed as mean \pm SD derived from 3 separate experiments (* P < 0.05 vs. LacZ). (b) IL-6 mRNA detected by Northern blot analysis in WT, LacZ, or Csk virus-infected synoviocytes. Significant decrease in IL-6 mRNA signals was observed in synovial cells infected with Csk virus at an moi of 10 or greater. Fold expression of IL-6 mRNA normalized by β -actin is shown below each blot. Data are expressed as the mean derived from 3 separate experiments (* P < 0.05 vs. LacZ).



tological examinations on day 35. These evaluations were performed by the same observer without knowledge of treatment. (a) Arthritis score. As described previously (27), with modifications, the severity was scored by appearance: 0 = normal, 1 = redness or mild swelling (confined to ankle or toes), 2 = mild swelling (extending from ankle to toes), 3 = moderate swelling, 4 = severe swelling, often with ulceration or ankylosis (maximum 4). (b) Paw volume. The hind-paw volume was measured with an instrument for plethysmography (TK-101; Unicom, Tokyo, Japan) by vertically immersing the paw to the level of the proximal end of the lateral malleolus in a water-filled tub. (c) Radiological score. Right hind paws were dissected on day 35 and radiologically examined by soft x-ray machine (CMB-2; Softex, Tokyo, Japan). A 0–3 subjective grading system (0 = normal, 1 = mild, 2 = moderate, 3 = severe) described previously (28), with modifications, was used to evaluate 5 different parameters including cartilage loss (joint space narrowing), subchondral bone erosion, osteoporosis, heterotopic ossification, and periosteal new bone formation. The radiological score refers to the sum of the subjective scores for each of these 5 parameters (maximum 15). (d) Histological evaluation. After radiography, specimens were placed in 3.7% formaldehyde for 24 hours and decalcified in 10% EDTA for 14 days, after which they were embedded in paraffin and longitudinally sectioned. A 0–3 subjective grading system (0 = normal, 1 = mild, 2 = moderate, 3 = severe) was used to evaluate 3 different parameters including periarticular inflammation (infiltration of mononuclear cells), synovial thickening (pannus formation), and subchondral bone erosion. The pathological score of a joint refers to the sum of the subjective scores for each of these 3 parameters (maximum 9). Each sample was evaluated for the pathological score in the talo-tibial, talo-calcaneal, and calcaneo-navicular joints (see Figure 7h, arrowheads), and the total pathological score equals the sum of scores of these 3 joints (maximum 27).

Imaging system and statistical analysis. The data of Western blots, Northern blots, and the *in vitro* kinase assay were quantified by scanning densitometry system (Luminous Imager; Aisin Cosmos Co., Aichi, Japan). All values were expressed as the mean \pm SD and were statistically analyzed by Mann-Whitney test.

Results

Efficiency of adenovirus-mediated gene transduction into human synoviocytes *in vitro*. Cultured rheumatoid synovial cells were infected with AxCASLacZ (LacZ virus) to evaluate the efficiency of adenoviral gene transduction into human rheumatoid synovial cells. Histochemical staining with the chromogenic substrate X-gal showed a blue nuclear staining accompanied by a perinuclear cytoplasmic staining in the cells expressing β -gal. The percentage of synoviocytes positive for β -gal increased in an moi-dependent manner (Figure 1a), and 95% of the cells were positively stained at the moi of 100.

The Src kinase activity was repressed in Csk-overexpressing human synoviocytes. To analyze the effect of Csk overexpression, human rheumatoid synoviocytes were infected with AxCATsk (Csk virus). Western blot analysis revealed that the level of expression of Csk protein was

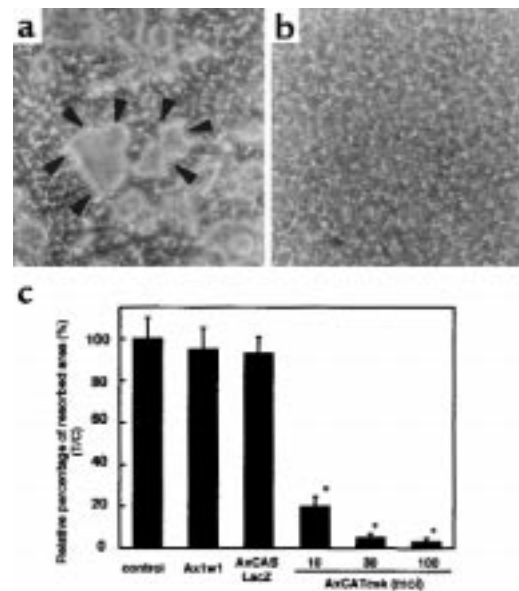


Figure 5

Effect of adenovirus-mediated Csk on bone-resorbing activity of osteoclasts. (a) Active resorption pit formation by WT virus-infected osteoclasts after removal of adherent cells. Arrowheads indicate 2 major pits surrounded by several other pits. (b) No resorption pit was seen when inoculated with Csk virus (moi = 100). (c) Relative resorbed area was drastically suppressed by Csk virus in an moi-dependent manner. Data are expressed as mean \pm SD of triplicate measurements (* P < 0.05 vs. LacZ).

very low in synovial cells infected with Ax1w1 (WT virus) or LacZ virus, but an moi-dependent expression of Csk was observed in Csk virus-infected cells, with no associated change in c-Src or actin expression (Figure 2a). Immunofluorescence staining showed a diffuse cytoplasmic expression of Csk in Csk virus-infected synoviocytes (Figure 2c), whereas only slight perinuclear staining was observed in WT virus-infected synoviocytes (Figure 2b). Although the expression of c-Src was unchanged by Csk expression, the kinase activity of c-Src was remarkably repressed in Csk-overexpressing synoviocytes (Figure 2d). Csk overexpression had repressive effects on the *in vitro* kinase activity of other members of Src family protein kinases, Fyn and c-Yes.

Adenovirus-mediated Csk overexpression inhibited cell growth of rheumatoid synoviocytes. Effect of adenovirus-mediated Csk on cell proliferation was evaluated by growth curve and by immunostaining for BrdU. Proliferation rate of Csk virus-infected synoviocytes was remarkably reduced compared with WT or LacZ virus-infected cells, as determined by the increase in cell number (Figure 3a). After inoculation with Csk virus, the percentage of proliferating (BrdU-positive) cells decreased in an moi-dependent manner (Figure 3b).

Adenovirus-mediated Csk overexpression reduced IL-6 production at mRNA level. Synovial cells derived from patients with RA spontaneously produce an elevated amount of IL-6 without external stimuli (29). To determine the effect of Csk overexpression on IL-6 production of rheumatoid synovial cells, IL-6 concentration in condi-

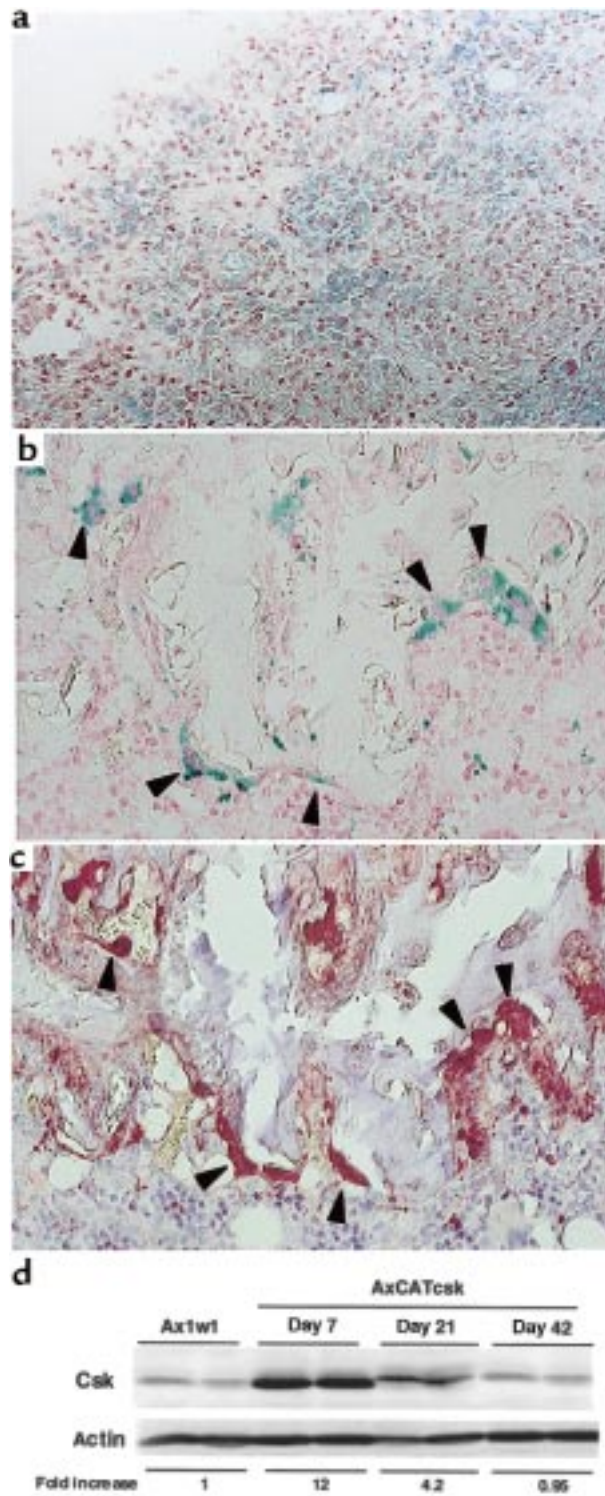


Figure 6
 In vivo expression of transgenes by adenovirus vectors. (a) In situ β -gal staining of the ankle synovial membrane, dissected 7 days after injection of LacZ virus, revealed a diffuse β -gal expression in the synoviocytes of rat ankles with adjuvant arthritis. (b) Multinucleated giant cells (arrowheads) at the bone/synovium interface were preferentially stained for β -gal. (c) These multinucleated cells (arrowheads) were positively stained for TRAP, a specific marker for osteoclasts. (d) In vivo expression of Csk protein in rat ankles detected by Western blotting ($n = 2$ each day). Fold increase in Csk expression normalized by β -actin is shown below each blot. The results were derived from 3 separate experiments, with duplicates performed in each experiment.

tioned medium was measured by ELISA, and IL-6 mRNA level was detected by Northern blot analysis in Csk-overexpressing synoviocytes. IL-6 concentration in conditioned medium of Csk virus-infected synoviocytes was reduced to almost 50% of noninfected synoviocytes at an moi of 10 or greater (Figure 4a). Northern blotting showed a dramatic decrease in IL-6 mRNA level in synovial cells infected with Csk virus at an moi of 10 or greater (Figure 4b).

Suppression of bone-resorbing activity of osteoclasts by adenovirus-mediated Csk. To evaluate the effect of Csk overexpression on bone-resorbing activity of human OCLs, pit-formation assay was performed on calcium phosphate-coated discs. WT or LacZ virus-infected OCLs formed numerous resorption pits, which were clearly observed after the cells were removed from the disc (Figure 5a, arrowheads). In contrast, pit formation was strongly inhibited by inoculation with Csk virus (moi = 100) (Figure 5b). Pit-forming activity quantified by measuring the resorbed area was drastically suppressed by Csk overexpression in an moi-dependent manner (Figure 5c).

Adenovirus vector-mediated gene transduction in vivo. LacZ virus was injected into rat ankles with adjuvant arthritis to determine the transgene expression in vivo. In situ staining for β -gal showed a diffuse blue staining in the synovial tissues and periarticular tissues of injected ankles. Light microscopy revealed the transgene expression in the synovial-lining and -sublining cells of the inflamed joints (Figure 6a). Immunohistochemical staining showed that both CD11b-positive and CD11b-negative synovial cells were stained for LacZ (data not shown). TRAP-positive OCLs (Figure 6c) at the pannus/bone interface were preferentially stained for β -gal (Figure 6b). LacZ expression macroscopically decreased in a time-dependent manner and was hardly detectable on day 42. Western blot analysis demonstrated that Csk virus injection led to a 12-fold increase in the expression of Csk protein in synovial tissues on day 7. On day 21, the expression was reduced to about 35% of day 7 levels; on day 42, it returned to the same level as that of the WT virus-injected rat (Figure 6d).

Csk virus injection ameliorated inflammation and suppressed bone destruction in adjuvant arthritis. Csk virus was directly injected into the inflamed ankle joints, and the severity of the disease was evaluated in comparison with WT or LacZ virus-injected rats by arthritis score, paw volume, and radiological and pathohistological examinations. On days 21–35, the arthritis score of Csk virus-injected right ankles was significantly improved compared with that of the WT and LacZ groups (Figure 7a). On days 14–35, the increase in paw volume was also significantly decreased by Csk virus injection into right ankles, compared with the WT and LacZ groups (Figure 7b). Anti-inflammatory effects of Csk virus injection into left ankles were shown by arthritis score (Figure 7c) and paw volume (Figure 7d), but the erosive changes in bone were not prominent in left ankles. Therefore, we evaluated the effects on bone erosion in the right ankles by radiological and pathological examinations. On day 35, the right ankles of WT or LacZ virus-injected rats showed the radiological findings of severe joint destruction — joint space nar-

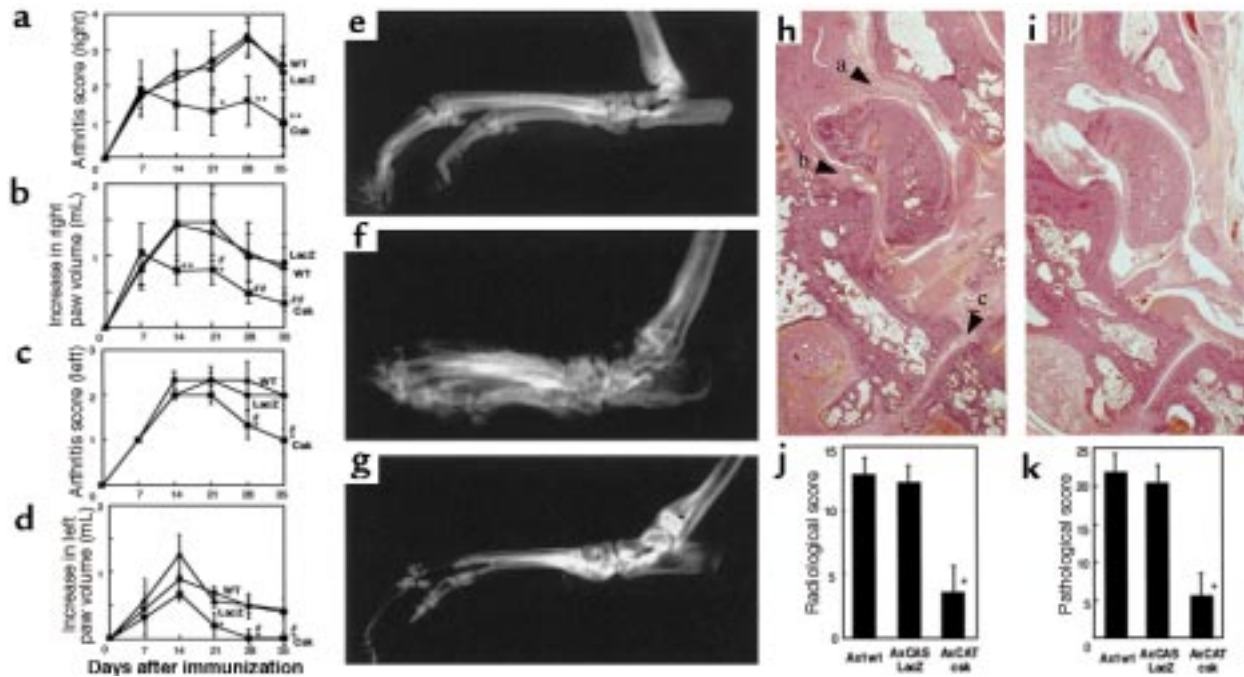


Figure 7

Therapeutic effects of Csk virus injection on rat adjuvant arthritis. All rats were immunized with adjuvant in the right foot-pad (day 0). Viruses were intra-articularly injected into ankles on day 7. Data are shown as mean \pm SD. (a) Effects of Csk virus injection into right ankles, as evaluated by arthritis score. The arthritis score of the Csk group ($n = 10$) was significantly lower than that of the WT ($n = 10$) and LacZ ($n = 10$) groups on days 21, 28, and 35 ($*P < 0.01$ Csk vs. LacZ, Csk vs. WT [day 21]; $**P < 0.001$ Csk vs. LacZ, Csk vs. WT [days 28 and 35]). (b) Effects of Csk virus injection into right ankles, as evaluated by increase in paw volume. The increase in paw volume of the Csk group was significantly less than that of the WT and LacZ groups on days 14, 21, 28, and 35 ($*P < 0.05$ Csk vs. WT; $*P < 0.01$ Csk vs. LacZ [day 21]; $**P < 0.01$ Csk vs. WT, Csk vs. LacZ [day 14]; $##P < 0.001$ Csk vs. WT, Csk vs. LacZ [days 28 and 35]). (c) Effects of Csk virus injection into left ankles, as evaluated by arthritis score. The arthritis score of the Csk group ($n = 10$) was significantly lower than that of the WT ($n = 10$) and LacZ ($n = 10$) groups on days 28 and 35 ($*P < 0.05$ Csk vs. LacZ; $#P < 0.05$ Csk vs. WT). (d) Effects of Csk virus injection into left ankles, as evaluated by increase in paw volume. The increase in paw volume of the Csk group was significantly less than that of the WT and LacZ groups ($*P < 0.05$ Csk vs. WT; $#P < 0.05$ Csk vs. LacZ). (e) On day 35, the right ankles of WT virus-injected rats showed the radiological findings of severe joint destruction — joint space narrowing, erosion, periarticular osteoporosis, and bone spur formation — compared with a normal rat ankle (e). (f) Radiological examination of Csk virus-injected right ankles showing minimal destructive changes. (g) The radiological score of Csk virus-injected right ankles was significantly decreased in comparison with WT and LacZ groups ($*P < 0.001$ Csk vs. WT, Csk vs. LacZ). (h) Pathohistological findings of WT virus-injected right ankles. Hyperplasia of synovial membrane occupied the articular spaces of talo-tibial (arrowhead a), talo-calcaneal (arrowhead b), and calcaneo-navicular (arrowhead c) joints. (i) Pathohistological findings of Csk virus-injected right ankles. Synovial hyperplasia and invasion into subchondral bone were markedly suppressed. (j) The pathological score of Csk virus-injected right ankles was significantly decreased in comparison with WT and LacZ groups ($*P < 0.001$ Csk vs. WT, Csk vs. LacZ).

rowing, erosion, and periarticular osteoporosis (Figure 7f) — compared with a normal rat ankle (Figure 7e), but these destructive changes were improved in Csk virus-injected rats (Figure 7g). Evaluation by radiological scoring confirmed the significant therapeutic effects (Figure 7j). Pathohistological examinations revealed that Csk virus injection obviously suppressed synovial hyperplasia, characterized by pannus formation and infiltration of inflammatory cells (Figure 7, h and i). Subchondral bone resorption and cartilage degeneration were also suppressed by Csk virus injection. Pathological scoring showed significant difference between Csk and WT (or LacZ) groups (Figure 7k).

Discussion

The first gene delivery system used in gene therapy for arthritis was the retrovirus vector (30), but it was

mainly used for ex vivo gene therapy, because retrovirus can deliver genes only into proliferating cells (31) and has a potential risk of insertional mutagenesis when integrated into the host genome. There are several advantages to the use of adenovirus vectors for gene therapy of arthritis. First, adenovirus vectors can easily be produced and purified at very high titers ($> 10^{10}$ PFU/mL) and can be used for in situ gene transfer. Second, the safety of the viral vector has been established from animal experiments; in fact, unattenuated adenoviruses have been successfully used for oral vaccine (32). Third, adenovirus vectors have been reported to be the most efficient vector in both in vitro and in vivo gene delivery to synovium (33). Using LacZ virus, we also confirmed that adenovirus vector can effectively transduce synoviocytes both in vitro and in vivo. Finally, adenovirus vectors can transduce nondi-

viding cells as well. This is particularly important because, as we recently reported (20), adenovirus vectors can efficiently transduce postmitotic osteoclasts, which play essential roles in bone destruction in RA. We also found, in the present study, that LacZ virus injected in the rat ankle joints can efficiently transduce osteoclasts present at the erosive bone surfaces, as well as synoviocytes (Figure 6, a and b). Several studies have used adenovirus vectors for gene therapy for animal models of arthritis. Transferred genes include inhibitory proteins of proinflammatory cytokines such as IL-1 and TNF- α (34, 35), cytokines such as IL-10 (36) and IL-12 (37), and the apoptosis-inducing molecule FasL (27). These attempts resulted in the successful amelioration of inflammatory reactions, although no clear effects have been described on inhibition of bone destruction.

Src family members of tyrosine kinases are involved in signal-transduction pathways that regulate a variety of biological activities (13, 38). They are implicated in the mitogenic response to several growth factors in fibroblasts; activation of lymphocytes, platelets and osteoclasts; and various cytokine signaling. Therefore, regulating Src family kinase activity can be a good therapeutic approach to RA. Csk is a cytoplasmic tyrosine kinase that specifically phosphorylates the carboxylterminus tyrosine residue of Src family tyrosine kinases and, thereby, negatively regulates their kinase activity (16). Csk overexpression reverts transformation of the cells transfected with *c-src* and *v-crk* (39) and negatively regulates antigen receptor-mediated activation of T lymphocytes (40). In addition, Csk overexpression reduces proinflammatory cytokine production in a macrophage cell line (41). These results suggest that the inflammatory reaction observed in RA synovial tissues can be reduced by *csk* gene transduction. Here, we demonstrated that the adenovirus vector carrying the *csk* gene (Csk virus) ameliorates the inflammatory reaction and bone destruction in experimental arthritis.

Csk virus induced an efficient expression of Csk protein in cultured synoviocytes in vitro and in synovial tissues in vivo. Csk overexpression in synoviocytes remarkably repressed the kinase activity of c-Src, with no quantitative changes in the amount of c-Src protein. Cell proliferation rate of rheumatoid synovial cells in vitro was strongly suppressed by overexpression of Csk, suggesting that the signaling mediated by Src family tyrosine kinases plays a crucial role in the proliferation of synoviocytes. Src family tyrosine kinases are involved in the intracellular signal transduction downstream of the receptors of growth factors such as PDGF, EGF, and FGF (12, 13). Therefore, the inhibitory effect of Csk on synoviocyte proliferation can partially be explained by blockade of the signal-transduction pathways of these growth factors. Because Src family kinases are implicated in integrin-mediated signal-transduction pathways (42, 43), Csk may modulate the adhesion property of synoviocytes and thereby inhibit anchorage-dependent cell growth.

IL-6 is a pleiotropic cytokine that has been suggested to be involved in the pathogenesis of RA. First, IL-6

levels are increased in synovial fluids and in the serum in RA, and correlate with the severity of the disease (44, 45). Second, synovial fibroblasts produce a great amount of IL-6 (29), and IL-6 stimulates the proliferation of synoviocytes and formation of osteoclasts in cooperation with soluble IL-6 receptor (46, 47). Third, IL-6 gene transcription is constitutively activated in rheumatoid synovial fibroblasts, owing to the activation of nuclear factor- κ B (NF- κ B) and C-promoter binding factor 1 (CBF1)(48). Finally, antigen-induced arthritis was poorly developed in IL-6-deficient mice (49), and blockage of IL-6 receptor ameliorated murine collagen-induced arthritis (50). These reports suggest the involvement of IL-6 in autoimmune arthritis. Our results demonstrated that adenovirus vector-mediated Csk overexpression suppressed IL-6 production by synoviocytes at the mRNA level, and protein production of IL-6 was significantly reduced, indicating that Src family protein kinases are also implicated in activated transcription of proinflammatory cytokines by rheumatoid synovial fibroblasts. The detailed mechanism by which Csk inhibits IL-6 expression remains elusive, but the inhibition could be explained by the suppression of Src-mediated activation of NF- κ B as recently described (51).

Importantly, adenovirus vector-mediated Csk overexpression strongly inhibited bone-resorbing activity of osteoclasts. Osteoclasts are primary cells responsible for bone resorption, and we recently reported that adenovirus vector can efficiently transfer genes into osteoclasts and modulate osteoclast function (23). Osteoclasts express a high level of c-Src in the ruffled border (23), and targeted disruption of *c-src* resulted in osteopetrosis due to dysfunction of osteoclasts, without any other critical abnormalities (14, 52). This shows that *c-src* is essential for osteoclastic function, and its function can be controlled by modulating the kinase activity of c-Src without affecting the normal function of other cells. In fact, Csk virus-infected osteoclasts, like *src*-deficient osteoclasts, were incapable of bone resorption.

The severity of inflammatory reactions in ankle joints of adjuvant arthritis rats, assessed by arthritis score and paw volume, was significantly improved by the local injection of Csk virus. The suppression of joint destruction was confirmed by radiological and pathohistological examinations. The results of in vitro study indicate that these in vivo effects of Csk virus on inflammatory reaction and bone destruction in arthritic rats result from direct inhibition of synoviocyte and osteoclast activity by the virus, although we need further investigations into the in vivo mechanism of therapeutic effects of Csk. Because we evaluated the effects of Csk on bone erosion in the ankles injected with adjuvant, and the efficiency of adenoviral gene transfer into lymphocytes is known to be very low, we conclude that Csk virus suppresses the effector phase of arthritic bone destruction, but it remains to be elucidated whether Csk has any effect on the onset of autoimmune arthritis. Csk injection into right ankles had no significant effects on noninjected left ankles in our experiments (data not shown), although it was reported that the adenoviral gene

transfer had effects on the contralateral knee in a rabbit model (35).

There are certain drawbacks in using adenovirus vectors for gene therapy of arthritis. First, adenovirus vector-mediated gene transduction is transient; therefore, repeated administration will be necessary to obtain further improvement of arthritis. In fact, as shown in Figure 6d, the expression level of Csk in the synovial cells was reduced on day 21 compared with that on day 7, although significant amounts of Csk protein could still be detected. Second, local injection of adenovirus vector sometimes induced inflammatory reactions because of the persistent expression of adenoviral proteins (33, 34). Although no significant difference was observed in the severity of arthritis between control (no virus injection) and WT or LacZ virus-injected groups (data not included) in our experiments, the development of a less immunogenic and higher-titer vector system is strongly expected.

In summary, intervention into intracellular signal-transduction pathways of synoviocytes and osteoclasts by adenovirus-mediated gene transfer of Csk resulted in suppression of bone destruction and amelioration of inflammation in rat adjuvant arthritis. These results suggest that signaling mediated by Src family kinases plays critical roles in the inflammation and joint destruction in RA. Adenovirus vector is useful for in vivo gene transfer to intra-articular tissues, and adenovirus-mediated gene transfer of *csk* gene is a promising means of preventing arthritic bone destruction. There will be no cure for RA until its etiology is elucidated, but suppression of synoviocyte and osteoclast activity by gene transfer might lead to a novel therapeutic strategy for preventing the joint breakdown associated with RA.

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