Mod Rheumatol (2001) 11:177–183

REVIEW ARTICLE

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The osteoclast: a potential therapeutic target of bone and joint destruction in rheumatoid arthritis

Abstract There is accumulating evidence that osteoclasts, the primary cells responsible for bone resorption, are involved in bone and joint destruction in rheumatoid arthritis (RA). Recent progress in bone cell biology has revealed the molecular mechanism of osteoclast differentiation and bone resorption by mature osteoclasts. We here highlight the potential role of RANKL–RANK pathways in bone destruction in RA. We also describe our recent trials on gene therapy of arthritic joint disease targeting osteoclasts by regulating Src kinase activity in the cells.

Key words Adenovirus · C-terminus Src family kinase (Csk) · Osteoclast recepter activator of NF-κB ligand (RANKL) · Src

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by invasive synovial hyperplasia. Proliferation of the synovial cells leads to pannus tissue that invades the bare area between cartilage and bone, finally resulting in progressive bone and joint destruction in the affected joints. Some of the therapeutic agents such as steroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatic drugs (DMARDs) can reduce the joint inflammation. However, very few of them can effectively suppress bone destruction in RA, and prolonged use of these drugs can be associated with side effects such as hepatotoxicity and stomach ulceration. Because ameliorating joint destruction is one of the most important issues in the treatment of RA, more efficient therapies against it are needed. There is accumulating evidence that osteoclasts, the primary cells responsible for bone resorption, are involved in bone and joint destruction in this disorder. In this article, we focus on the role of osteoclasts in the bone pathology of RA, and propose that they can be a potential target for RA treatment.

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Possible involvement of osteoclasts in bone destruction in RA

Osteoclasts are multinucleated giant cells (50–100 μm on average) with a very short life span (1–2 weeks in vivo) specifically differentiated for bone resorption[1] (Fig. 1). Bone resorption is necessary for normal skeletal development, adaptability, and maintenance. This process is critical for the growth, modeling, and remodeling of bone, and under normal conditions, it is tightly linked to bone formation by osteoblasts (coupling). On the other hand, the disruption of this coupling process between bone resorption and formation usually leads to abnormal bone resorption under pathological conditions. RA is characterized by abnormal synovial proliferation leading to erosive bone destruction originating from the interface of cartilage and bone (the bare area) in the affected joints. Synovial tissues of RA joints produce various kinds of inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), as well as the matrix metalloproteases and cathepsins, which have been believed to play important roles in joint destruction in the disease.[2] The cellular mechanism underlying the bone and cartilage destruction in RA has remained unclear, although recent studies have revealed the essential role of osteoclasts in this destruction. Bromley and Woolley[3] observed a number of acid phosphatase-positive multinucleated cells (chondroclasts and osteoclasts) in the erosive surfaces of RA joints obtained at the time of joint replacements. In collagen-induced arthritis, multinucleated giant cells were observed at the bone–pannus junctions of arthritic joints, and cells isolated from the lesions can differentiate into mature osteoclasts.[4] Gravallese et al.[5] also found multinucleated cells present on the surface of subchondral bone, especially in

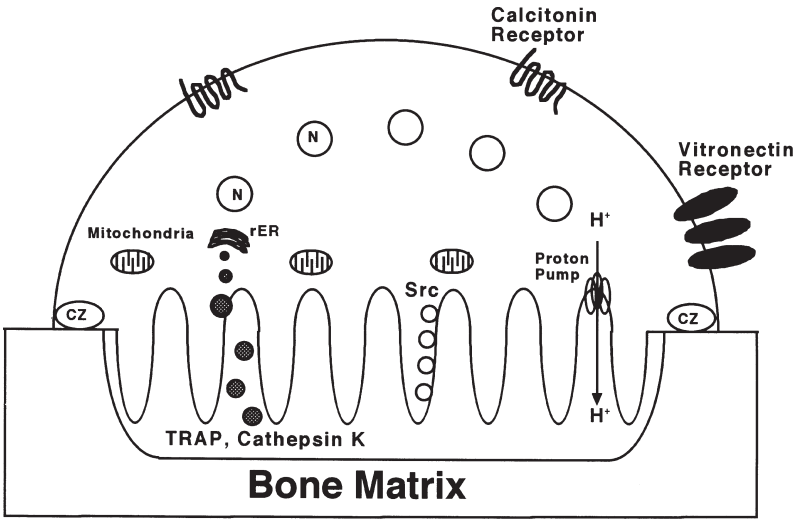

Fig. 1. A schematic representation of typical structures and functions of osteoclasts. *CZ*, clear zone; *rER*, rough endoplasmic reticulum

the areas of direct invasion of pannus into this bone. Their most important finding was that those multinucleated cells were positive for unique markers of osteoclasts such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and calcitonin receptors, satisfying the major criteria of mature osteoclasts. Interestingly, some TRAP-positive multinucleated cells and mononuclear cells in synovium could be observed apart from the bone surface. These findings suggest the possible involvement of synovial tissues in osteoclastogenesis in RA. In an attempt to reveal the osteoclastogenic potential of RA synovial tissues, synovial cells obtained from RA joints were cultured in the presence of the osteotropic factors 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and macrophage colony-stimulating factor (M-CSF). After 3 weeks of culture, we observed many multinucleated giant cells which were TRAP-positive, possessed abundant calcitonin receptors, and made resorption pits on dentine slices.[6] We also found that peripheral monocytes can differentiate into osteoclast-like cells when cocultured with synovial fibroblasts obtained from RA synovial tissues in the presence of $1,25(OH)_2D_3$ and M-CSF.[7] Similar results were reported by Fujikawa et al.,[8] who found that synovial macrophages isolated from RA synovial tissues can differentiate into osteoclast-like cells when cocultured with UMR106 rat osteoblast-like cells. These results suggest that RA synovial tissues have the potential for osteoclastogenesis in suitable conditions, at least in vitro, and that synovial macrophages (B cells) are the source of osteoclasts.

Role of RANKL/RANK pathways in bone destruction in RA

Remarkable progress has been made during the last few years in the field of osteoclast research, primarily owing to the discovery of potent osteoclast differentiation signaling, i.e., the receptor activator of the NF-κB ligand (RANKL)–RANK system.[9] RANKL is a member of the tumor necrosis factor (TNF) superfamily of cytokines, and was originally identified as a membrane-bound survival factor for dendritic cells produced by activated T cells.[10] The expression of RANKL is upregulated in osteoblasts and bone marrow stromal cells by osteotropic factors such as $1,25(OH)_2D_3$ and parathyroid hormone, and in the presence of M-CSF, RANKL can stimulate osteoclast differentiation from hematopoietic precursor cells in vitro.[11] RANKL also acts on mature osteoclasts and activates the bone-resorbing activity and survival of the cells. RANKL binds to its receptor RANK, a transmembrane receptor belonging to the TNF receptor superfamily, which is expressed in monocyte–macrophage-lineage osteoclast precursor cells as well as in mature osteoclasts and dendritic cells.[10,12,13] The binding of RANKL to RANK induces intracellular signals, including NF-κB activation and c-Jun N-terminus kinase (JNK) activation, which are mediated by TRAF (TNF receptor-associated factor) 6. The other important actor in this signaling system is osteoprotegerin (OPG), a soluble receptor of RANKL, which also belongs to the TNF receptor superfamily. OPG specifically binds to RANKL, and inhibits its RANKL activity by preventing its binding to RANK.[9]

The essential role of RANKL–RANK signaling pathways in osteoclast development in vivo has been established by a series of targeted gene disruption experiments. In short, the targeted disruption of either RANKL or RANK induced osteopetrosis in mice, a pathological bone disease which is characterized by an increased bone mass due to a deficiency in osteoclast differentiation.[9,14] In addition, we and another group found that mice deficient in TRAF6 also showed osteopetrotic phenotypes.[15,16] On the other hand, the targeted disruption of OPG induces reduced bone mass, a bone pathology reminiscent of osteoporosis, in mice due to the increased number and activity of osteoclasts.[17] These results clearly demonstrate the essential role of RANKL–RANK pathways in osteoclast development and activation in vivo. The next question is whether the RANKL–RANK system is also involved in pathological bone destruction

such as RA. We recently reported that RANKL is highly expressed in synovial fibroblasts by Northern blotting and immunocytochemistry, and similar results have been reported by other groups.[7,18,19] $1,25(OH)_2D_3$ treatment increased the expression of RANKL in synovial fibroblasts and reduced the expression of OPG in the cells. RANKL expression was also detected in $CD4^+$ T lymphocytes in RA synovial tissues by in situ hybridization, and Kong et al.[21] demonstrated that activated T lymphocytes can support osteoclast differentiation through the surface-bound and soluble-type RANKL they produce. They also showed the expression of RANKL on the cell surface of activated T lymphocytes in synovial tissues of adjuvant arthritis rats.[19] These results indicate the important function of activated T lymphocytes in bone and joint destruction in RA through RANKL production. However, the role of T cells in osteoclast development in RA is still controversial because activated T cells also produce many cytokines which strongly inhibit osteoclast differentiation, such as interferon-γ and interleukin-10.[20] In either case, these studies suggest that RANKL produced by synovial fibroblasts and/or activated T lymphocytes in RA synovial tissues plays an essential role in osteoclast development and bone destruction in RA (Fig. 2). Based on these findings, Kong et al.[21] proposed that OPG could be a possible therapeutic agent against bone destruction in RA.[21] They showed that exogenous administration of recombinant OPG suppressed bone destruction in rat adjuvant arthritis. Interestingly, not only bone destruction but also cartilage destruction was prevented by OPG treatment. Clinical studies exploring the effect of OPG treatment on RA joint destruction are currently being conducted in the USA.

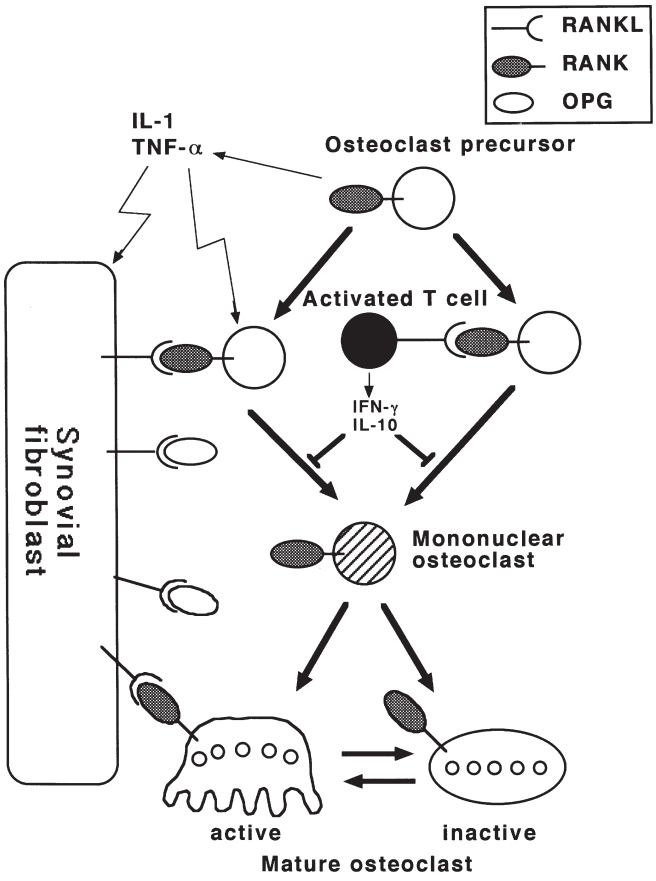

Fig. 2. Involvement of RANKL–RANK pathways in osteoclast differentiation and bone destruction in RA

Efficiency of adenovirus vectors in transducing osteoclasts

As mentioned above, the important role of osteoclasts in bone and joint destruction in RA is now widely recognized, so that pharmacological agents regulating osteoclast differentiation and/or function, such as OPG and bisphosphonates, can be potent therapeutic agents for RA. One alternative is gene therapy, where genes or cDNAs are directly transferred to target cells. In preclinical studies, ex vivo and in vivo gene transfer methods have been used successfully to reduce the joint destruction in experimental arthritis, and the first clinical trial, in which the IL-1 receptor antagonist gene was delivered to synoviocytes ex vivo, was started in 1996 in the USA.[22,23] Although modulating osteoclast function by gene therapy can be a good therapeutic approach for treating RA, transducing foreign genes into osteoclasts, even in vitro, is extremely difficult, mainly because osteoclasts are terminally differentiated nonproliferating cells with a very short life span. Antisense technology has been utilized successfully to suppress particular gene expression in osteoclasts. We previously reported that osteoclast function was successfully suppressed by inhibiting the expression of c-*src* or c-*cbl* by antisense oligodeoxynucleotides.[24] The shortcoming of antisense methods is that the antisense inhibition is not always successful, and it does not allow us to "overexpress" any genes. Retrovirus vectors can be used to transduce osteoclast precursors, and Matsuo et al.[25] reported that the retrovirus vector-mediated c-*fos* gene or *fra-1* gene transfered into osteoclast precursors from c-*fos*-deficient mice, which have an impaired ability to differentiate into mature osteoclasts, can rescue osteoclast differentiation. However, retrovirus vectors cannot transduce postmitotic cells such as mature osteoclasts. We recently reported that adenovirus vectors can be utilized efficiently as gene transfer agents for postmitotic osteoclasts both in vitro and in vivo.[26] Recombinant adenovirus carrying the *lacZ* gene can infect human osteoclast-like cells (OCLs) obtained from giant cell tumors as well as mouse OCLs formed in vitro. At an MOI of 100, more than 85% of OCLs were positively stained by β-galactosidase activity with no apparent morphological changes or cellular toxicity (Fig. 3). When the virus was injected into the bone marrow cavity of adult mouse femur, many osteoclasts present on the bone surface became β-galactosidase-positive. Adenovirus vectors have several advantages in introducing foreign genes into mature osteoclasts. First, these vectors are capable of infecting a variety of terminally differentiated cells, such as neurons and hepatocytes. Second, recombinant adenoviruses can easily be amplified to a very

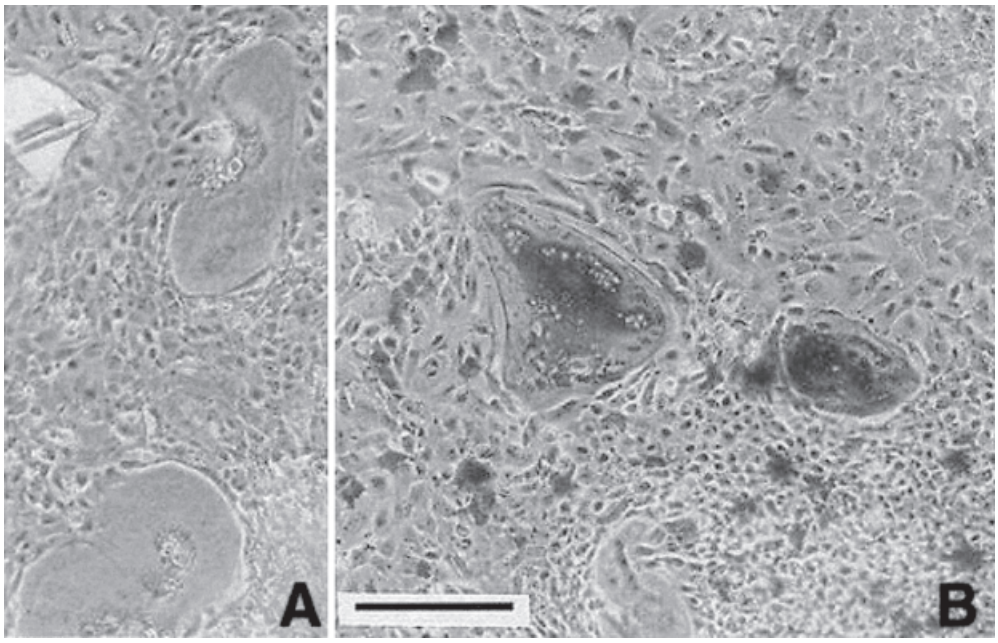

Fig. 3. Effective gene transduction into mature osteoclasts by adenovirus vectors. Human osteoclast-like cells obtained from giant cell tumors were infected with either control virus (A) or LacZ virus, and stained for β-galactosidase activity. Many multinucleated cells infected with LacZ virus were positively stained, indicating an efficient gene transduction. *Bar* 100 μm[26]

high titer in vitro. Third, adenovirus infection in the cells has been reported to require the interaction of the RGD sequence in the penton base of the virus with the cell-surface vitronectin receptors (αvβ3 or αvβ5 integrins), which are expressed at very high levels on the cell surface of osteoclasts. Our results suggest that the recombinant adenovirus vector system is suitable for gene transduction into mature osteoclasts and for modulation of their function.

Essential role of c-Src in osteoclast function

C-*src* was first identified as the normal cellular counterpart of the oncogene encoded by Rous sarcoma virus, v-*src*. The protooncogene product c-Src is a 60-kD protein and belongs to the nonreceptor-type tyrosine kinase family, which includes eight members (Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk) in addition to c-Src itself. The c-*src* protooncogene has been highly conserved throughout its evolution and widely expressed. Although the level of expression is low in most cell types, some cell types, particularly neurons and blood platelets, express high levels of c-Src protein. It is known that c-Src and the other members of the Src family, which share highly conserved sequences both within and outside the kinase catalytic domain, play important roles in signal transduction mechanisms that contribute to the regulation of cell growth and development. However, the physiological role of the c-*src* gene had not been clarified until Soriano et al.[27] successfully performed the targeted disruption of the gene by homologous recombination in mouse embryos in 1991. Unexpectedly, cell proliferation and other basal functions did not appear to be impaired in c-*src*-deficient animals, and no obvious phenotypic or functional abnormalities were observed in neuronal tissues or in platelets, probably because other Src family members are expressed in these cells, and they might impart a degree of functional redundancy with c-*src*. Surprisingly, the mice showed striking skeletal abnormalities with a phenotype of osteopetrosis. These included a failure of the incisors to erupt, a slower growth, shorter and abnormally shaped long bones, and decreased bone marrow cavities, which are all attributed to reduced bone resorption. In vitro osteoclast formation experiments and in vivo bone marrow transplantation studies revealed that osteoclast differentiation was not impaired, but that the bone-resorbing activity of mature osteoclasts was greatly reduced in c-*src* knock-out (KO) mice.[28] A morphological characteristic of the KO mouse osteoclasts was their disorganized ruffled border structure. This ruffled border is the apical membrane of the osteoclast, which is extensively folded due to the intense vesicular traffic associated with proton and lysosomal enzyme secretion. We and others have demonstrated that c-*src* is highly expressed in osteoclasts, and the c-Src protein is concentrated on the ruffled border membranes and intracellular membranes.[29,30] This, in addition to the association of this molecule with secretory granules and vesicles in platelets, chromaffin cells, and neurons, indicates that c-Src may contribute to vesicle targeting or membrane fusion in osteoclasts. The fact that no other abnormalities in c-*src* KO mice were found outside the skeletal tissues leads us to believe that c-Src can be an ideal therapeutic target for suppressing pathological bone resorption by inhibiting osteoclast function without affecting other tissues or cells. In fact, several specific inhibitors to c-Src kinase have been developed, and have been reported to be effective in suppressing animal bone resorption models.[31,32]

Modulation of osteoclast function by regulating c-Src activity

The tyrosine kinase activity of c-Src is strictly regulated by phosphorylation and dephosphorylation of the tyrosine residue located close to the C-terminus, which corresponds to tyrosine 527 (Tyr527) in chicken c-Src. Phosphorylation of Tyr527 inhibits Src catalytic activity by creating an intramolecular binding site for the Src homology (SH) 2 domain. This interaction is thought to result in autoinhibition by locking the molecule in an inactive state. Deletion of the C-tail, as in the v-Src oncoprotein, or mutation of the tyrosine residue to phenylalanine creates an active oncoprotein and causes a 10–20-fold increase in the kinase activity. C-terminus Src family kinase (Csk) is a cytoplasmic tyrosine kinase which specifically phosphorylates the C-terminus tyrosine residue of c-Src, thereby negatively regulating its kinase activity. In order to modulate osteoclast function by regulating c-Src kinase activity, we constructed adenovirus vectors encoding wild-type *csk* gene (Csk virus) or a kinase-deficient mutant of the gene (Csk-KD virus). These viruses efficiently transduced the genes in osteoclasts in vitro, and the catalytic activity of c-Src in osteoclasts was dose-dependently inhibited by Csk virus, whereas Csk-KD virus infection tended to increase the kinase activity, probably by working as a dominant negative molecule against intrinsic Csk protein in osteoclasts. Adenovirus vector-mediated Csk overexpression caused a dramatic morphological change in osteoclasts, disorganized the cytoskeletal organization of the cells, and abrogated the pit-forming activity on dentin slices.[33] To examine the effect of these adenoviruses on in vivo bone resorption, we utilized a modified bone resorption model reported by Uy et al.[34] Either Csk virus or Csk-KD virus was injected onto the calvaria of adult mice together with interleukin-1α (IL-1α) twice a day for 3 days. The mice were then killed and the calvaria were examined histologically. IL-1α injection induced a significant increase in the resorbed area and in the number of osteoclasts, and Csk virus injection significantly reduced the bone resorption induced by IL-1α, while Csk-KD virus injection, in contrast, exacerbated it.[33] It should be noted that IL-1α also induced periosteal inflammatory reaction, and this too was reduced by Csk virus injection. This suggests that Csk virus can efficiently suppress bone resorption in vivo as well.

We next examined the effect of Csk virus on pathological bone resorption in an animal model of RA.[35] There are two types of gene transduction strategies for RA gene therapy: systemic administration and local administration. The

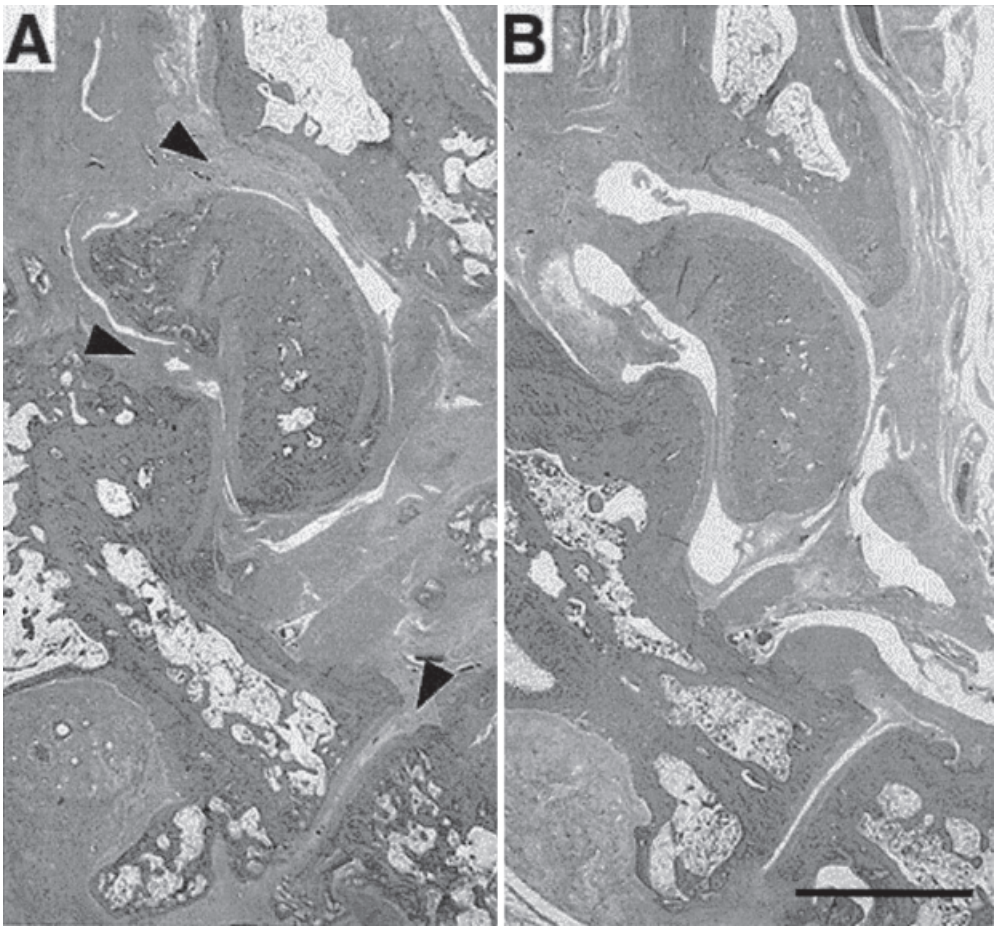

Fig. 4. Amelioration of bone destruction in rat adjuvant arthritis by Csk adenovirus injection. Ankle joints of adjuvant arthritis rat injected with (**A**) control virus, or (**B**) Csk virus. Hyperplasia of synovial membrane occupied the articular spaces (*arrowheads*) in the control virus-injected joint, and Csk virus injection significantly reduced it. *Bar* 1 mm[35]

efficient in vivo gene delivery to synovial cells by local administration of adenovirus vectors has been well established. When adenovirus vectors encoding *lacZ* gene were injected intraarticularly, strong β-galactosidase (β-gal) activity was observed in synovial lining cells. In addition, TRAP-positive osteoclasts on the erosive bone surface demonstrated strong β-galactosidase staining, as shown in serial tissue sections, indicating that intraarticular injection of adenovirus vectors can transduce osteoclasts on the erosive surface of arthritic joints. The effect of Csk adenovirus on inflammatory joints in adjuvant arthritis rats was evaluated by direct administration of the virus on their ankle joints.[35] Western blot analysis demonstrated that Csk virus injection led to a 12-fold increase in the expression of Csk protein in the injected joints on day 7, and the expression returned to the control level on day 42. Not only was the bone destruction suppressed by Csk virus injection, but the synovial inflammatory reaction detected by visual examination or paw swelling was also reduced (Fig. 4). The mechanism of this anti-inflammatory effect of the virus is probably the inhibitory effect of the virus on the proliferation of synovial fibroblasts and the production of inflammatory cytokines such as interleukin-6.

Conclusion

The ultimate goal of the treatment of RA is to prevent bone and joint destruction and preserve the daily activity of the patients. Recent studies have showed that osteoclasts are involved in the pathogenesis of bone and joint destruction and can be a potent therapeutic target of this disease, and that therapies that inhibit osteoclast formation or function can at least ameliorate the progression of these bone changes. Kong et al.[21] clearly demonstrated that blocking RANKL–RANK pathways by systemic administration of OPG could suppress not only bone destruction but also cartilage destruction in rat adjuvant arthritis, and bisphosphonates, potent antiresorptive agents, were reported to be possible therapeutic agents for arthritic joint destruction.[36] However, inhibition of osteoclast function by bisphosphonates or calcitonin alone did not completely prevent bone erosion in RA patients in spite of their preventive effects against systemic bone loss.[37] Therefore, a combination of antiresorptive and anti-inflammatory therapy is ideal for RA. Regulating Src family kinase activity can be a good therapeutic approach to RA because Src family members of tyrosine kinases are involved in signal transduction pathways that regulate various aspects of the pathology of the disease. Adenovirus vector-mediated *csk* gene expression can be a promising means of preventing arthritic bone destruction by suppressing osteoclast function as well as by inactivating synovial fibroblasts.

Acknowledgments We would like to thank Tsuyoshi Miyazaki and Hiroshi Takayanagi for their contributions in the *csk* gene therapy experiments.

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