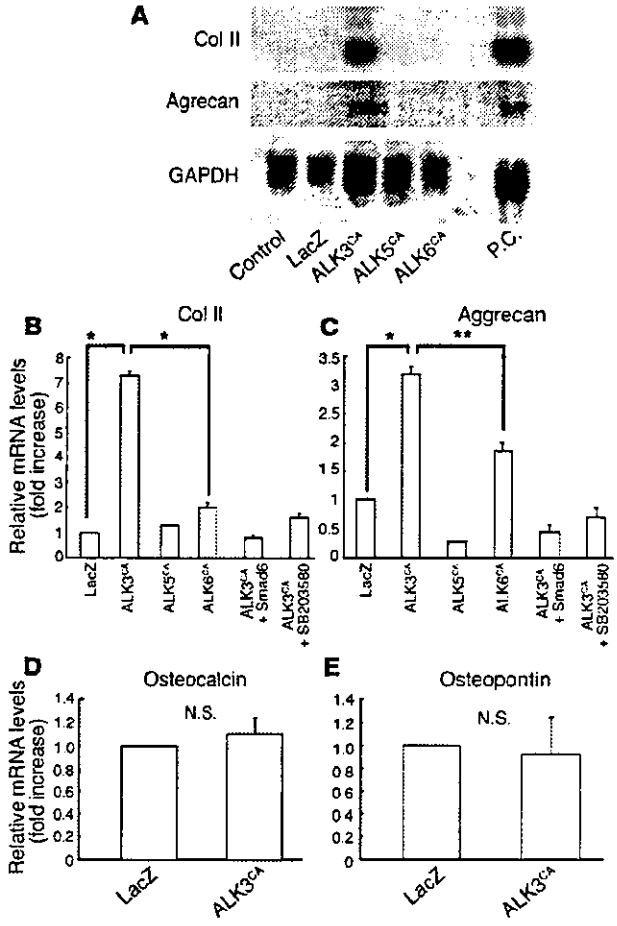


**Figure 2**
Effects of ALK3$^{CA}$, ALK5$^{CA}$, and ALK6$^{CA}$ expression on chondrocyte-specific gene expression in SFs. (**A**–**E**) Gene expression in SFs, as determined by Northern blot analysis (**A**) and real-time PCR analysis (**B**–**E**). Subconfluent monolayer SF cultures were infected with adenovirus vectors and they were then subjected to pellet culture 24 hours after viral infection; mRNA extracted from the pellets after 7 days of culture was then analyzed. Expression of type II collagen (Col II) and aggrecan was clearly induced in ALK3$^{CA}$-expressing cultures, as shown by Northern blot analysis (**A**) and real-time PCR analysis (**B** and **C**); this was suppressed by Smad6 coexpression and SB203580 (**B** and **C**). Expression of type II collagen and aggrecan was also observed in ALK6$^{CA}$-expressing cultures, albeit less efficiently, as shown in **B** and **C** by real-time PCR. Neither the osteocalcin nor the osteopontin gene was induced by ALK3$^{CA}$ virus infection (**D** and **E**). P.C., positive control, which represents the Northern blotting using mRNA of primary chondrocytes. N.S., not significant; $^{*}P < 0.001$; $^{**}P < 0.005$ (significantly different).

of osteocalcin or osteopontin was hardly detectable in the cells (Figure 2, C and D), indicating that hypertrophic and osteogenic differentiation were somehow blocked in these cultures. In contrast, neither type II collagen nor aggrecan gene expression was observed in ALK5$^{CA}$ virus-infected cells (Figure 2, A–C). Type II collagen and aggrecan expression induced by ALK3$^{CA}$ transduction was completely suppressed by coexpression with Smad6 or by SB203580 (Figure 2, B and C).

*ALK3 gene transduction increases Alcian blue–positive matrix and type II collagen deposition in pellet cultures of SFs.* For histological analysis, cells were subjected to pellet culture 24 hours after the viral infection. After 3 weeks of pellet culture, cells were fixed and examined by Alcian blue staining (Figure 3, A, D, G, and I) and Alizarin red staining and type II collagen immunostaining (Figure 3, B, E, G, and J) and type X collagen immunostaining (Figure 3, C and F). ALK3$^{CA}$ virus-infected cultures showed cartilageous matrix production that was strongly positive for Alcian blue staining (Figure 3D), while no positive staining was observed in LacZ virus-infected cultures (Figure 3A) or ALK5$^{CA}$ virus-infected cultures (Figure 3G), and only weak staining was observed in ALK6$^{CA}$ virus-infected cultures (Figure 3H). No Alizarin red staining was observed in ALK3$^{CA}$-infected cultures (not shown), indicating that mineralization associated with osteogenic differentiation was not induced. ALK3$^{CA}$ virus-infected SFs showed an oval shape, morphologically reminiscent of chondrocytes (Figure 3D). Immunostaining with anti-type II collagen showed positive staining in ALK3$^{CA}$ virus-infected pellet cultures (Figure 3E) and weak staining in ALK6$^{CA}$ virus-infected cultures (Figure 3H), while we failed to detect type X collagen in ALK3$^{CA}$ virus-infected cultures (Figure 3F), which suggests an absence of terminal differentiation to hypertrophic chondrocytes. No positive type II collagen immunostaining was detected in LacZ virus-infected cultures (Figure 3B) or ALK5$^{CA}$ virus-infected cultures (Figure 3H).

*ALK3$^{CA}$-transduced SFs after pellet culture form cartilage matrix in vivo.* To study chondrogenic differentiation of SFs in vivo, we subcutaneously transplanted the pellets into nude mice. Mice were sacrificed 3 weeks after the transplantation and the pellets were recovered and subjected to histological analysis. The transplanted SF pellets expressing ALK3$^{CA}$ were positively stained for toluidine blue (Figure 4C), which detects proteoglycan components, as does Alcian blue staining. Type II collagen immunostaining was also positive (Figure 4D), indicating the cartilaginous differentiation of the cultures in vivo, while Alizarin red staining was almost undetectable (data not shown). ALK6$^{CA}$ expression also induced chondrogenesis, albeit much less prominently (not shown), while neither LacZ (Figure 4, A and B) or ALK5$^{CA}$ (not shown) expression could induce chondrogenic phenotypes in the cultures. The histological observation was further confirmed by real-time PCR; expression of type II collagen and aggrecan was significantly higher in ALK3$^{CA}$-transduced pellets (Figure 4, E and F). These results suggest that ALK3$^{CA}$

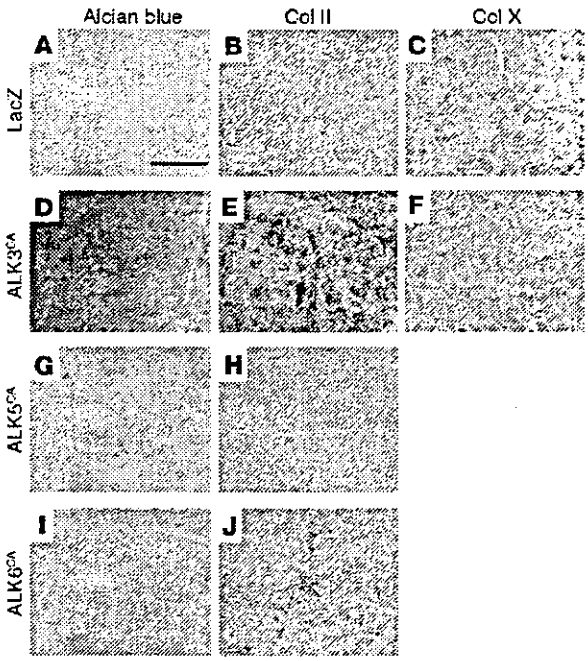


**Figure 3**
ALK3$^{CA}$ gene transduction increases Alcian blue–positive matrix and type II collagen deposition in pellet cultures of SFs. (**A**–**J**) Adenovirus–infected SF pellets were fixed with 3.7% formaldehyde after 3 weeks of culture and then were subjected to Alcian blue staining (**A**, **D**, **G**, and **I**) or immunostaining with anti–type II collagen (**B**, **E**, **H**, and **J**) or anti–type X collagen (Col X) (**C** and **F**). Distinct Alcian blue (**D**) and type II collagen (**E**) staining was observed in ALK3$^{CA}$-expressing cultures. ALK6$^{CA}$-expressing cultures showed weaker staining (**I** and **J**), and no positive staining was observed in ALK5$^{CA}$ virus–infected (**G** and **H**) or LacZ virus–infected (**A** and **B**) cultures. No type X collagen immunostaining was observed in cultures expressing LacZ or ALK3$^{CA}$ (**C** and **F**). Scale bar: 100 μm.

overexpression was able to target cartilage formation without subsequent bone formation in vivo.

*Segregation of ALK signaling pathways.* ALK signaling is known to be mediated by both the Smad pathways and MAP kinase pathways, especially the p38 pathways (31–33). We therefore attempted to distinguish the roles of the Smad pathways and p38 pathways from each other using a specific p38 inhibitor or adenovirus vectors. Smad6 coexpression or treatment of the cultures with the p38 inhibitor SB203580 completely abrogated the chondrogenic gene expression induced by ALK3$^{CA}$ (Figure 2, B and C). These results indicate that both the Smad pathways and the p38 MAP kinase pathways are required for the differentiation. Although Smad1 expression alone (MOI = 20) or a small amount of ALK$^{CA}$ virus (MOI = 2) failed to induce type II collagen expression in SFs, both had synergistic effects, and robust upregulation of type II collagen gene was observed by coinfection of Smad1 virus (MOI = 20) and ALK3$^{CA}$ virus (MOI = 2) (Figure 5A). Interestingly, activation of p38 pathways alone by MKK6$^{CA}$ expression in SFs induced rapid induction of Sox9 and type II collagen, which declined rapidly, however, and type X collagen expression was subsequently increased (Figure 5B). Coexpression of Smad1 together with MKK6$^{CA}$ not only reduced type X collagen expression but also maintained type II collagen expression in the cells (Figure 5B). Pellet cultures infected

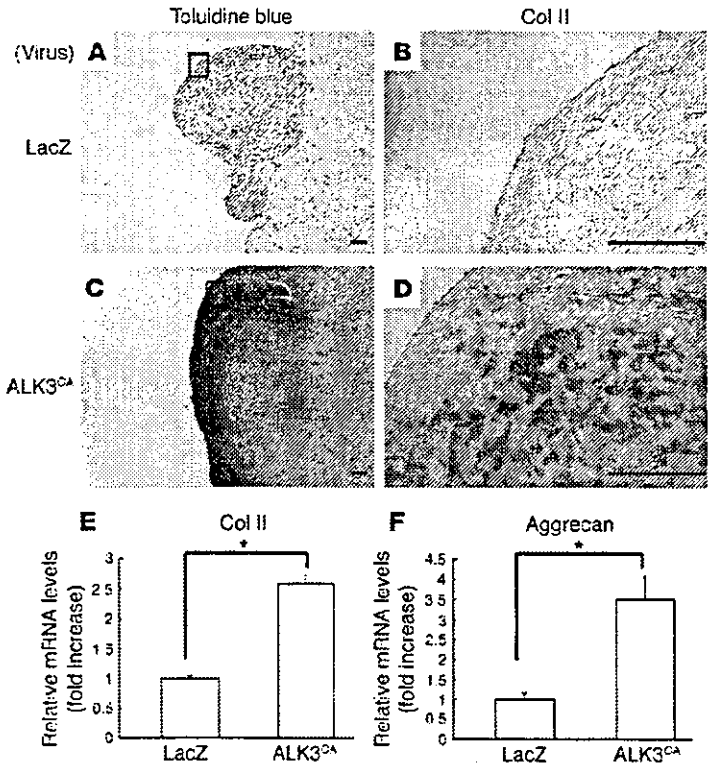


**Figure 4**
ALK3$^{CA}$-transduced SFs form cartilage matrix in vivo. (**A**–**D**) Three weeks after transplantation into nude mice, pellets were recovered and stained with toluidine blue (**A** and **C**) and immunostained with anti–type II collagen (**B** and **D**). Type II collagen immunohistochemistry was shown in the enlarged features of the rectangular area in the toluidine blue staining. Distinct positive staining was observed in ALK3$^{CA}$-expressing cultures (**B** and **D**) in contrast to LacZ virus–infected cultures (**A** and **C**). Scale bars: 100 μm. (**E** and **F**) Real-time PCR analysis of type II collagen and aggrecan. Their expression was significantly higher in ALK3$^{CA}$-expressing pellets than in LacZ-expressing pellets. *$P$ < 0.001 (significantly different).

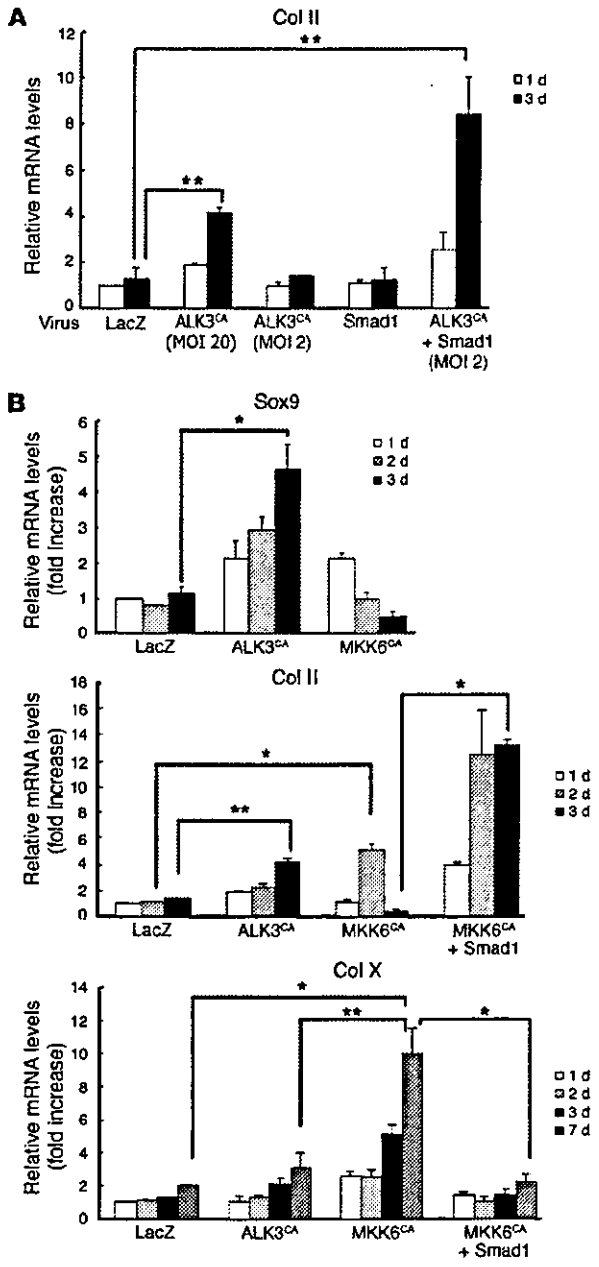


**Figure 5**
Segregation of downstream signaling pathways of ALK3. (**A**) Synergistic effect of Smad1 expression on the chondrogenic effects of the ALK3$^{CA}$ virus. Expression of Smad1 (MOI = 20) together with ALK3$^{CA}$ virus (MOI = 2) strongly induced expression of type II collagen in SFs. White bars indicate type II collagen expression on day 1 of cultures, and black bars indicate that on day 3. $^{**}P < 0.005$ (significantly different). (**B**) MKK6-p38 pathways promote terminal chondrocytic differentiation of SFs. Mandatory activation of p38 pathways by expression of MKK6$^{CA}$ using adenovirus vectors rapidly activated expression of the Sox9 and type II collagen genes, which rapidly declined, while expression of a terminal chondrocytic differentiation marker, type X collagen, was gradually increased. Adenovirus vector–mediated overexpression of Smad1 together with MKK6$^{CA}$ suppressed type X collagen expression and maintained type II collagen expression in SFs. $^{*}P < 0.001$; $^{**}P < 0.005$ (significantly different).

with MKK6$^{CA}$ virus were positively stained by type X collagen immunostaining as well as Alizarin red staining, which was suppressed by Smad1 virus coinfection (Figure 6).

*Type X collagen expression and p38 activation in synovial cells in osteoarthritic joints.* To examine the role of p38 activation in the development of degenerative changes in the articular cartilage, we next analyzed synovial tissues in the mouse model of osteoarthritis. After ACL and MM resection, the animals developed degenerative joint changes mimicking osteoarthritis. Osteochondrophytes were formed at the posterior edge of the femoral condyle and they were positively stained by anti-type X collagen as well as toluidine blue (rectangular areas in Figure 7, A and C) 4 weeks after the operation (corresponding to the stage of moderate osteoarthritis). Clusters of migrating synovial cells were observed adjacent to the osteochondrophytes (Figure 7B, arrowheads), where future osteochondrophytes will develop, and they were weakly stained by toluidine blue and anti-type X collagen at the marginal area between synovium and osteophytes (rectangular areas in Figure 7, B and D). This region was also positively stained by anti-phospho-p38 (Figure 7F). No positive staining was observed in the normal synovium, however (data not shown).

## Discussion

The signaling events leading to chondrogenesis still remain elusive, although there is accumulating evidence that TGF-β superfamily cytokines may play an important role (19–22). The receptors of TGF-β family members are composed of two different types of serine/threonine kinase receptors, known as type I and type II (31, 34, 35). Type II receptors are constitutively active kinases and phosphorylate type I receptors, also called ALKs. Type I receptors in turn mediate specific intracellular signaling pathways and therefore determine the specificity of the downstream signaling. So far, seven type I receptors have been identified, ALKs 1–7. ALK3 (BMPR-IA) and ALK6 (BMPR-IB) are structurally similar to each other and function as BMP receptors, while ALK5 and ALK4 work as type I TGF-β receptors. Using the adenovirus vector system, Fujii et al. reported that ALK1$^{CA}$, ALK2$^{CA}$, ALK3$^{CA}$, and ALK6$^{CA}$ induced osteoblastic differentiation of C2C12 myoblasts and that ALK3$^{CA}$ or ALK6$^{CA}$ introduction induced chondrocytic differentiation of ATDC teratocarcinoma cells (27).

In the present study, we focused on the regulation of chondrogenic differentiation of primary SFs obtained from rheumatoid arthritis patients. SFs have chondrogenic potential (15, 16) and can migrate into articular cartilage defects, where they deposit a scar-like tissue as Hunziker et al. pointed out (14), suggesting that SFs have anabolic effects on joint homeostasis and are involved in the restoration process of articular cartilage. We demonstrated that adenovirus vector–mediated ALK3$^{CA}$ gene expression induced robust induction of chondrocyte-specific gene expression in SFs in a ligand-independent manner. Clear induction of Sox9, a key transcription factor regulating chondrogenesis (36, 37), followed by type II collagen and aggrecan expression, was observed in the ALK3$^{CA}$-expressing cultures, while type X collagen was only weakly induced in the cultures and no osteocalcin expression could be found (Figures 2 and 5). Induction of these chondrocyte-specific genes through ALK3$^{CA}$ expression was not observed in skin fibroblasts, suggesting the cell specificity of the events (data not

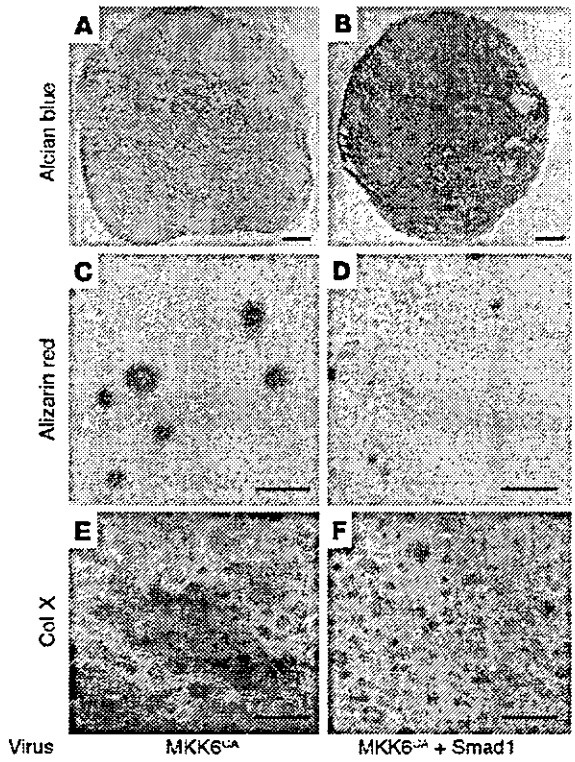


**Figure 6**
Induction of Alizarin red staining and type X collagen in MKK6-transduced SFs in pellet cultures. (**A–F**) SFs infected with MKK6$^{CA}$ virus alone (**A**, **C**, and **E**) or together with Smad1 virus (**B**, **D**, and **F**) were subjected to pellet culture. Cultures were fixed with 3.7% formaldehyde 3 weeks later, and then stained with Alcian blue (**A** and **B**), Alizarin red (**C** and **D**) or anti–type X collagen (**E** and **F**). Note the increased Alcian blue staining and the reduced Alizarin red activity and type X collagen immunoactivity with Smad1 coexpression. Scale bars:100 μm (**A–D**) and 50 μm (**E** and **F**).

shown). The chondrogenic effect of ALK3$^{CA}$ virus was further confirmed histologically by pellet cultures performed in vitro and in vivo (Figures 3 and 4). Induction of neither the osteoblast markers osteopontin and osteocalcin nor the terminal chondrocyte differentiation markers type X collagen and mineralization was observed in ALK3$^{CA}$-expressing cells (Figures 2, 3, 5, and 6). These results suggest that ALK3 signaling, that is, BMP signaling, has both stimulatory and regulatory roles in chondrogenesis: to induce the chondrogenic differentiation of SFs and at the same time to block their osteoblastic or hypertrophic differentiation. Despite the structural similarity between ALK3 and ALK6, the ALK6$^{CA}$ virus was much less efficient in chondrogenesis, the reason for which remains to be clarified. Although many studies have demonstrated a prochondrogenic effect for TGF-β (15, 16, 20–22), we failed to find an anabolic effect for ALK5$^{CA}$ which is expected to mimic TGF-β signaling, on the chondrogenic differentiation of SFs. We cannot fully explain the discrepancy between our results and those of previous studies, but Robbins and coworkers recently reported that adenovirus vector-mediated TGF-β gene transduction into arthritic joints in fact exacerbated cartilage degradation (38), raising the possibility that sustained activation of TGF-β signaling, via ALK5, has instead a negative effect on chondrogenesis. Further study will be required to elucidate the difference between TGF-β and BMP signaling.

The signaling of TGF-β/BMPs is transduced by Smad family members (31, 34, 35). Receptor-regulated Smads (R-Smads) are direct substrates of type I receptors and are phosphorylated at the C-terminal SSV/MS motif. R-Smads then form heteromeric complexes with common-mediator Smads and translocate into the nuclei, where they regulate transcription of target genes. In addition to Smad pathways, there is evidence that MAP kinase cascades are also implicated in ALK signaling, in which TGF-β–activating kinase (TAK1), a member of the MAP kinase kinase kinase family, plays a key role. TAK1 activates MAP kinase kinase in combination with an adaptor molecule, TAB1, which leads to JNK and p38 activation (32). The role of p38 in chondrogenesis has recently attracted particular interest because p38 inhibitors such as SB203580 suppress the chondrogenic differentiation of ATDC5 cells induced by growth/differentiation factor-5 (33, 39). However, the exact roles of the Smad pathways and p38 pathways in chondrocyte differentiation are not yet fully clarified. We used a combination of adenoviral gene delivery and a chemical inhibitor to segregate the roles of these two pathways downstream of ALK3 activation and found that (a) inhibitory Smad (Smad6) expression or treatment with the p38 inhibitor SB203580 suppressed the effect of ALK3$^{CA}$ expression (Figure 2) (b) Smad1 synergistically augmented the effect of ALK3$^{CA}$ (Figure 5A), and (c) activation of p38 pathways alone by MKK6$^{CA}$ expression induced the hypertrophic differentiation markers type X collagen and mineralization in SFs, which was suppressed by Smad1 coexpression (Figures 5B and 6). These results suggest that although both Smad and p38 activation is necessary for chondrogenic differentiation of SFs, sustained activation of p38 pathways alone prompts the terminal differentiation of the cells. Consistent with our results, Zhen et al. (40) reported that parathyroid hormone inhibits type X collagen expression in hypertrophic chondrocytes by suppressing p38 pathways. Von der Mark et al. (41) reported the focal appearance of type X collagen in osteoarthritic cartilage, which may be involved in the degenerative changes of the articular cartilage and in the pathogenesis of osteoarthritis. Using the mouse model of osteoarthritis, we found that activated p38 is associated with type X colla-

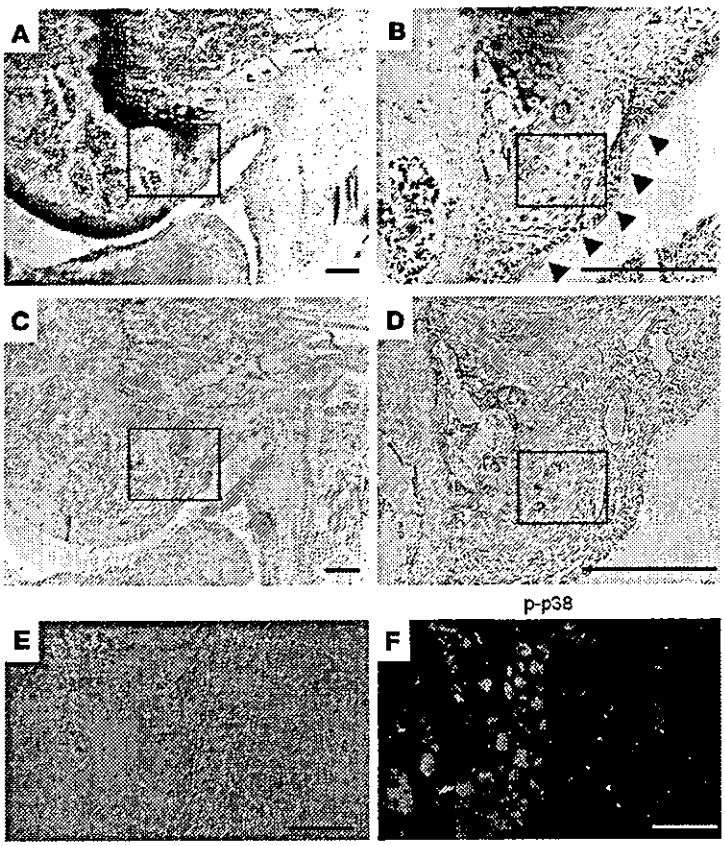


**Figure 7**
Histological analysis of knee joints in the mouse ACL and MM resection model. (**A–F**) Toluidine blue staining (**A** and **B**) and type X collagen immunostaining (**C** and **D**) at the marginal area between the articular cartilage and synovium. **B** and **D** present higher-magnification views of **A** and **C**, respectively. Osteochondrophytes were formed at the posterior edge of the femoral condyle, and they were positively stained by anti–type X collagen as well as toluidine blue (rectangular areas in **A** and **C**). Clusters of migrating synovial cells were observed adjacent to the osteochondrophytes (**B**, arrowheads) where future osteochondrophytes will develop, and they were positively stained by anti–type X collagen at the marginal area between synovium and osteophytes (rectangular area in **D**). This region was also positively stained by anti–phospho-p38 (**F**). **E** and **F** represent phase-contrast microscopy (**E**) and immunostaining with anti–phospho-p38 (**F**) of the rectangular area in **D**. Positive phospho-p38 staining was observed at the area of osteochondrophytes as well as the marginal synovium. Scale bars: 500 μm (**A–D**) and 50 μm (**E** and **F**).

gen expression in the synovial tissues adjacent to osteochondrophytes as well as in the degenerative cartilage (Figure 7).

Smad pathways not only are required for chondrogenic differentiation of SFs but also critically regulate the stage of differentiation of the cells and suppress their terminal differentiation process. Consistent with our findings, Scharstuhl recently reported inhibitory action of Smad7 in TGF-β–induced chondrocyte proliferation and proteoglycan production (42), indicating a critical role for Smad pathways. Hidaka and coworkers (43) demonstrated that adenovirus vector–mediated BMP-7 expression in chondrocytes accelerates the cartilage repair process. More recently, Lories and colleagues (44) demonstrated that BMP-2 and BMP-6 expressed in arthritic synovium are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis, and Fukui et al. (45) found that BMP-2 expression was increased by proinflammatory cytokines in normal and osteoarthritis chondrocytes. These findings, combined with our observations, suggest that although BMPs have favorable effects on the repair process of articular cartilage, they may have proapoptotic and/or degenerative effects on the cells when p38 pathways are overactivated. Our findings suggest an important role for p38 signal transduction pathways in chondrocytes and SFs, leading to degenerative joint disorders, and suggest the potential utility of p38 modifiers in the treatment of rheumatoid arthritis and/or osteoarthritis. In fact, p38 kinase modifiers are now in clinical trials to treat rheumatoid arthritis (46). Based on our observations, we would like to propose that SFs are an excellent source for chondroprogenitors, which can be differentiated into chondrocytes via ALK3 activation, and that activation of the Smad pathway while controlling the degree of p38 activation may be a way to generate committed chondrocytes for the repair and/or replacement of cartilage.

## Acknowledgments

The authors thank R. Yamaguchi and M. Ikeuchi (Department of Orthopaedic Surgery, The University of Tokyo), who provided expert technical assistance; K.L. Insogna (Yale University) for critical reading of the manuscript; and Y. Iwamoto (Thomas Jefferson University) for type II collagen and aggrecan probes. ALK and Smad adenovirus vectors were kindly provided by K. Miyazono (The University of Tokyo) and T. Imamura (The Cancer Institute of the Japanese Foundation for Cancer Research). This work was in part supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Health Science research grants from the Ministry of Health and Welfare of Japan and an Uehara Memorial Award to S. Tanaka.

Received for publication August 28, 2003, and accepted in revised form January 6, 2004.

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DOI 10.1007/s10165-004-0358-6



REVIEW ARTICLE

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# Intracellular signal transduction pathways: good therapeutic targets for joint destruction in rheumatoid arthritis

**Abstract** Preventing joint destruction is one of the most challenging issues in treating patients with rheumatoid arthritis (RA), and I propose that intracellular signaling pathways in osteoclasts and synovial fibroblastic cells (SFCs) can be good therapeutic targets. Osteoclasts are primarily involved in the bone destruction in RA joints, and SFCs support osteoclast differentiation and activation by producing various proinflammatory cytokines including receptor activator of NF-κB ligand (RANKL), the osteoclast differentiation factor belonging to the tumor necrosis factor-α superfamily. Suppressing c-Src pathways by adenovirus vector-mediated C-terminal Src family kinase (Csk) gene or Ras/extracellular-regulating kinase (ERK) pathways by introducing dominant negative Ras ($Ras^{DN}$) adenovirus reduced osteoclastic bone resorption as well as the abnormal proliferation and interleukin-6 production of SFCs, and the local injection of these viruses ameliorated the joint destruction in adjuvant arthritis rats. Moreover, chondrogenic differentiation of SFCs could be induced by stimulating activin receptor-like kinase 3 pathways.

**Key words** Adenovirus · Osteoclast · Rheumatoid arthritis (RA) · Synovial fibroblast cells (SFCs)

## Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder with an unknown etiology characterized by the invasive synovial hyperplasia leading to the progressive joint destruction.[1] Radiographic studies have shown that the bone erosion in RA begins at the early stage of the disease, and gradually exacerbates. Bone erosion results in the severe deformity of the affected joints and impairs the normal activity and the quality of life of the RA patients, and hereby, preventing such devastating states is one of the most challenging issues in treating them. Because the exact etiology and pathology of RA remains unknown, most treatments of RA just treat symptoms of the disease. Non-steroidal anti-inflammatory drugs, including cyclooxygenase 2 inhibitors, have been prescribed to reduce the painful symptoms of the disease, but they have little effect on stopping the progression of the joint destruction. Recent studies have revealed that some disease-modifying antirheumatic drugs and biological agents such as anti-tumor necrosis factor (TNF)-α antibody ameliorate the progression of the joint destruction in RA.[2] However, the bone-protective effect of these reagents is limited in most cases, and their long-term effects have not been established yet. Moreover, the prolonged usage of these medicines sometimes causes severe side effects. Therefore, novel therapeutic interventions specifically targeting the joint destruction in RA are greatly expected.

Proliferating synovium produces an elevated amount of proinflammatory cytokines interleukin (IL)-1, IL-6, IL-17, and TNF-α, and matrix-degenerating enzymes matrix metalloproteinases and cathepsins, which are involved in the bone and cartilage destruction.[1,3] Considerable data have demonstrated that synovial fibroblastic cells (SFCs), type B synovial cells with fibroblastic morphology, are one of the principal cells implicated in the arthritic conditions in RA.[4] In RA, SFCs markedly increase in number and display transformed phenotypes, and the activation of various protooncogenes including *myc*, *ras*, and *fos* is involved in the abnormal growth rate and transcriptional activity of the cells.[5] Bone erosion usually begins at the interface of the cartilage and the proliferating synovium, and bone-resorbing osteoclasts can be observed at the erosive synovium/bone interfaces in RA joints. Accumulating evidence has revealed that osteoclasts, primary cells responsible for bone resorption, are involved in the bone destruction in RA, and recent progress in the molecular biology and biochemistry has elucidated the molecular mechanism of the osteoclast differentiation and activation. In contrast to such catabolic

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actions, recent studies have revealed that synovial cells have anabolic effects on the joint homeostasis, and differentiate into osteoblasts or chondrocytes under proper conditions.

In this review, I would like to introduce the role of osteoclasts in the joint destruction in RA, and the critical involvement of SFCs on the osteoclast differentiation in RA. Adenovirus vectors can efficiently transduce osteoclasts and SFCs both in vitro and in vivo. By modulating intracellular signaling pathways in these types of cells using adenovirus vectors, we could regulate the joint destruction in the experimental animal models of arthritis. We also successfully induced chondrogenic differentiation of SFCs both in vitro and in vivo by stimulating transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) signaling pathways.

## Involvement of osteoclasts in bone destruction in RA

The cellular mechanism underlying the bone and cartilage destruction in RA is still unclear, but emerging evidence has revealed the essential role of osteoclasts. Bromley and Woolley[6] observed a number of acid phosphatase-positive multinucleated cells (chondroclasts and/or osteoclasts) in the erosive joint areas of RA patients. Gravallese et al.[7] found that multinucleated cells present on erosive bone surface and in the areas of the direct invasion of pannus into the subchondral bone. Abundant multinucleated giant cells were also observed at the bone-pannus interfaces of arthritic joints in collagen-induced arthritis rats.[8] 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.[7] Interestingly, some multinucleated cells and mononuclear cells apart from the bone surface were also TRAP-positive, suggesting the possible involvement of synovial tissues in the osteoclastogenesis in RA. To analyze the osteoclastogenic potentiality of RA synovial tissues, synovial cells were isolated from RA synovium at the time of knee replacement surgeries, and the cells were cultured in the presence of 1α,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] and macrophage colony-stimulating factor (M-CSF). After 3 weeks of culture, there appeared many multinucleated giant cells, which were TRAP-positive, possessed abundant calcitonin receptors, and made resorption pits on dentine slices.[9] We also found that peripheral monocytes can differentiate into osteoclast-like cells when cocultured with SFCs. These results suggest that RA SFCs can support osteoclast differentiation from monocyte-macrophage lineage precursor cells.

## Role of RANKL/RANK pathways in bone destruction in RA

Receptor activator of NF-κB ligand (RANKL) is a member of the TNF superfamily cytokines, which was originally identified as a membrane-bound survival factor for dendritic cells produced by activated T cells.[10,11] The expression of RANKL is also induced in osteoblasts or bone marrow stromal cells by various hormones or cytokines. In cooperation with M-CSF, RANKL stimulates osteoclast differentiation from hematopoietic precursor cells in vitro.[12] RANKL also acts on mature osteoclasts and promotes their bone-resorbing activity and survival. RANKL binds to its specific receptor RANK, the type I membrane receptor belonging to TNF receptor superfamily. RANK is expressed in wide range of cells including monocyte-macrophage lineage osteoclast precursor cells, mature osteoclasts and dendritic cells. Upon binding to its ligand RANKL, RANK recruits an adaptor molecule TNF receptor-associated factor (TRAF) 6, which subsequently activates downstream signaling pathways NF-κB, c-Jun N-terminus kinase (JNK), p38 mitogen-activated protein (MAP) kinase, and nuclear factor of activated T cells (NFAT) c1.[13] Another important actor in the RANKL/RANK pathway is osteoprotegerin (OPG), a soluble decoy receptor of RANKL which belongs to TNF receptor superfamily.[14,15] Osteoprotegerin specifically binds to RANKL and inhibits RANKL activity by competitively preventing its binding to RANK.

The essential role of RANKL/RANK signaling pathways in osteoclast development was further established by a series of gene knockout mice.[16] 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.[11,16] We and another group found that mice deficient in TRAF6 also showed osteopetrotic phenotypes.[17] In contrast, OPG-deficient animals demonstrated osteopenia due to the increased number and activity of osteoclasts.[18,19] These results clearly demonstrate the essential role of RANKL/RANK pathways in osteoclast development and activation in vivo.

Not only is the RANKL/RANK pathway critical for normal bone development and growth, but also is implicated in the pathological bone resorption observed in RA (Fig. 1). We and other groups found a high level expression of RANKL in RA synovial tissues.[20,21] Enhanced expression of RANKL is observed in SFCs as well as in $CD4^+$ T lymphocytes in synovial tissues of collagen-induced arthritis rats as shown by in situ hybridization.[22] Expression of RANKL is increased by T-cell proinflammatory cytokine IL-17, and IL-17 enhanced RANKL expression and strongly upregulated the RANKL/OPG ratio in the synovium.[23] Osteoprotegerin treatment ameliorates the arthritic bone destruction in adjuvant arthritis rats[24] and TNF-α transgenic animals,[25] and the bone erosion in serum transfer-induced arthritis was markedly reduced in RANKL-deficient animals.[26] Recent studies also demonstrated that the systemic bone loss as well as the local bone erosion in TNF-α transgenic mice was reversed by OPG injection in combination with anti-TNF-α antibody therapy.[27,28] These studies indicate that RANKL produced by SFCs and/or activated T lymphocytes in RA synovial tissues plays an essential role in the osteoclast development and the joint destruction, and therefore, the RANKL/RANK pathway can be a good therapeutic target.

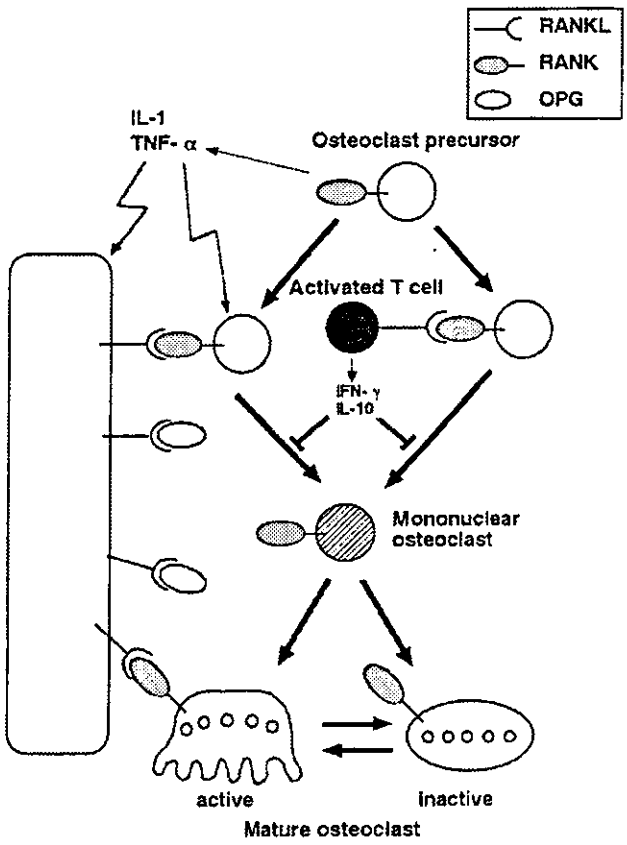


Fig. 1. Involvement of receptor activator of NF-κB ligand (*RANKL*)–receptor activator of NF-κB (*RANK*) pathways in osteoclast differentiation and bone destruction in RA. RANKL is highly expressed in synovial fibroblastic cells (SFCs) and activated T cells, and binds to its specific receptor RANK, which is expressed in monocyte-macrophage lineage osteoclast precursor cells. The interaction between RANKL and RANK is blocked by osteoprotegerin (*OPG*), a physiological inhibitor of RANKL. *IL*, interleukin; *TNF*, tumor necrosis factor; *IFN*, interferon

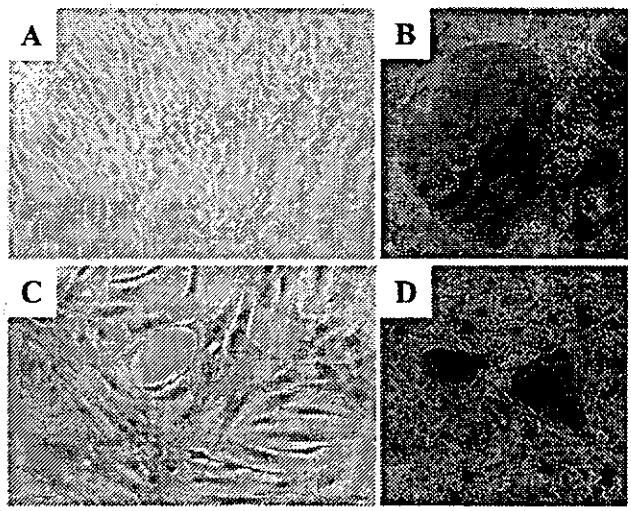


Fig. 2A–D. Effective gene transduction into SFCs and mature osteoclasts by adenovirus vectors. Human SFCs obtained from synovial tissues of rheumatoid arthritis patients (**A** and **C**) or osteoclast-like cells from giant cell tumors (**B** and **D**) were infected with the control virus (**A** and **B**) or LacZ virus (**C** and **D**), and stained for β-galactosidase activity 1 day after infection. Both SFCs and osteoclasts infected with LacZ virus were positively stained, indicating an efficient gene transduction

## Efficiency of adenovirus vectors in transducing SFCs and osteoclasts

As mentioned above, osteoclasts play pivotal roles in the bone and joint pathology in RA, and SFCs support osteoclast differentiation and activation by producing RANKL. Therefore, pharmacological agents targeting these cells can be potent therapeutic candidates for the treatment of 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 United States.[29,30] The proper selection of the target cells and target genes has been a continuous matter of interest for successful gene therapies. The target cells for the RA gene therapy include SFCs and osteoclasts, and we previously reported that adenovirus vectors efficiently transduce foreign genes into these cells both in vitro and in vivo.[31,32] As shown in Fig. 2, recombinant adenovirus vectors carrying the *lacZ* gene can infect human SFCs and osteoclast-like cells obtained from giant cell tumors.[31,32] At a multiplicity of infection of 100, almost 100% of SFCs and more than 85% of osteoclast-like cells were positively stained by β-galactosidase (β-gal) activity with no apparent morphological changes or cellular toxicity. When injected into knee joints of adjuvant arthritis rats, synovial lining cells and osteoclasts present on bone surface were positively stained for β-gal activity (Fig. 3).[32] These results suggest that the adenovirus vector system is suitable for gene therapies targeting SFCs and osteoclasts. As for target molecules, we focused on the intracellular signaling pathways which are important for both SFCs and osteoclasts, i.e., c-Src pathways and Ras/ERK pathways.

## Adenovirus vector-mediated regulation of c-Src pathways in SFCs and osteoclasts

c-Src was first identified as the normal cellular counterpart of the transforming protein encoded by Rous sarcoma virus, v-Src.[33] The protooncogene product c-Src is a 60 kDa protein and belongs to non-receptor-type tyrosine kinase family, i.e., Src family tyrosine kinase family. The *c-src* protooncogene is highly conserved throughout evolution and widely expressed. 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 mecha-

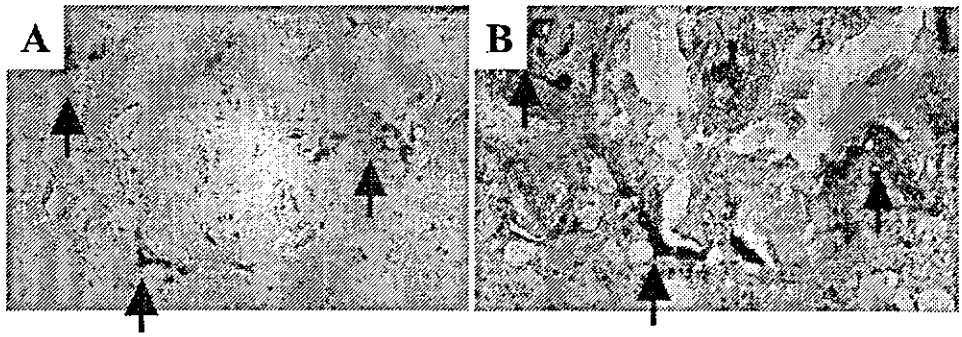


**Fig. 3A,B.** Adenovirus-mediated gene transduction in osteoclasts in vivo.[41] LacZ virus was injected into the inflammatory ankle joint of an adjuvant arthritis rat, and the expression *lacZ* gene in osteoclasts was determined in the serial sections by enzyme histochemistry of β-galactosidase (**A**) and tartrate-resistant acid phosphatase (TRAP) (**B**) after 1 week of the viral injection. Most of the TRAP-positive osteoclasts were positively stained for β-galactosidase activity

nisms that contribute to the regulation of cell growth and development.[33] The physiological role of the c-*src* gene had not been clarified until Soriano et al. successfully performed the targeted disruption of the gene by homologous recombination in mouse embryos in 1991.[34] To everyone's surprise, the mice showed striking skeletal abnormalities with a phenotype of osteopetrosis. In vitro osteoclast formation experiments and in vivo bone marrow transplantation studies have revealed that osteoclast differentiation was not impaired, but that bone-resorbing activity of mature osteoclasts was much reduced in c-*src* knockout (KO) mice.[35,36] The morphological feature of the KO osteoclasts was their disorganized ruffled border structure.[36] The 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. c-Src is highly expressed in osteoclasts, and highly concentrated on ruffled border membranes and intracellular membranes.[37,38] The fact that no other abnormalities in c-*src* KO mice were found outside the skeletal tissues leads us to consider that c-Src can be an ideal therapeutic target for suppressing pathological bone resorption by inhibiting osteoclast function without affecting other tissues or cells.

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.[39] Dephosphorylation of this residue causes a 10- to 20-fold increase in the kinase activity of c-Src. C-terminus Src family kinase (Csk) is a cytoplasmic tyrosine kinase which specifically phosphorylates Tyr527 of c-Src, thereby negatively regulate its kinase activity.[40] To regulate c-Src kinase activity in SFCs and osteoclasts, we constructed adenovirus vectors encoding *csk* gene (Csk virus). Csk virus efficiently infected SFCs and osteoclasts, and dose-dependently inhibited the kinase activity of c-Src in these cells.[41] Adenovirus vector-mediated Csk overexpression in RA SFCs suppressed the proliferation of the cells, and reduced their IL-6 production.[32] Csk virus also induced dramatic cytoskeletal disorganization in osteoclasts, and strongly inhibited pit formation on dentine slices.[41]

## Important role of Ras/ERK pathways in SFC activation and osteoclast survival

The other signaling pathway we focused on as a therapeutic target is the Ras/ERK pathway. Small GTPase Ras, the protein product of proto-oncogene *ras*, is ubiquitously found in eukaryotic organisms.[42] Ras is known to function as a downstream effector of cell surface receptor tyrosine kinases (RTKs) and leads to the activation of ERK pathways, which in turn regulates the activities of nuclear transcription factors and gene transcriptions. In human cancer cells, oncogenic mutations of Ras protein are frequently observed and contribute to the malignant growth properties of the cells. In RA and animal models of arthritis, synovial cells with large pale nuclei, prominent nucleoli and abundant cytoplasm are found adjacent to the affected cartilage and bone of the joint, and these cells in culture have a tendency to grow in disorganized monolayers, proliferate in an anchorage-independent manner, lack contact inhibition, and form microfoci, exhibiting a morphologically transformed appearance.[4] Although the expression of Ras and its oncogenic mutations were reported in RA synovial cells, the precise role of Ras in RA pathology remains unclear.[43] To analyze the role of Ras and its downstream signaling in osteoclasts as well as in SFCs, we constructed a replication-deficient adenovirus vector carrying the dominant negative mutant of *ras* gene ($Ras^{DN}$). In SFCs, adenovirus-mediated overexpression of $Ras^{DN}$ dramatically decreased the proliferation rate of the cells. Interleukin-1-induced upregulation of IL-6 production was also decreased by the viral infection, which was supposedly mediated by the downregulation of IL-1-induced ERK activation.[44]

In addition, the life span of osteoclasts was markedly decreased by the adenovirus, while activating Ras/ERK pathways by constitutively active mutant of ERK expression prolonged the survival of osteoclasts.[45] These results suggest that Ras/ERK pathways are critically involved in SFC activation and osteoclast survival.

## Amelioration of arthritic bone destruction by adenovirus vector-induced gene expression

The efficient in vivo gene delivery to synovial cells by local administration of adenovirus vectors has been well established.[32,46] In addition, TRAP-positive osteoclasts along the erosive bone surface demonstrated strong β-gal staining as shown in the serial tissue sections, indicating that intra-articular injection of adenovirus vectors can transduce osteoclasts in vivo (Fig. 3). The effect of Csk and $Ras^{DN}$ adenovirus administration into inflammatory ankle joints of adjuvant arthritis rats was investigated.[32,44] Not only was the bone destruction by osteoclasts suppressed by Csk or $Ras^{DN}$ virus injection, but also the synovial inflammatory reaction detected by arthritis score or paw swelling was reduced (Fig. 4). These results lead us to conclude that regulating c-Src and/or Ras/ERK pathways in SFCs and osteoclasts can be a novel therapeutic approach to treat RA joint destruction (Fig. 5).

## Stimulating chondrogenic differentiation of SFCs

In contrast to such catabolic actions, recent studies have revealed that SFCs have anabolic effects, leading to the bone and cartilage production. Hunziker and Rosenberg[47] reported that synovial cells can migrate into partial-thickness articular cartilage defects, where they proliferate and subsequently deposit a scar-like tissue. Nishimura et al.[48] demonstrated SFCs to show chondrogenic differentiation after being cultured in the presence of TGF-β, and De Bari et al.[49] recently demonstrated that multipotent mesenchymal stem cells were isolated from human synovial tissues, which differentiated into chondrocytes as well as osteoblasts, adipocytes, and myotubes under proper culture conditions. These observations lead us to speculate that synovial tissues contain multipotent cells with osteogenic and/or chondrogenic potential that can be involved in the repair process of destroyed joints and therefore might provide a good source for engineering the bone and cartilage.

There is accumulating evidence that TGF-β superfamily cytokines play an essential role in bone and cartilage development. We analyzed the role of TGF-β/BMP signaling on chondrogenic differentiation of human SFCs, and found that the introduction of an activated mutant of ALK3 (con-

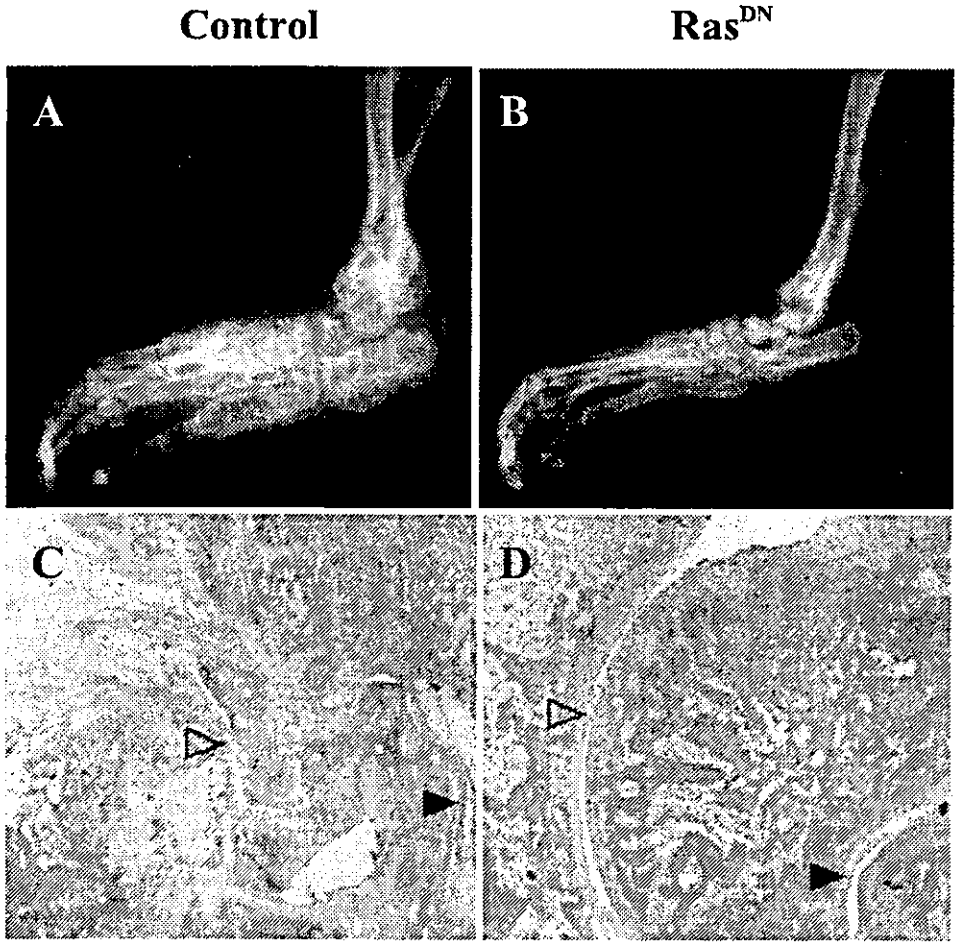


**Fig. 4A–D.** Therapeutic effects of dominant negative Ras (*$Ras^{DN}$*) adenovirus injection on rat adjuvant arthritis. **A,B** The radiological findings of the ankle of LacZ virus- **(A)** and $Ras^{DN}$ virus- **(B)** injected rats. Severe joint destruction could be seen in LacZ virus-injected rats, while $Ras^{DN}$ virus-injected ankle joints show minimal destructive changes. **C** Pathohistological findings of the LacZ virus-injected ankles show synovial hyperplasia and destructive change of articular cartilage and bone. *Open arrowhead* and *closed arrowhead* indicate talo-tibial and talo-calcaneal joint, respectively. **D** Pathohistological findings of the $Ras^{DN}$ virus-injected ankle. Synovial hyperplasia with invasion into subchondral bone and the destruction of bone and cartilage were markedly suppressed. **C,D**: H&E staining

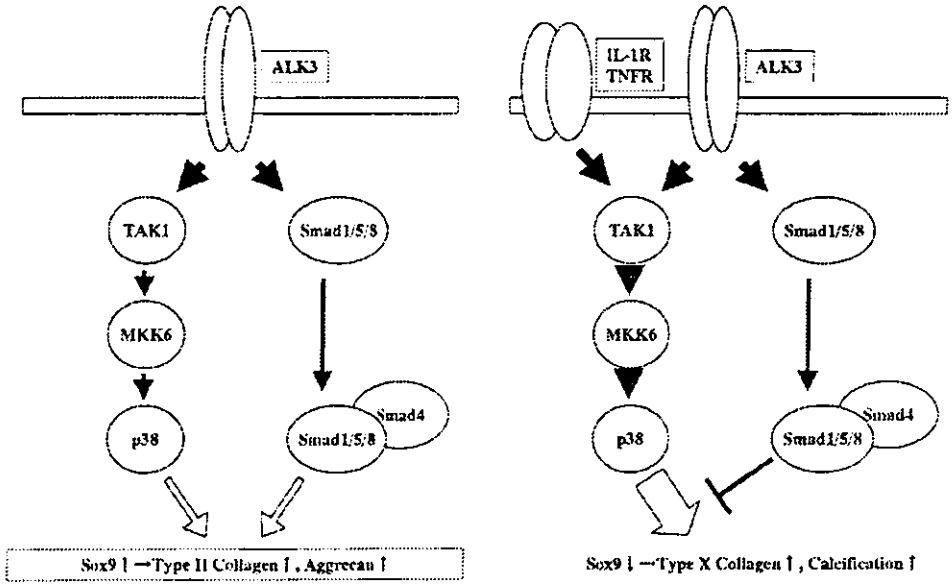


**Fig. 7.** The role of the Smad pathway and p38 mitogen-activated protein kinase pathway on chondrogenic differentiation of SFCs. Although both pathways are necessary for chondrocyte-specific marker expression in the cells (*left panel*), overactivation of p38 pathways alone lead to the terminal chondrocytic differentiation of the cells, leading to the articular cartilage degeneration. The proper balance between these two pathways is required for maintaining the articular cartilage integrity. *IL*, interleukin; *TNFR*, tumor necrosis factor receptor; *ALK*, activin receptor-like kinase

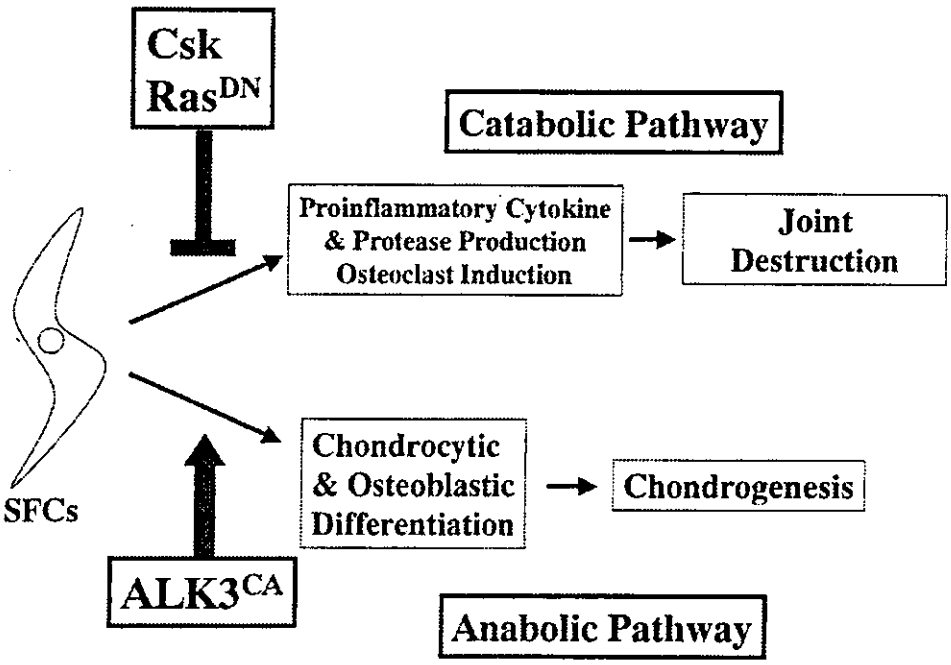


**Fig. 8.** Schematic representation of the therapeutic strategies targeting synovial fibroblastic cells (*SFCs*). Suppressing Src pathways by C-terminal Src family kinase (*Csk*) expression or Ras/ERK pathways by dominant negative Ras ($Ras^{DN}$) expression suppresses the catabolic pathways of the cells which lead to the bone and joint destruction in RA, while stimulating ALK3 pathways activates anabolic pathways leading to chondrogenic differentiation of the cells. $ALK3^{CA}$, constitutively active activin receptor-like kinase

differentiation of SFs, but also regulate the stage of differentiation of the cells and suppress their terminal differentiation process. It should be noted that the proinflammatory cytokines IL-1 and TNF-α, which are known to have catabolic effects on joint integrity, induced p38 activation in SFs.[52] These cytokines may stimulate terminal chondrogenic differentiation of SFs which are involved in the repair process, and lead to the articular cartilage degradation. Based on our observation, we would like to propose that SFs are an excellent source from which to obtain chondroprogenitors, which can be differentiated into chondrocytes via ALK3 activation, and that stimulating Smad pathways and controlling p38 activation to the proper level can be a good therapeutic strategy for maintaining the healthy joint homeostasis and treating degenerative joint disorders.

## Concluding remarks

The ultimate goal of the treatment of RA is to preserve the daily activity of the patients by preventing bone and joint destruction. Recent studies have revealed that osteoclasts are involved in the pathogenesis of the bone and joint destruction in RA, and SFCs are critically involved in the differentiation and activation of osteoclasts by producing various catabolic factors including TNF-α, IL-1, and RANKL, which makes osteoclasts and SFCs good therapeutic targets for bone and joint destruction in RA. We demonstrated that suppressing Src pathways by introducing Csk and/or Ras/ERK pathways by the $Ras^{DN}$ adenovirus in osteoclasts reduces bone resorption both in vitro or in vivo by suppressing osteoclast activity or survival (Fig. 5). Csk virus and $Ras^{DN}$ virus also suppress catabolic actions of SFCs by inhibiting abnormal proliferation of the cells and their IL-6 production (Fig. 5). We also succeeded to stimulate chondrogenic differentiation of SFCs by introducing $ALK3^{CA}$, and the proper balance of the Smad pathway and the p38 MAP kinase pathway is critical downstream of ALK3. Based on these observations, I would like to propose that modulating intracellular signaling in osteoclasts and/or SFCs by adenovirus vectors can be good therapeutic approach for treating RA patients with bone and joint destruction (Fig. 8).

Of course, we have to realize the disadvantages as well as advantages of using adenovirus vectors as therapeutic reagents.[53] The disadvantages of the adenovirus vectors include the transient gene expression because they do not integrate the transgene into target cell chromosome, immunological reaction such as neutralizing antibody response and cytotoxic T-lymphocyte responses against the virus, and the dissemination of the vectors from the site of local injection.[54] The safety issue is particularly important regarding its clinical application, and in fact, the first case of fatality induced by the infusion of adenovirus vectors into hepatic artery was recently reported. Therefore, development of a new generation of adenovirus vectors or finding substitutes for gene therapy is absolutely necessary for the clinical application of the signaling molecule-targeting strategies.

**Acknowledgments** This work was in part supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health Science Research Grants from the Ministry of Health, Labour and Welfare, to S.T.

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Osteoporos Int (2005) 16: 64–70
DOI 10.1007/s00198-004-1642-1

ORIGINAL ARTICLE

# Significance of intima-media thickness in femoral artery in the determination of calcaneus osteo-sono index but not of lumbar spine bone mass in healthy Japanese people

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Received: 16 April 2003 / Accepted: 19 March 2004 / Published online: 27 May 2004


**Abstract** The aim of this cross-sectional study was to investigate whether physical activity and local arterial thickening may affect bone metabolism. To analyze the effects of physical activity and atherosclerosis on bone in healthy Japanese people, health-related quality of life (HRQL) and local arterial thickening were assessed by means of the MedicalOutcomes Study 36-item Short Form (SF-36), and intimal-medial thickness (IMT) in common carotid artery (CA) and femoral artery (FA), respectively. Bone mineral density (BMD) in lumbar spine was measured by dual X-ray absorptiometry and the osteo-sono assessment index (OSI) of the calcaneus by ultrasound. Healthy subjects (106 male and 154 female) were recruited from those who participated in a local health check program at the Osaka City University Hospital. A significant correlation existed between lumbar spine BMD and calcaneus OSI ($r=0.551$, $P<0.0001$). Among various scores in SF-36, only physical functioning score correlated weakly but significantly in a positive manner with lumbar spine BMD ($\rho=0.156$, $P=0.0147$) and calcaneus OSI ($\rho=0.190$, $P=0.0024$). Lumbar spine BMD correlated negatively with FA IMT ($\rho=-0.191$, $P=0.0027$) whereas calcaneus OSI with FA IMT ($\rho=-0.199$, $P=0.0014$). Multiple regression analyses revealed a significant association between FA IMT and calcaneus OSI, whereas lumbar spine BMD did not correlate significantly with FA or CA IMT. When subjects were restricted to female, FA IMT, but not CA IMT, still showed tendency against independent factors negatively associated with calcaneus OSI. Furthermore, lumbar spine BMD, but not calcaneus OSI, was weakly but significantly associated with increased physical functioning score independently. In conclusion, it was suggested that physical activity may affect bone strength in lumbar spine and calcaneus and that FA IMT might be a significant determinant of bone strength in calcaneus, but not in lumbar spine, in healthy Japanese subjects.

**Keywords** ADL · Atherosclerosis · Bone mineral density · Osteoporosis · Quality of Life

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## Introduction

In healthy individuals, the relationship between atherosclerosis and bone mass has not been extensively studied. The hypothesis that reduced blood flow to the lower extremities may affect bone remodeling, resulting in a decrease in BMD, has been proposed [1]. Prospective community-based study showed that decreased vascular flow in the lower extremities may be associated with an increased rate of bone loss at the hip and calcaneus among relatively healthy older women [2]. Measurement of the far-wall intima-media thickness (IMT) of the common carotid artery (CA) and femoral artery (FA) by high-resolution ultrasonography has been established as a clinically useful index for identifying early-stage general and local atherosclerosis in lower extremities [3, 4, 5, 6, 7, 8, 9], since CA IMT is strongly correlated with the presence of coronary artery diseases [3, 4, 5, 6, 7, 8, 9], and FA IMT with local atherosclerosis [9].

Physical activity has been identified as having a favorable effect on bone status [10, 11] and atheroscle-

rosis [12]. We have reported that, in patients with rheumatoid arthritis, physical activity assessed by the modified Health Assessment Questionnaire (M-HAQ) score [13] correlated significantly in a positive manner with the osteo-sono assessment index (OSI) of the calcaneus by ultrasound [14]. Recently, the Medical Outcomes Study 36-item Short Form (SF-36), which is a self-administered questionnaire containing 36 items that, when scored, yield eight domains considering physical, cognitive, emotional, and social aspects [15, 16], has emerged as an valuable index to assess health-related quality of life (HRQL) [17].

This background prompted us to examine the influence of general and local atherosclerosis in lower extremities as reflected by CA IMT and FA IMT, respectively, on calcaneus OSI in comparison with lumbar spine BMD in healthy Japanese subjects. Furthermore, the modulatory effect of physical activity, as reflected by SF-36 score, was investigated on bone quality in calcaneus and lumbar spine, and arterial thickening.

## Materials and methods

### Subjects

Healthy subjects ($n=260$), 106 males and 154 females, were recruited from people who participated in a local health check program at the Osaka City University Hospital after written informed consent was obtained. The mean ages of healthy subjects were 51.4 ± 12.5 years. Exclusion criteria are the subjects who are known to be suffering from any major disease which might affect atherosclerosis and bone metabolism, such as hypertension, hyperlipidemia, diabetes mellitus, cerebral vascular accident impairing seriously activity of daily life (ADL), osteoporosis, and osteomalacia. The subjects who have been continuously taking medicines were also excluded from the present study.

### Assessment of health-related quality of life (HRQL)

HRQL was assessed by means of SF-36 [15, 16]. The questionnaire consists of 36 items and measures three aspects of health: functional ability, well-being and overall health. These are quantified using eight multi-item domains (physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health). The physical functioning domain assesses limitations in physical activities such as walking and climbing stairs. The role-physical and role-emotional domains measure problems with work or other daily activities as a result of physical health or emotional problems. Bodily pain assesses limitations resulting from pain; vitality measures energy and tiredness. The social functioning domain examines the effect of physical and emotional health on normal social activities, and mental health assesses happiness, nervousness, and depression. The general health perceptions domain evaluates the personal opinion of one's health compared with that of one's peers, as well as the expectation of changes in health. All domains are scored on a scale from 0 to 100, with 100 representing the best possible health state. Two summary scales (physical and mental component) can also be derived [16, 18].

The SF-36 has been validated for use to assess HRQL in osteoporotic patients [2, 19].

### BMD measurement at lumbar spine

BMD was measured in the lumbar spine (L2–L4) in the anterior-posterior projection by dual-energy X-ray absorptiometry (DXA; QDR-4500A, Hologic Inc., Waltham, Mass., USA), essentially as previously described [20]. The precision of the measurement of lumbar spine BMD using DXA was less than 1.8%.

### Quantitative ultrasound assessment of calcaneus

Quantitative ultrasound assessment of calcaneus was performed using an ultrasound system [Acoustic Osteo-Screener (AOS-100), Aloka Co. Ltd, Tokyo, Japan], as previously described [12, 14]. Briefly, the AOS-100 measures both speed of sound (SOS) and an attenuation-related parameter called the transmission index (TI). These measurements yield a derived parameter, the osteo sono-assessment index (OSI), which has been proposed to be an estimate of the elastic modulus of the calcaneus [22]. Precision of the OSI parameter was 2.2% [23].

### Ultrasonographic examination of CA and FA IMT

Ultrasonographic examination of the CA and FA was performed in the supine position by high-resolution ultrasonography with a 10 MHz in-line Sectascanner (SSD 610 CL; Aloka), as previously described [12, 21, 24, 25, 26, 27]. To avoid inter-observer variability, all measurements were performed by the same examiner (H.Y.) who was unaware of subject characteristics. Briefly, CA and FA were scanned at the level of the bifurcation on both the right and left sides. IMT was measured in the far wall of the CA and FA at sites of the most advanced arterial thickening as diffuse and continuous projection with the greatest distance between the lumen-intimal interface and the media-adventitial interface but without atherosclerotic plaque, which was defined as localized lesions of thickness $\geqq$2.0 mm, from digitized still images of the arteries during scanning [27, 28]. These interfaces were all manually traced on the same day to avoid possible variation during the study period and the mean value calculated as the mean of at least three still images obtained from the same section of the CA and FA [21, 24]. Reproducibility of the IMT measurement was acceptable as shown by coefficients of variation (CV) of

2.8% and 3.4% for CA IMT and FA IMT, respectively. These were calculated from the 40 measurements performed in 20 RA patients on two different occasions according to Bland and Altman [29] using the formula: $CV(\%) = 100(SD/\sqrt{2})/x$, where SD is the standard deviation of absolute differences between the two repeated measurements, and x is the pooled mean value.

### Statistical analysis

Values are expressed as mean ± SD unless otherwise indicated. Statistical analysis was performed with the Stat View V system (Abacus Concepts, Berkeley, Calif., USA) for the Apple computer. The correlation coefficients were calculated by Pearson and Spearman Rank correlation analyses due to abnormal distribution of various clinical variables. *P*-values of $< 0.05$ were considered as statistically significant. Multiple regression analysis was performed to assess independent association with lumbar spine BMD and calcaneus OSI.*P*-values of $< 0.05$ were considered as statistically significant.

## Results

### Clinical variables, IMTs and bone density of healthy subjects

Clinical characteristics of healthy subjects ($n = 260$, M/F 106/154 enrolled in this cross-sectional study are shown in Table 1. CA and FA IMT were 0.603 ± 0.166 mm and 0.840 ± 0.396 mm, respectively. Lumbar spine BMD and calcaneus OSI were 0.918 ± 0.151 g/cm$^2$ and 2.69 ± 0.38×10$^6$, respectively. IMT at CA and FA and physical functioning score did not differ significantly between male and female, although lumbar spine BMD and calcaneus OSI were significantly lower in female than in male. As shown in Fig. 1, calcaneus OSI correlated significantly by Pearson correlation analysis in a positive manner with lumbar spine BMD in total subjects ($r = 0.551, P < 0.0001$).

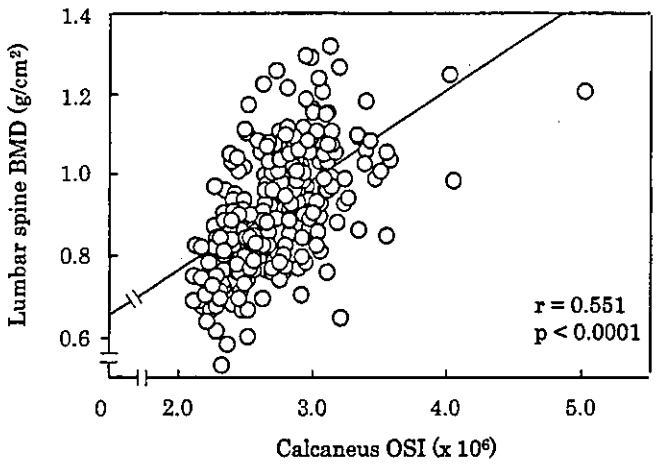


**Fig. 1** Positive correlation between lumbar spine BMD and calcaneus OSI in 260 healthy Japanese subjects. A significant positive correlation was found between lumbar spine BMD and calcaneus OSI ($r = 0.551$, $P < 0.0001$) by Pearson analysis

### Unadjusted domain scores of the eight subscales and adjusted domain scores of two summary scales of the SF-36 scores

Table 2 represents the HRQL scores assessed by SF-36. Adjusted domain scores of two summary scales were both around 50 points, confirming that the subjects enrolled in the present study have not been suffering from major health problems.

### Correlation of lumbar spine BMD and calcaneus OSI with the SF-36 score

Among the eight subscales and two summary scales of the SF-36 scores, physical functioning emerged as the only factor which correlated significantly by Pearson correlation analysis in a positive manner with both lumbar spine BMD (Fig. 2) and calcaneus OSI (Fig. 3).

**Table 1** Clinical characteristics of 260 healthy individuals enrolled in the present study. Data are expressed as mean ± SD, *BP* blood pressure, *LDL* low-density lipoprotein, *PF* physical functioning, *BMD* bone mineral density, *OSI* osteo-sono assessment index, *CA IMT* common artery intima-media thickness, *FA IMT* femoral artery intima-media thickness

| | Total | Male | Female |
|---|---|---|---|
| Number | 260 | 106 | 154 |
| Age (years) | 51.4 ± 12.5 | 47.4 ± 11.8 | 54.2 ± 12.2 |
| Body weight (kg) | 58.1 ± 11.3 | 67.1 ± 9.7 | 51.9 ± 7.5 |
| Height (cm) | 160.4 ± 9.6 | 169.4 ± 6.3 | 154.3 ± 6.1 |
| Body mass index (kg/m$^2$) | 22.5 ± 3.0 | 23.4 ± 2.7 | 21.8 ± 3.1 |
| Postmenopausal | – | – | 107 |
| Smoking index | 203.7 ± 371.4 | 461.2 ± 457.4 | 34.9 ± 140.6 |
| Smoking habit | 89 | 72 | 17 |
| Systolic BP (mmHg) | 123.9 ± 18.5 | 128.0 ± 15.3 | 121.1 ± 20.0 |
| Diastolic BP (mmHg) | 69.1 ± 10.6 | 71.2 ± 11.1 | 67.5 ± 10.0 |
| LDL-cholesterol (mg/dl) | 128.7 ± 32.4 | 121.0 ± 32.0 | 134.0 ± 31.7 |
| PF score (SF-36) | 90.9 ± 9.6 | 93.1 ± 8.2 | 89.5 ± 10.2 |
| Lumbar 2–4 spine BMD (g/cm$^2$) | 0.918 ± 0.151 | 0.979 ± 0.141 | 0.876 ± 0.145 |
| Calcaneus OSI (×10$^6$) | 2.69 ± 0.38 | 2.90 ± 0.39 | 2.55 ± 0.29 |
| CA IMT (mm) | 0.603 ± 0.166 | 0.600 ± 0.151 | 0.605 ± 0.176 |
| FA IMT (mm) | 0.840 ± 0.396 | 0.834 ± 0.398 | 0.845 ± 0.396 |

**Table 2** Unadjusted domain scores of the eight subscales and two summary scales of the SF-36 scores. Data are expressed as mean ± SD

| *Scores in SF-36* | |
|---|---|
| Physical functioning | 91.0 ± 9.6 |
| Role physical | 91.2 ± 22.8 |
| Bodily pain | 76.9 ± 19.3 |
| General health perceptions | 64.4 ± 16.3 |
| Vitality | 64.4 ± 20.4 |
| Social functioning | 88.2 ± 17.6 |
| Role emotional | 87.0 ± 28.0 |
| Mental health | 73.2 ± 19.0 |
| *Summary scales in SF-36* | |
| Physical components | 52.4 ± 7.1 |
| Mental components | 48.3 ± 9.3 |

## Correlations of lumbar spine BMD and calcaneus OSI with clinical variables including CA and FA IMT

Table 3 shows the summary of correlations of lumbar spine BMD and calcaneus OSI with clinical characteristics including IMTs at CA and FA by Spearman Rank correlation because of abnormal distributions of samples. Among the clinical variables included, gender, age, and BMI but not systolic blood pressure, were correlated significantly with both lumbar spine BMD and calcaneus OSI. Although FA IMT and physical functioning score of SF-36 correlated significantly with both lumbar spine BMD and calcaneus OSI, CA IMT did not correlate. Serum LDL cholesterol was negatively correlated with lumbar spine BMD but not calcaneus OSI.

## Multiple regression analysis of factors independently associated with lumbar spine BMD and calcaneus OSI in male plus female subjects

Table 4 represents the results of multiple regression analysis of various clinical variables to evaluate their independent association with lumbar spine BMD and

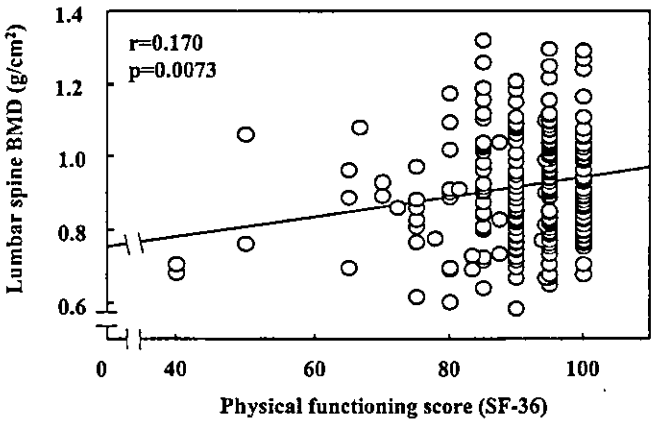


**Fig. 2** Correlation of physical functioning score of SF-36 with lumbar spine BMD in 260 healthy Japanese subjects. A significant positive correlation was found between physical functioning and lumbar spine BMD ($r = 0.170$, $P = 0.0073$) by Pearson analysis

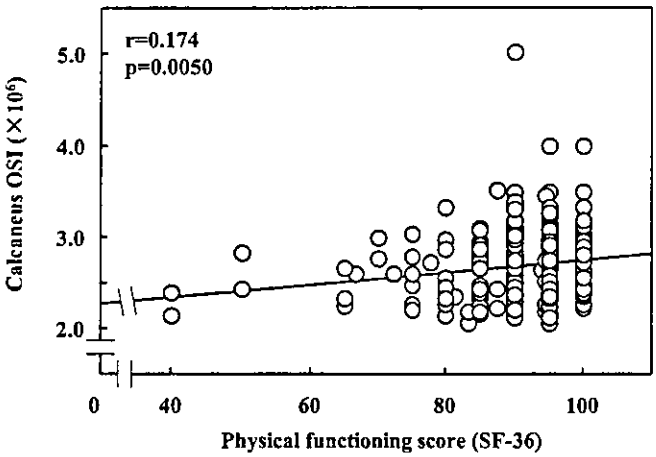


**Fig. 3** Correlation of physical functioning score with calcaneus OSI in 260 healthy Japanese subjects. A significant positive correlation was found between physical functioning and calcaneus OSI ($r = 0.174$, $P = 0.0050$) by Pearson analysis

**Table 3** Correlations of calcaneus OSI and lumbar spine BMD with clinical characteristics by Spearman rank correlation

| Clinical variables | Lumbar spine BMD | | Calcaneus OSI | |
|---|---|---|---|---|
| | $\rho$ | $P$ | $\rho$ | $P$ |
| Gender | −0.331 | < 0.0001‡ | −0.481 | < 0.0001‡ |
| Age (years) | −0.459 | < 0.0001‡ | −0.374 | < 0.0001‡ |
| Body mass index (kg/m²) | 0.186 | 0.0038‡ | 0.411 | < 0.0001‡ |
| Systolic BP (mmHg) | −0.622 | 0.6868 | 0.075 | 0.2273 |
| LDL-cholesterol (mg/dl) | −0.141 | 0.0266† | −0.071 | 0.2526 |
| PF score (SF-36) | 0.156 | 0.0147† | 0.190 | 0.0024‡ |
| CA IMT (mm) | −0.098 | 0.1225 | −0.017 | 0.7867 |
| FA IMT (mm) | −0.191 | 0.0027‡ | −0.199 | 0.0014‡ |

‡$P < 0.01$, †$P < 0.05$

**Table 4** Multiple regression analysis of factors independently associated with calcaneus OSI and lumbar spine BMD in male plus female. Values are standard regression coefficients ($\beta$). $R^2$ multiple coefficient of determination

| Independent variables | Lumbar spine BMD | | Calcaneus OSI | |
|---|---|---|---|---|
| | Model 1 | Model 2 | Model 1 | Model 2 |
| Gender | −0.038 | −0.040 | −0.273‡ | −0.272‡ |
| Age (years) | −0.470‡ | −0.442‡ | −0.312‡ | −0.263‡ |
| Body mass index (kg/m²) | 0.233‡ | 0.235‡ | 0.312‡ | 0.301‡ |
| Systolic BP (mmHg) | 0.035 | 0.051 | 0.005 | −0.007 |
| Smoking index | 0.025 | 0.023 | 0.042 | 0.077 |
| LDL-cholesterol (mg/dl) | −0.034 | −0.021 | 0.065 | 0.084 |
| PF score (SF-36) | 0.101§ | 0.102§ | 0.055 | 0.041 |
| CA IMT (mm) | −0.037 | – | 0.017 | – |
| FA IMT (mm) | – | −0.043 | – | −0.117† |
| $R^2$ | 0.296‡ | 0.296‡ | 0.355‡ | 0.365‡ |

‡$P < 0.01$, †$P < 0.05$, §$P < 0.10$

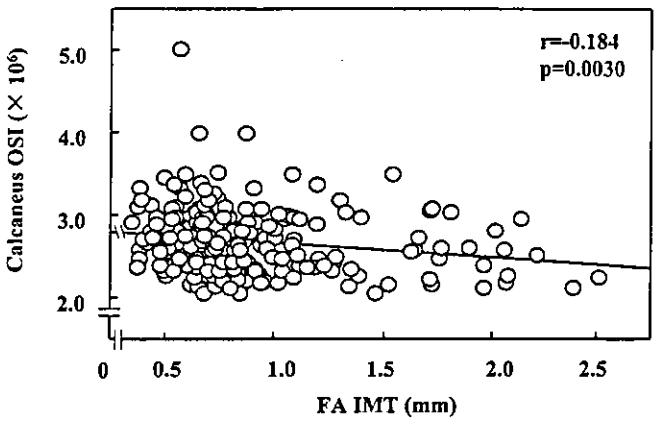


Fig. 4 Correlation of FA-IMT with calcaneus OSI in 260 healthy Japanese subjects. Negative correlation was found between FA-IMT and calcaneus OSI ($r=-0.184$, $P=0.0030$) by Pearson analysis

Table 5 Multiple regression analysis of factors independently associated with calcaneus OSI and lumbar spine BMD in female subjects. Values are standard regression coefficients ($\beta$). $R^2$ multiple coefficient of determination

| Independent variables | Lumbar spine BMD | | Calcaneus OSI | |
|---|---|---|---|---|
| | Model 1 | Model 2 | Model 1 | Model 2 |
| Age (years) | −0.203 | −0.190 | −0.170 | −0.069 |
| Body mass index ($kg/m^2$) | 0.196† | 0.190† | 0.370‡ | 0.351‡ |
| Menopause | −0.418‡ | −0.422‡ | −0.377‡ | −0.389‡ |
| Systolic BP (mmHg) | 0.089 | 0.086 | −0.001 | −0.017 |
| Smoking index | −0.068 | −0.066 | 0.091 | 0.116 |
| LDL-cholesterol (mg/dl) | 0.009 | 0.008 | 0.092 | 0.103 |
| PF score (SF-36) | 0.084 | 0.088 | 0.056 | 0.059 |
| CA IMT (mm) | −0.012 | – | 0.040 | – |
| FA IMT (mm) | – | −0.021 | – | −0.139§ |
| $R^2$ | 0.319‡ | 0.320‡ | 0.338‡ | 0.351‡ |

‡$P<0.01$, †$P<0.05$, §$P<0.10$

calcaneus OSI. In model 1, which included gender, age, BMI, systolic BP, smoking index, serum LDL cholesterol, physical functioning score of SF-36 and CA IMT, only age and BMI were independent factors significantly associated with lumbar spine BMD. There was a tendency toward higher physical functioning score in those with higher lumbar BMD. In case of calcaneus OSI, gender, in addition to age and BMI, emerged as the independently associated factor. CA IMT failed to be significantly associated with both lumbar spine BMD and calcaneus OSI. In model 2, which included FA IMT in the place of CA IMT, FA IMT, of great interest, emerged as an independent factor significantly associated with calcaneus OSI, but not lumbar spine BMD (Table 4 and Fig. 4).

Multiple regression analysis of factors independently associated with lumbar spine BMD and calcaneus OSI in female subjects

Table 5 represents the results of multiple regression analysis when the subjects were restricted to females only. The results were essentially the same, indicating the tendency towards independent association of FA IMT selectively with calcaneus OSI but not with lumbar spine BMD, although the association did not reach statistical significance ($P=0.0787$). It was reasonable that menopause was negatively associated with both lumbar spine BMD and calcaneus OSI.

## Discussion

Since the physical functioning domain of SF-36 scores assesses limitations in physical activities such as walking and climbing stairs, it is reasonable that it showed a significant and positive correlation with lumbar spine BMD and calcaneus OSI, since a large body of literature supports the positive impact of physical activity on bone mass, particularly in weight-bearing bones such as lumbar spine and calcaneus [30, 31, 32, 33, 34, 35, 36, 37]. By multiple regression analysis, physical functioning score showed a tendency of independently associating with lumbar spine BMD. However, it did not associate with calcaneus OSI, in spite of the positive impact on bone mass. One possibility for the lack of its positive associations with calcaneus OSI might be ascribed to the inclusion criteria that the subjects enrolled in the present study were restricted to those in good health and were eager to join the health programs spontaneously and thus that the distribution of HRQL scores in the subjects fell within the narrower ranges. Alternatively, it might be explained by less precision of calcaneus OSI compared to that of lumbar spine BMD. When we extended the ranges of ADL of the subjects to include patients with early rheumatoid arthritis, we found a significant correlation of ADL score assessed using a self-administered questionnaire modified from the Stanford Health Assessment Questionnaire, named MHAQ [13] with calcaneus OSI [14].

The most important finding in the present study is that FA IMT emerged as an independent factor that significantly associated with calcaneus OSI but not with lumbar spine BMD (Table 4). When the subjects were restricted to female patients, FA IMT was independently associated with calcaneus OSI, but not with lumbar spine BMD, although the association was not significant due probably to smaller numbers of subjects because of higher $\beta$-value than that in whole subjects (Table 5).

We have previously observed a significant correlation between peak wave velocity, an index of atherosclerosis, and bone mineral content (BMC) in the paretic lower limbs in hemiparetic patients [37]. Although bone loss and arterial disease of the lower limbs certainly shares common risk factors, such as tobacco

consumption and sedentary life style, bone loss in the affected lower limbs seems to be related with a direct local effect of atherosclerosis and not with associated general risk factor since the more ischemic atherosclerosis limb also showed greater bone loss [38]. These considerations are also supported from the present study demonstrating that CA IMT, in contrast to FA IMT, failed to be independently associated with calcaneus OSI (Table 4). The reason for greater bone loss in the less-perfused lower limbs is explained by several mechanisms. Since a correlation exists between intraosseous blood flow and bone remodeling in humans [39], it is conceivable that reduced intraosseous blood flow secondary to arterial disease induced osteopenia by suppressing bone formation and stimulating bone resorption. In fact, it was demonstrated that atherosclerotic lesions in the intraosseous arterioles were indentical to those in the cutaneous, muscular or myocardial vessels [40]. It is possible that hindlimb unloading-induced decreases in blood flow and increase in shear stress alter vascular endothelial cell release of nitric oxide (NO) and prostaglandin $I_2$ ($PGI_2$), which could subsequently modify the focal balance between osteoblast and osteoclast activity [41]. These data may suggest that enhanced local atherosclerosis, as reflected by increased FA IMT, may facilitate local bone loss by reducing intraosseous blood flow even in normal population of Japanese people, resulting in accelerated bone loss. The clinical significance of the present study is the association of local blood flow in lower extremities with reduction in calcaneus OSI. Therefore, these data may suggest that we should be careful of bone loss in calcaneus in those whose blood flow in the lower extremities may be impaired.

The limitation of the present study is that the multiple regression model explained only 35.5–36.5% of the variance of calcaneus OSI. This may indicate the presence of other factors affecting calcaneus OSI, that were not included in the models. Furthermore, due to small numbers of subjects, analyses were performed in subjects including male and female, although FA IMT emerged as a factor independently associated with calcaneus OSI even after adjustment for gender in the multiple regression analysis. Another limitation of the present study is that we have no direct histopathological demonstration that increased IMT is due to atherosclerosis. The arterial thickening might have been due to another, non-atherosclerotic arteriopathy. However, IMT measurement is still useful in that IMT is strongly correlated with the presence of coronary artery diseases.

In summary, it was suggested that the beneficial influence of physical activity on bone status as measured by calcaneus OSI and lumbar spine BMI was evident in healthy Japanese population. Furthermore, as reflected by the importance of FA IMT in the maintenance of calcaneal ultrasonographic values, but not lumbar spine BMD, it was strongly suggested that the importance of local blood flow in the maintenance of bone quality even in apparently healthy Japanese population.

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