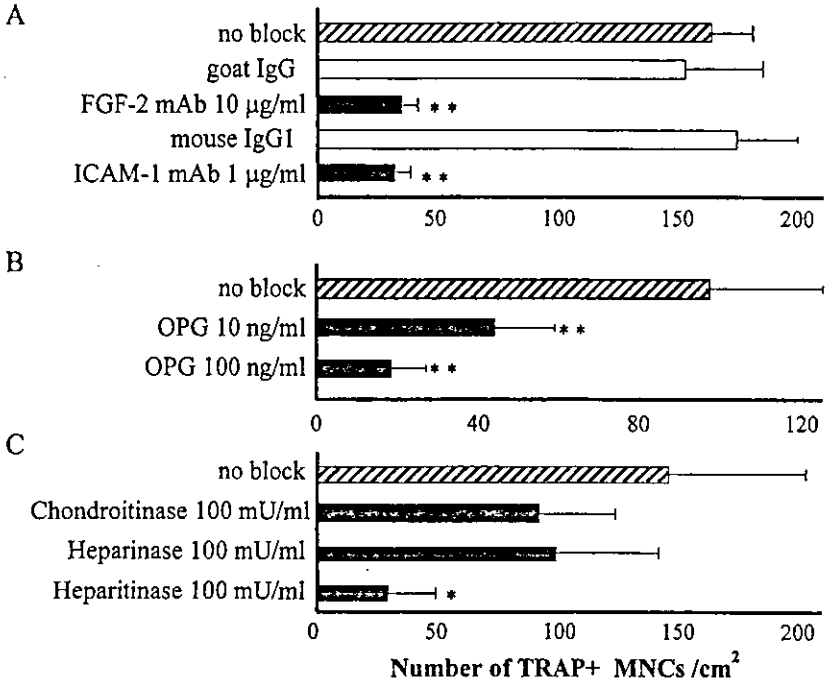

Figure 6. Inhibitory effects of anti–fibroblast growth factor 2 (anti–FGF-2) monoclonal antibody (mAb), anti–intercellular adhesion molecule 1 (anti–ICAM-1) mAb, osteoprotegerin (OPG), or heparitinase on FGF-2–induced tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells (MNCs) from coculture of rheumatoid arthritis synovial fibroblasts (RASFs) and peripheral blood mononuclear cells (PBMCs). On day 4, anti–FGF-2 antibody (10 μg/ml), goat IgG (control), ICAM-1 mAb 84H10 (1 μg/ml), or mouse IgG1 (control) (A), OPG (10 ng/ml or 100 ng/ml) (B), or RASFs pretreated with a heparitinase mixture (100 mU/ml), heparinase (100 mU/ml), or chondroitinase (100 mU/ml) (C) were added to the coculture system. Values are the mean and SD. * = $P < 0.05$ and ** = $P < 0.01$ versus no block, by unpaired t-test.

gesting that FGF-2 plays a pivotal role in osteoclastogenesis through the up-regulation of ICAM-1 and RANKL on RASFs (Figure 6A). The formation of TRAP-positive multinucleated cells from PBMCs in the presence of RASFs was also abrogated by the addition of anti–ICAM-1 mAb. The addition of 10 ng/ml of OPG to the cocultures on the fourth day also reduced the number of TRAP-positive multinucleated cells in a dose-dependent manner, reaching basal levels with the addition of 100 ng/ml of OPG (Figure 6B). These results imply that the high-affinity adhesion of PBMCs and RASFs is a prerequisite for the efficient signaling of RANKL on RASFs during osteoclast maturation. Furthermore, pretreatment of RASFs with 100 mU/ml of heparitinase, but not the same concentration of heparinase or chondroitinase, significantly reduced the formation of TRAP-positive multinucleated cells from the RASF and PBMC coculture (Figure 6C), suggesting that the heparan sulfate on RASFs, which holds FGF-2 as a coreceptor and sends it to the FGFR-1 signaling receptor, plays a pivotal role in the osteoclastogenesis involving FGF-2/RANKL on RASFs.

In fibroblast proliferation, activation of MAPKs followed by autophosphorylation of FGFR is known to be involved in FGF-2 signaling. To study the association between MAPK activation and FGF-2-mediated RANKL expression on RASFs, we pretreated RASFs with PD 98059 (a specific inhibitor of ERK) and SB 202190 (a specific inhibitor of p38 MAPK). PD 98059, but not SB 202190, inhibited the up-regulation of RANKL on RASFs in a dose-dependent manner (Figure 7A). These results suggest that the FGF-2–mediated RANKL expression on RASFs requires activation of the ERK cascade. Furthermore, when we added RASFs pretreated with PD 98059 or SB 202190 to cocultures, we found that the formation of TRAP-positive multinucleated cells was also inhibited by PD 98059 in a dose-dependent manner (Figure 7B).

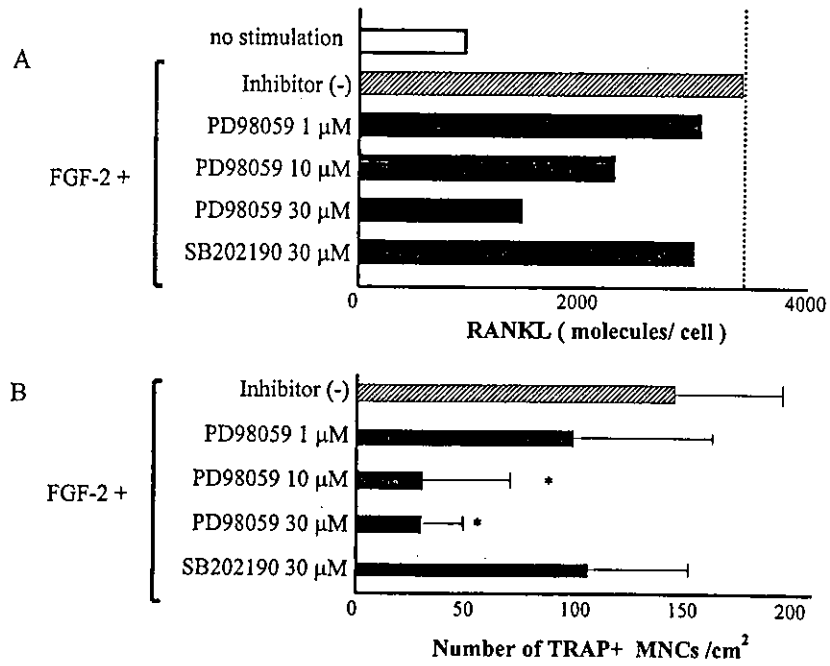

Figure 7. Involvement of MAPK activation in fibroblast growth factor 2 (FGF-2)–mediated RANKL expression on rheumatoid arthritis synovial fibroblasts (RASFs) and on osteoclast formation in a coculture system. A, RASFs were treated with PD 98059 (1, 10, or 30 μM) or SB 202190 (30 μM) at 37°C for 30 minutes. After washing, RASFs were incubated with or without FGF-2 (10 ng/ml) for 6 hours. RANKL expression was determined by FACScan. Values are from a representative experiment of cells from 3 rheumatoid arthritis patients. Dotted vertical line indicates without treatment with MAPK inhibitor. B, For coculture of RASFs and peripheral blood mononuclear cells (PBMCs) for the generation of osteoclasts, RASFs were treated with PD 98059 or SB 202190 at 37°C for 30 minutes. After washing, RASFs were added to adherent PBMCs. Cocultures were maintained in the presence of 50 ng/ml of macrophage colony-stimulating factor and $10^{-7}M$ 1,25-dihydroxyvitamin D_3 for 9 days, and then the dishes were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells (MNCs) that contained >3 nuclei were identified as osteoclasts, and these were counted by light microscopy. Values are the mean and SD of triplicate measurements. * = $P < 0.05$ versus no inhibitor, by unpaired *t*-test.

DISCUSSION

Prevention of bone and cartilage destruction is the most important issue in the treatment of RA, but it has never been completely achieved by conventional drug therapy, which mainly targets the suppression of inflammation. Recently, some centers have used bisphosphonates to inhibit osteoclast activity, but this is not a specific therapy for the pathologic process of RA. In this regard, the mechanisms underlying osteoclast differentiation within the synovium, away from osteoblasts or bone marrow stromal cells, which are thought to be indispensable for osteoclastogenesis, have not yet been delineated. However, it has been reported that RASFs in coculture with PBMCs induce osteoclast formation and that this system requires cell-to-cell interaction between RASFs and PBMCs (17), but the underlying mechanism(s) of this interaction is not yet clear. In the present study, we showed that FGF-2, which is produced by RA synovial tissue, induced ICAM-1 and RANKL expression on RASFs.

We and other investigators have reported that osteoblasts, which express ICAM-1 (14) and RANKL (18), promote osteoclast formation by adhesion to PBMCs. It was suggested that in RA synovial tissue, RASFs direct osteoclast formation under the same mechanism as osteoblasts. Furthermore, we found that differences in RANKL and ICAM-1 expression levels on RASFs and OASFs by FGF-2 were not due to differ-

ences in levels of FGFR-1 expression, but rather, differences in levels of HSPG expression, which is a coreceptor of FGFR-1. These results suggest that control of the expression of HSPG is linked to the specific control of bone resorption in RA.

It has been reported that the actions of FGF-2 save RASFs from apoptosis (19), that RASFs express the HOXD9 gene, and FGF-2 promotes the autonomous production of FGF-2 (20), and that the synovial fluid concentration of FGF-2 correlates with the degree of bone destruction more so than do the synovial fluid concentrations of inflammatory cytokines, such as tumor necrosis factor α, interleukin-1α, and interleukin-6 (6). According to these reports, FGF-2 plays a leading role in the pathologic changes of RA, such as cell proliferation and bone and cartilage destruction, and is thought to be an attractive target in any disease-specific treatment.

Since Yayon et al (8) first demonstrated the importance of HSPG for high-affinity binding of FGF-2 to FGFR-1, extensive biochemical and biologic data have been reported. These data indicate that HSPG is essential for FGF/FGFR signaling (9–11,21,22). Although several models have been proposed from binding studies, functional analyses, and crystal structure analyses, the mechanism(s) through which HSPG/heparin assists FGF/FGFR signaling remains poorly understood.

Preincubation of RASFs with heparinase and heparitinase strongly inhibited FGF-2–stimulated expression of RANKL on RASFs. Furthermore, preincubation of RASFs with heparitinase strongly controlled osteoclast formation, whereas preincubation of RASFs with heparinase failed to control osteoclastogenesis.

Extrapolating from the current structural models, we suggest that FGFR dimerization and autophosphorylation is supported by cooperative "heparin-like end structures," and that cell surface association and concentration of these end structures compensate for the relative scarcity of such end structures in native HSPG (23,24). Heparitinase catalyzes the eliminative cleavage of α-*N*-acetyl-D-glucosaminidic linkage in heparan sulfate, whereas heparinase mainly acts on heparin. In this osteoclast formation assay, we found that heparitinase resolved the heparan sulfate chains of cell surface HSPG most effectively and inhibited FGF receptor dimerization, activation, and signaling.

FGF-2/FGFR signaling enhanced cell proliferation to fibroblasts through ERK (25,26). In studies of the osteoclastogenesis of the murine monocytic cell line RAW264.7, it was reported that the ERK pathway negatively regulated RANKL-induced osteoclast maturation, whereas the p38 MAPK pathway positively regulated it (27,28). Our results showed that activation of ERK was necessary for RANKL expression on RASFs. Osteoclast formation was suppressed, a finding similar to that when RASFs were preincubated with heparitinase under the control of ERK activation. Previous studies indicated that FGF-2 acts on osteoblasts through activation of FGFR-1 and ERK, causing the expression of RANKL and the stimulation of bone resorption at physiologic or pathologic concentrations (29). It is possible that the same mechanism also exists in RASFs.

In conclusion, the present study demonstrated that FGF-2 binds to FGFR-1 through HSPGs, which are characteristically expressed on RASFs, and results in RANKL- and ICAM-1–mediated maturation of osteoclasts via ERK activation. Thus, FGF-2 is involved in osteoclast maturation, which leads to bone destruction and osteoporosis in RA. These results suggest that FGF-2– and HSPG-mediated signaling could be a suitable target for the treatment of RA.

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