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ORIGINAL ARTICLE

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Osteoclast differentiation factor induces synovial macrophage–osteoclast differentiation in rheumatoid arthritis

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Abstract The aim of this study was to clarify the role of osteoclast differentiation factor (ODF) and osteoprotegerin (OPG) in synovial macrophage–osteoclast differentiation. Synovial macrophages were cultured in the presence of macrophage-colony-stimulating factor (M-CSF) and/or ODF. OPG was added to cocultures of synovial macrophages and UMR106. The cultures on glass coverslips were stained with osteoclast-associated markers, tartrate-resistant acid phosphatase (TRAP), and vitronectin receptor (VNR), as well as macrophage-associated markers CD11b and CD14. Functional evidence of osteoclast formation was determined by a resorption pit assay. To investigate whether rheumatoid arthritis (RA) synovial cells expressed messenger RNA (mRNA) for ODF, OPG, and the receptor activator of NF-κB (RANK), we performed a polymerase chain reaction (PCR) analysis. The addition of M-CSF or ODF alone induced TRAP-positive multinucleated cell formation. Resorption pits were rarely detected with M-CSF alone. ODF was capable of inducing bone resorption and enhancing osteoclastogenesis, as well as bone resorption in the presence of M-CSF. In the coculture system, both osteoclast formation and bone resorption were inhibited by OPG in a dose-dependent manner. In all experiments, synovial cells, including macrophages and fibroblasts, expressed the mRNA for RANK, ODF, and OPG. Our findings suggest that ODF plays a role in regulating RA synovial macrophage–osteoclast differentiation, and that synovial cells might have the ability to produce ODF. OPG might be further developed as a new strategy for treating bone destruction in RA joints.

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease characterized by the destruction of bone and cartilage. While most studies of the pathogenetic mechanism of RA have focused on synovial hyperplasia and the infiltration of inflammatory cells, the cellular mechanism of pathological bone resorption has not been well established. Classically, the erosion of marginal and subchondral bone usually occurs at the attachment of the synovium, directly invading and destroying the underlying articular cartilage and bone. Multinucleated cells (MNCs) with the phenotypic characteristics of osteoclasts have been found in the area of subchondral bone erosion.[1] Isolated cells from rheumatoid synovium have phenotypic and functional characteristics of osteoclasts.[2–4] These findings led us to consider that osteoclasts may contribute to pathological bone resorption in RA.

We have demonstrated that RA synovial macrophages are capable of differentiating into mature osteoclasts using the coculture of UMR106 (rat osteosarcoma cell line) in the presence of human macrophage-colony-stimulating factor (M-CSF), dexamethasone, and $1,25(OH)_2D_3$.[5] This evidence suggests that osteoclast precursors are present in RA synovium. However, the precise mechanism of synovial macrophage–osteoclast differentiation is unknown.

In 1997, osteoclast differentiation factor (ODF), also known as osteoprotegerin ligand (OPGL) and the receptor activator of NF-κB ligand (RANKL), was identified as a member of the tumor necrosis factor (TNF) ligand family and a membrane-bound protein expressed on osteoblasts/stromal cells.[6,7] ODF stimulates osteoclast development and activation in the absence of supporting osteoblasts/stromal cells.[8–11] In addition, ODF-deficient mice have severe osteopetrosis because of a lack of osteoclasts.[12] This evidence

indicates that ODF plays a crucial role in osteoclastogenesis and bone resorption.

The Receptor activator of NF-κB (RNAK), which is a member of the TNF receptor family, is the sole signaling receptor for ODF.[13,14] ODF mediates a signal through RANK expressed on osteclast precursors and induces osteoclast differentiation. Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitor factor, has been found to be a secreted member of the TNF receptor family.[15,16] It has been shown that OPG is a potent inhibitor of the differentiation and activation of osteoclasts by acting as a decoy receptor for ODF to compete against RANK.[17,18] ODF, RANK, and OPG are key regulators of physiological bone resorption.

Kong et al.[19] have recently shown that activated T cells contribute to bone destruction through the production of ODF in adjuvant arthritis. Horwood et al.[20] have also shown that ODF produced by activated T cells induces osteoclast formation and bone resorption in vitro. Thus, we hypothesized that ODF and OPG play a role in regulating the osteoclastic bone resorption in RA. The aim of our study was to clarify the role of ODF and OPG in synovial macrophage–osteoclast differentiation in RA. Our study demonstrated that ODF is sufficient to induce the differentiation of synovial macrophages into osteoclasts, and that OPG inhibits osteoclast formation in synovial macrophage–UMR106 cocultures. Synovial cells expressing ODF mRNA might support osteoclast formation and lead to bone destruction in RA.

Material and methods

Reagents

Recombinant human M-CSF was purchased from R&D Systems (Abingdon, UK). A recombinant human-soluble form of ODF was purchased from Pepro Tech. EC (London, UK). Dexamethasone was purchased from Sigma Chemical (St. Louis, MO, USA), and $1,25(OH)_2D_3$ was purchased from Roussel Uclaf (Romainville, France). OPG was kindly provided by Japan Tobacco (Tochigi, Japan).

Patients

Synovial tissues were obtained from five female patients (age range 60–72 years), who satisfied the American Rheumatism Association criteria for RA, when they underwent total joint arthroplasty.

Isolation and culture of synovial cells

The synovial tissues were washed in phosphate-buffered saline (PBS) and dissected. The tissues were mixed with 10 ml of collagenase (0.1%) and trypsin, and the mixture was incubated with shaking for 90 min at 37°C. The cell suspension was filtered through a 70-μm cell strainer and centrifuged (693 *g*) for 5 min. The isolated synovial cells were finally resuspended in α-minimal essential medium (α-MEM, GibcoBRL, Grand Island, NY, USA) containing penicillin 100 U/ml, streptomycin 100 μg/ml (GibcoBRL), and 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA) (MEM/FCS).

Effect of ODF on osteoclast formation and bone resorption

Isolated synovial mononuclear cells (1×10^6/well) were seeded on dentine slices (4 mm) or glass coverslips (6 mm) in 96-well plates. After 2-h incubation, the dentine slices and glass coverslips were washed vigorously to remove nonadherent cells, and the adherent cells were used as synovial macrophages. The synovial macrophages were placed in 1 ml MEM/FCS with M-CSF (25 ng/ml) and/or synovial ODF (30 ng/ml). All incubation was carried out at 37°C in 5% CO_2, and the medium was replenished with factors every 3–4 days.

Effect of OPG on osteoclast formation and bone resorption in cocultures

Isolated synovial mononuclear cells (5×10^5/well) were added to 7 mm in diameter wells of 96-well plates containing dentine slices or glass coverslips, on which 1×10^4 osteoblast-like UMR106 cells (rat osteosarcoma cell line) had previously been cultured for 24 h in MEM/FCS. After 2-h incubation, the adherent cells on the dentine slices and glass coverslips were cultured in MEM/FCS containing M-CSF (25 ng/ml), dexamethasone (10^{-8} M), and $1,25(OH)_2D_3$ (10^{-8} M). OPG was added to the cocultures at concentrations of 1, 10, or 100 ng/ml at the beginning of each experiment and at every medium change. All incubation was carried out in the same manner as in the above experiment.

Histochemical and immunohistochemical characterization of cultured cells

After 1 and 7 days of incubation, the cells on the glass coverslips were stained histochemically for osteoclast-associated enzyme tartrate-resistant acid phosphatase (TRAP) using a kit from Sigma Chemicals (UK). For immunohistochemical staining, the cells were fixed with cold methanol–acetone (50:50, vol/vol) for 10 min and incubated with CD11b and CD14, which are macrophage-associated markers, as well as vitronectin receptor (VNR) (23C6), which is an osteoclast marker and is known not to be expressed on macrophages, using an indirect immunoperoxidase technique. Cells containing more than three nuclei were counted as multinucleated cells (MNCs).

Preparation of dentine slices for examination by light microscopy

On day 14, the dentine slices were placed in NH_4OH (1 N) for 30 min and then cleaned by ultrasonication to remove

adherent cells. The dentine slices were then washed with distilled water and stained with toluidine blue. Bone resorption was evaluated by scanning the area of resorption pits with a digital analyzer.

RNA isolation and analysis

Synovial cells were grown to confluence in a 5-cm dish in MEM/FCS. The synovial cells, including mainly macrophages (type A) and fibroblasts (type B), were harvested after experimentation by trypsinization. Total cellular RNA was used in reverse transcriptase polymerase chain reaction (RT-PCR). PCR was performed using a three-step protocol with 30 cycles, and samples were denatured at 94°C (1 min), annealed at 60°C (1 min), and extended at 72°C (1 min). Specific primers for PCR were the following: ODF (335 bp) forward; 5′-TTTGAGACTCCATGAAAATGC-3′ and reverse; 5′-TAACTATTAGTTTTCCATTGC-3′; RANK (410 bp) forward; 5′-TTTGCAGATCGCTCCTCCAT-3′ and reverse; 5′-AGGCATCAGAGAAGTAGCT-3′; OPG (484 bp) forward; 5′-TGACAAATGTCCTCCTGGTA-3′ and reverse; 5′-ACACAGGGTAACATCCATTTTTG AGTTG-3′; β-actin (867 bp) forward; 5′-TGACGGGG TCACCCACACTGTGCCCATCTA-3′ and reverse; 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′. PCR products were analyzed by running them on a 3% agarose gel stained with ethidium bromide and photographed under UV illumination.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *P* values <0.05 were considered to be significant.

Results

Effect of ODF on RA synovial macrophage–osteoclast formation

On day 1, the cells on the glass coverslips strongly expressed both CD11b and CD14 antigens, which are known to be macrophage-associated markers. The cells were entirely negative for osteoclast markers.

After 7-day incubation, no TRAP-positive MNCs were observed on the glass coverslips when synovial macrophages were cultured in the absence of sODF and M-CSF (Fig. 1A). A few TRAP-positive MNCs were found on the glass coverslips in the culture treated with M-CSF alone (Fig. 1B). In contrast, sODF alone induced the formation of numerous TRAP-positive MNCs (Fig. 1C), and the addition of synovial ODF (sODF) to the M-CSF-treated cultures resulted in an increase in the formation of TRAP-positive MNCs (Fig. 1D). The TRAP-positive MNCs induced by sODF were also positively stained for anti-VNR antibody (Fig. 2).

On day 14, no resorption pits were observed on the dentine slices when synovial macrophages were cultured in the absence of sODF and M-CSF (Fig. 3A). In two out of five experiments, treatment with M-CSF alone induced a few resorption pits (Fig. 3B). When sODF was added to the cultures, all dentine slices had resorption pits on up to 15% of their surface. In combination, sODF and M-CSF stimulated the formation of extensive resorption pits (Fig. 3D). As seen in Fig. 4, the extent of the resorption pits in the cultures treated with both sODF and M-CSF was significantly increased compared with sODF treatment alone ($P < 0.05$).

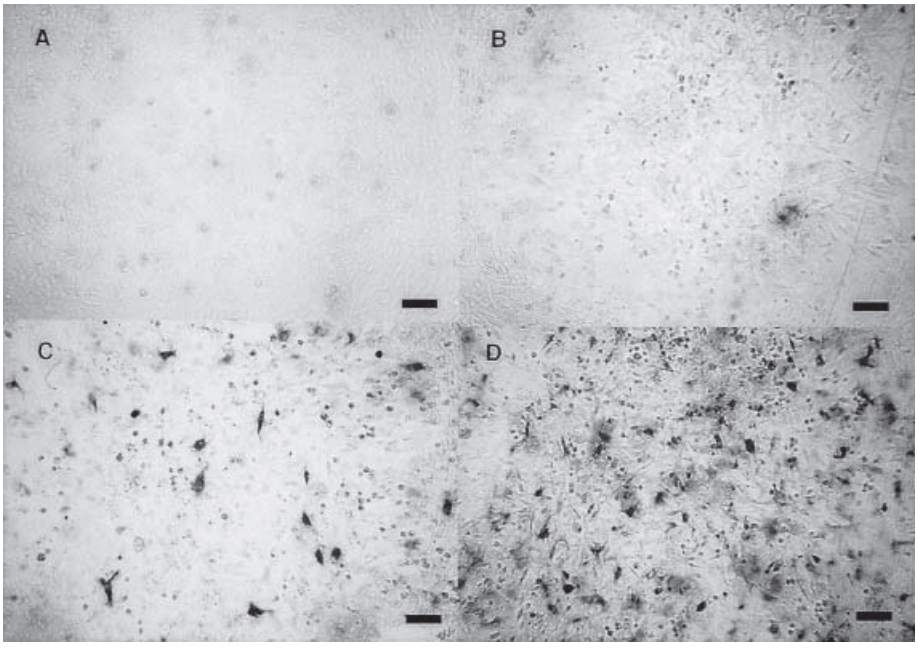

Fig. 1. Tartrate-resistant acid phosphatase-positive multinucleated cells in the culture of rheumatoid arthritis (RA) synovial macrophages. **A** Control. **B** With macrophage-colony-stimulating factor (M-CSF) (25 ng/ml). **C** With synovial osteoclast differentiation factor (sODF) (30 ng/ml). **D** With M-CSF (25 ng/ml) and sODF (30 ng/ml). *Bar* 100 μm

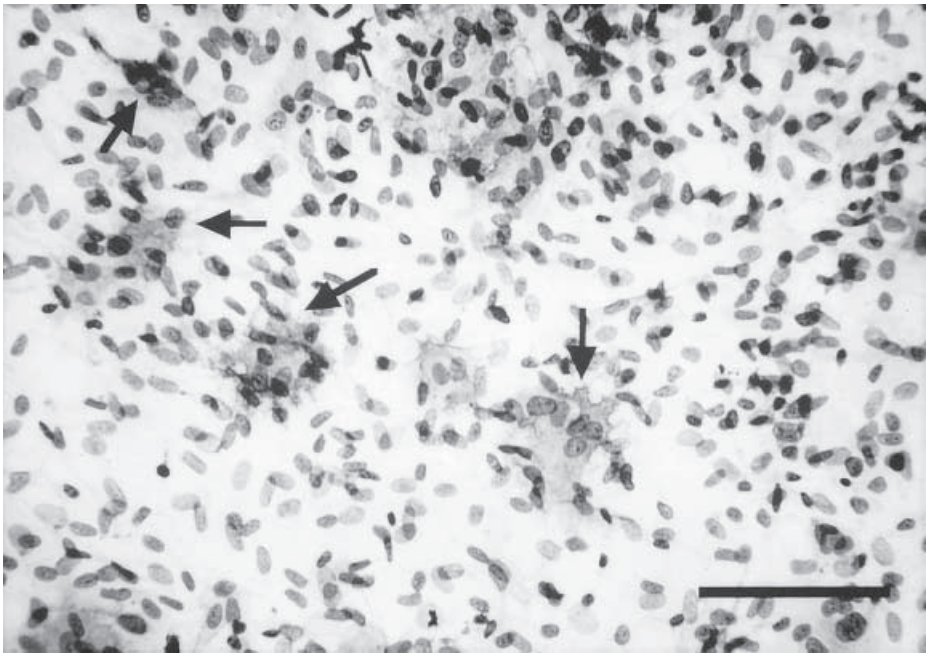

Fig. 2. Vitronectin receptor (VNR)-positive multinucleated cells on coverslips in the presence of M-CSF and sODF (*arrows*). *Bar* 100 μm

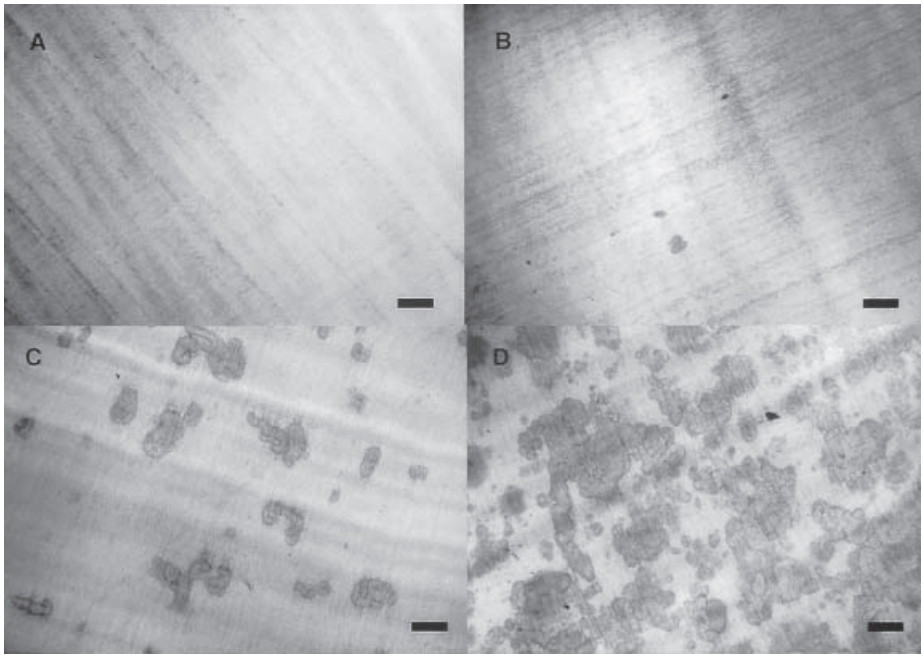

Fig. 3. Resorption pits on dentine slices in the culture of synovial macrophages for 14 days. **A** Control. **B** With M-CSF (25 ng/ml). **C** With sODF (30 ng/ml). **D** With M-CSF (25 ng/ml) and sODF (30 ng/ml). *Bar* 100 μm

Inhibitory effect of OPG on RA macrophage–osteoclast differentiation in cocultures of synovial macrophages and UMR106

To investigate the mechanism of synovial macrophage–osteoclast differentiation in cocultures of synovial macrophages and UMR106, we examined the effect of OPG on osteoclast formation and bone resorption. On day 7, a number of osteoclast-like cells exhibiting TRAP as well as VNR with the ability to form resorption pits were found in the cocultures of synovial macrophages and UMR106 with M-CSF, dexamethasone, and $1,25(OH)_2D_3$.

The addition of OPG to the cocultures resulted in strong inhibition of osteoclast formation. The number of TRAP- and VNR-positive MNCs and the extent of resorption pits decreased in a dose-dependent manner (Fig. 5). OPG 100 ng/ml completely abolished pit formation in the coculture of human peripheral blood mononuclear cell cultures (data not shown) and UMR106. In the cocultures of synovial macrophages and UMR106, however, there

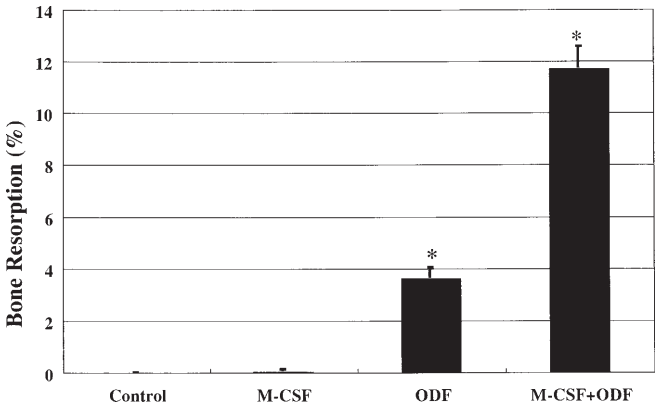

Fig. 4. Osteoclast differentiation of synovial macrophages after 14 days incubation in response to M-CSF (25 ng/ml) or sODF (30 ng/ml), showing the proportion of the dentine slice surface area resorbed (mean ± SE). $*P < 0.01$ compared with control cultures

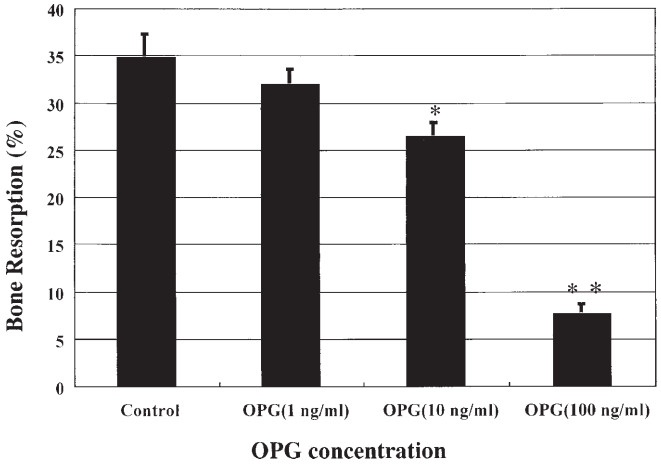

Fig. 5. Pit formation by the cocultures of synovial macrophages and osteoblastic cells (UMR106) in the presence of dexamethasone and $1,25(OH)_2D_3$, after 14 days incubation, in response to increasing concentrations of osteoprotegerin (OPG) (shown in ng/ml). $*P < 0.05$, $**P < 0.01$ as compared with control cultures; i.e., in the absence of OPG

were few pits when OPG 100 ng/ml was added to the cocultures.

Detection of ODF, OPG, and RANK mRNA expression in RA synovial cells

To demonstrate that synovial cells involve osteoclast precursors, we examined whether synovial cells express mRNA for RANK using the RT-PCR approach. As shown in Fig. 6, synovial cells expressed RANK mRNA in all samples.

We also examined whether the mRNA expression of ODF or OPG is detected in synovial cells. RA synovial cells expressed mRNA for ODF as well as OPG in all samples (Fig. 6). In addition, the expression of both ODF and OPG was regulated by osteotropic factors, including prostaglandin E_2, dexamethasone, and $1,25(OH)_2D_3$, as in osteoblastic cells (data not shown).

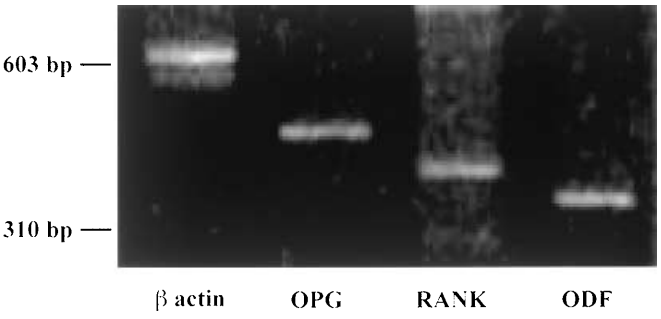

Fig. 6. Messenger RNA (mRNA) expression of *ODF*, *OPG*, the receptor activator of NF-κB (*RANK*), and β-actin were detected by reverse transcriptase-polymerase chain reaction. RA synovial cells were cultured to confluence in minimal essential medium/fetal calf serum for 7–10 days

Discussion

In this study, we demonstrated that ODF is capable of inducing osteoclast formation as well as bone resorption in synovial mononuclear cell cultures in vitro. This study also indicated that osteoclast formation in our cocultures is dependent on an ODF signaling mechanism, and that this ODF signaling might work in RA synovial tissues. Some of our results confirm those in a previous report.[21]

ODF induced the formation of the MNCs, which exhibited TRAP, VNR, and the evidence of pit formation on the dentine slices. Thus, the MNCs induced by ODF were authentic osteoclasts. OPG significantly inhibited this effect (data not shown). These results suggest that ODF is directly capable of inducing synovial macrophage–osteoclast differentiation.

M-CSF is an essential factor for osteoclast development,[22–26] but it does not have the ability to activate osteoclasts.[27,28] In the presence of M-CSF alone, a few resorption pits were observed in two experiments. It was reported by Shalhoub et al.[9] that M-CSF induced the formation of a few pits in human peripheral blood mononuclear cell (PBMC) cultures without ODF, following 37 days of incubation. By RT-PCR, RA synovial cells, including macrophages and fibroblasts, expressed both ODF mRNA and OPG mRNA in all experiments (Fig. 6). Faust et al.[29] have shown that human PBMCs express low levels of ODF mRNA as well as OPG mRNA. These results agree with studies in which ODF mRNA expression was detected in several different cells, such as T cells in rheumatoid synovium as well as macrophage-like and fibroblast-like synovial cells.[20,30,31] Our findings suggest the possibility that synovial cells produce ODF in an autocrine fashion and trigger osteoclastogenesis and bone resorption.

ODF is not sufficient to induce osteoclast formation from hematopoietic precursors in the absence of M-CSF.[32] However, our data demonstrate that synovial macrophages differentiate into osteoclasts in the presence of ODF with-

out adding further M-CSF. M-CSF has been detected in the synovial fluids of RA patients.[33,34] The addition of M-CSF significantly stimulated ODF-induced bone resorption. Thus, this result may be explained by the ability of synovial macrophages to produce small amounts of endogenous M-CSF.

It is known that OPG plays a key role in maintaining the normal bone metabolism by inhibiting the action of ODF.[35,36] The addition of OPG to the cocultures of synovial macrophages and UMR106 inhibited osteoclast formation and bone resorption in a dose-dependent manner. This result indicates that RA synovial macrophage–osteoclast differentiation in this coculture system is an ODF-dependent mechanism. OPG at a concentration of 100 ng/ml did not completely block bone resorption in this coculture system, while bone resorption was abolished by OPG (100 ng/ml) in the cocultures of human PBMCs with UMR106 (data not shown). This probably happens for two reasons. First, the synovial macrophages may have the ability to produce ODF protein. Second, there may be another pathway that is independent of ODF signaling. It has recently been reported that in mouse bone marrow cell cultures, TNF-α directly induces osteoclast formation and IL-1α stimulates osteoclast activation, and that this osteoclast formation and activation are independent of the ODF signaling system.[37,38]

Interestingly, RA synovial cells expressed mRNA not only for ODF but also for OPG. Various osteotropic factors regulate ODF mRNA[39] as well as OPG mRNA and protein production on osteoblastic cells.[40–43] Osteotropic factors such as prostaglandin E_2, $1,25(OH)_2D_3$, and interleukin-1 increased ODF mRNA levels in synovial cells as it does in RA synovial fibroblast cultures.[31] Therefore, RA synovial cells as well as osteoblastic cells may have the ability to regulate ODF and OPG production under hormonal conditions. Further studies are needed to clarify how synovial cells regulate ODF/OPG protein.

It has been shown that RANK expressed on osteoclast precursors is the signaling receptor for ODF in osteoclastogenesis.[15,16] The evidence that synovial cells express RANK mRNA strongly supports the suggestion that osteoclast precursors are present in synovial cells,[5] since osteoclast precursors express RANK but macrophages do not.

This study clearly demonstrates that ODF plays a major role in RA synovial macrophage–osteoclast differentiation. RA synovial cells might have the ability to contribute to osteoclastic bone resorption through regulation of the ratio of ODF/OPG production. Therefore, OPG might be proved to be a potential therapeutic strategy for bone destruction in RA.

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