Tumor Necrosis Factor-α Promotes the Expression of Osteoprotegerin in Rheumatoid Synovial Fibroblasts

AYAKO KUBOTA, KAZUHISA HASEGAWA, TORU SUGURO, and YASUKO KOSHIHARA

ABSTRACT. ***Objective.*** To clarify the regulation of osteoprotegerin (OPG) expression in rheumatoid synovial fibroblasts by investigating the effect of tumor necrosis factor-α (TNF-α) and the mechanism of TNF-α-induced OPG expression.

Methods. OPG expression was examined by Northern blot hybridization and reverse transcriptase-polymerase chain reaction in synovial fibroblasts from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and subjects with no inflammatory condition. Amounts of OPG in conditioned medium were determined by ELISA. The effect of OPG on TNF-α-induced osteoclastogenesis was investigated in primary cultures of RA synovial cells.

Results. OPG was highly expressed in RA synovial fibroblasts compared to OA and noninflammatory synovial fibroblasts. Different levels of OPG expression were found among patients with RA. TNF-α induced OPG expression in all synovial fibroblasts, even OA and noninflammatory fibroblasts, and expression occurred to a remarkable degree in RA fibroblasts. The OPG expression was upregulated by TNF-α in a time- and dose-dependent manner. TNF-α-induced OPG expression was inhibited by hymenialdisine, a nuclear factor-κB inhibitor, in a dose-dependent manner, and expression was inhibited by soluble TNF receptor/Fc fusion protein I (TNFs-RI/Fc), not by TNFs-RII/Fc. In contrast, TNF-α-induced osteoclastogenesis in primary cultures of RA synovial cells was inhibited by the addition of OPG.

Conclusion. These results suggest that OPG is highly expressed and is upregulated by TNF-α in rheumatoid synovial fibroblasts. TNF-α-induced OPG expression is mediated predominately through TNF-RI. Although TNF-α is known to stimulate bone destruction, TNF-α-induced upregulation of OPG may contribute to self-protection from the bone destruction in RA. (J Rheumatol 2004;31:426–35)

Key Indexing Terms:
OSTEOPROTEGERIN RANKL RHEUMATOID ARTHRITIS
SYNOVIAL FIBROBLAST TUMOR NECROSIS FACTOR-α

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive bone destruction, which promotes severe arthralgia. Recent studies suggest that osteoclasts induced by synovial fibroblasts play a key role in bone destruction of RA. Synovial fibroblasts, which express receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) on the membrane, support osteoclastogenesis[1,2]. Various cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1α (IL-1α), and IL-6, which are produced by synovial tissue, induce the formation and activation of osteoclasts under inflammatory conditions. Of these cytokines, TNF-α may be the most potent osteoclastogenic cytokine produced in inflammation. TNF-α is localized in the lining and deeper layers of RA synovium. In contrast to RA, a lower proportion is observed in osteoarthritis (OA) and noninflammatory synovial fibroblasts[3,4]. Elevated TNF-α has been detected in RA synovial fluid[5,6]. Recent clinical reports note that anti-TNF-α therapy is effective for aggressive RA[7,8]. Inhibition of the action of TNF-α may not only reduce the symptoms, but it may also prevent or slow the progression of bone destruction. Based on our observations, we hypothesized that TNF-α is a pivotal cytokine in the pathogenesis of RA.

TNF-α is a pleiotropic molecule that has a variety of contrasting biological effects. In some circumstances, TNF-α enhances inflammation or inhibits cell apoptosis; in others it acts as an immunosuppressive or promotes cell apoptosis. TNF-α mediates its diverse biological effects via 2 receptors, TNF-RI (p55) and TNF-RII (p75)[9]. Both receptors exist in membrane-bound and soluble forms. The 2 receptors work differently and in some ways have overlapping actions[10-12], but the precise functions of each of the receptors are not known.

Osteoprotegerin (OPG), a novel member of the TNF receptor superfamily, inhibits bone resorption and is a soluble receptor for RANKL in osteoblasts and stromal cells[13,14]. Numerous studies have reported that proinflammatory cytokines or osteotropic hormones upregulate

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RANKL mRNA concentrations and downregulate OPG mRNA levels[15]. In contrast, a few studies report that TNF-α, IL-1α, and IL-1ß upregulate OPG mRNA levels in periodontal disease[16] or osteosarcoma[17,18] and human vascular endothelial cells[19]. Together with these reports, the fact that the expression of RANKL and OPG are upregulated under pathological conditions led us to conclude that cytokine-induced OPG expression may serve a homeostatic function.

We investigated the regulation of OPG expression in rheumatoid synovial fibroblasts by examining the effect of TNF-α and the mechanism of TNF-α induced OPG expression.

MATERIALS AND METHODS

Patients. Synovial tissues from 6 patients with RA were obtained during total knee or elbow arthroplasty at the Department of Orthopaedic Surgery, Toho University. The RA patients, who had advanced endstage disease, were 6 women with a median age of 56 years (range 36–76). All patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) revised criteria for RA[20]. Among the samples, 4 patients with RA (lanes 3–6 in Figure 1) were having a disease flare (knee pain, swelling, stiffness, clinical evidence of effusion), while the remaining 2 RA patients (lanes 7 and 8, Figure 1) were quiescent. Control synovial tissues were obtained from 2 noninflammatory subjects who underwent arthroscopy for meniscus injury and 3 patients with OA who underwent total knee arthroplasty. Each patient was receiving nonsteroidal antiinflammatory drugs (NSAID). Each patient gave informed consent for subsequent experiments.

Isolation of synovial cells. Synovial cells were isolated from synovial tissues as described[21], with modification. Briefly, the synovial tissue was dissected away, washed twice with α-minimum essential medium (α-MEM, Institute Scientific, Santa Ana, CA, USA), and mixed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS, pH 7.4) containing 1 mg/ml collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.15 mg/ml DNase I (Sigma, St. Louis, MO, USA), and incubated with shaking for 90 min at 37°C. The incubation mixture was filtered through a 100 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). The resulting cell suspension was centrifuged at 1400 rpm for 20 min at 4°C and washed 3 times with α-MEM with 5% heat-inactivated horse serum (Morgate, Australia) by centrifugation at 1400 rpm for 10 min at 4°C. The cells were finally suspended in α-MEM containing 20% horse serum. Isolated synovial cells were used for primary synovial cell culture, and adherent synovial fibroblasts were cultured through 5–8 passages for the RNA extraction.

Osteoclast formation in primary cultures of RA synovial cells. Freshly isolated synovial cells were seeded in 4-well tissue culture plates (Becton Dickinson) and cultured in medium containing $1,25(OH)_2D_3$ (10^{-7} M) (kindly supplied by Teijin Company, Tokyo, Japan) or TNF-α (R&D Systems, Minneapolis, MN, USA) in the presence of recombinant human macrophage-colony stimulating factor (M-CSF) (2 ng/ml) (R&D Systems). To investigate the inhibitory effect of OPG on TNF-α-induced osteoclast formation in the presence of M-CSF (2 ng/ml), OPG (a generous gift from Sankyo Company, Tokyo, Japan) was added to the cultures at a concentration of 20 ng/ml. The medium was changed every 3 days. After 3 weeks, all plates were treated with pronase E solution (0.002% pronase E and 0.02% EDTA in PBS) for 5 min to remove synovial fibroblasts, and adherent cells were used for cytochemical staining and RNA extraction.

Determination of osteoclast characteristics. The adherent cells were fixed and stained with an acid phosphatase detection kit (Sigma) for detecting tartrate resistant acid phosphatase (TRAP) activity, as described[22]. TRAP-positive multinucleated cells (MNC) with more than 3 nuclei were counted as osteoclasts. The value is represented by the average number of TRAP-positive MNC from 3 wells in 2 random areas per well. For staining F-actin fiber, osteoclasts detached from the dish by exposure to pronase E solution for 20 min were seeded on cover glasses and incubated 1 day. Cells were washed with PBS and fixed with PBS containing 10% formalin. F-actin fibers were detected by staining with rhodamine-conjugated phalloidin solution[23]. To assay the resorption by MNC, we established primary

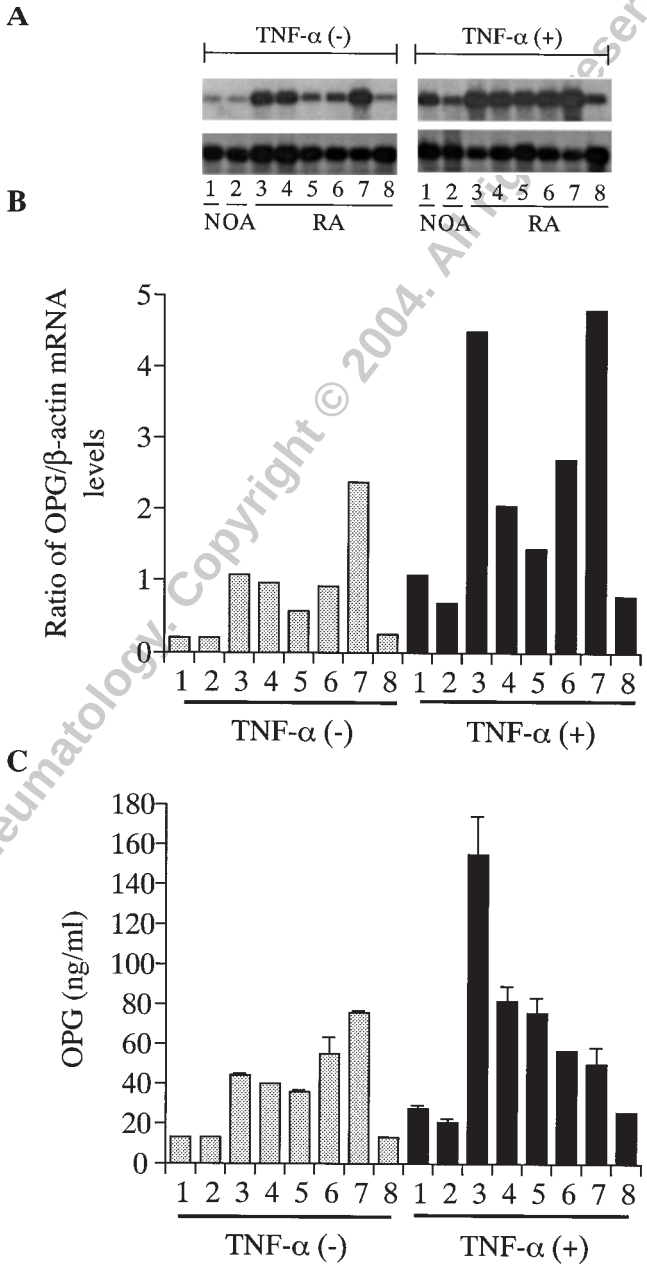

Figure 1. Expression of OPG mRNA and production of OPG in RA, OA, and noninflammatory synovial fibroblasts. Total RNA was extracted from cultured synovial fibroblasts in the absence (left side) or presence (right side) of TNF-α (10 ng/ml) for 24 h. Total RNA (4 µg) was analyzed by Northern blot hybridization (A). Level of OPG mRNA was compared to the level of ß-actin mRNA by densitometry, as shown in the graph (B). Levels of OPG in conditioned medium were measured by ELISA (C). Left side indicates the absence and right side the presence of TNF-α (10 ng/ml). Lane 1, noninflammatory synovial fibroblasts; lane 2, OA fibroblasts; lanes 3–8, RA fibroblasts.

cultures of RA synovial cells on osteologic discs (Millenium Biologix Inc., Kingston, ON, Canada), which provided a smooth-surfaced mineralized substrate for the assessment of pit formation[24], for 3 weeks in the presence of M-CSF (2 ng/ml). Resorption pits were visualized by Von Kossa staining[25] after removing cells.

After RNA extraction from TNF-α-induced MNC, mRNA expression of osteoclast-specific genes was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR), as described below.

RNA extraction from synovial fibroblasts. Total RNA was extracted using the AGPC method[26]. Total RNA was extracted from RA, OA, and noninflammatory synovial fibroblasts after 24 h incubation in the presence of TNF-α, soluble TNF receptor/Fc fusion protein I [TNFs-RI/Fc; recombinant mature human TNF-RI/Fc chimera is a disulfide-linked homodimeric protein (R&D Systems)], or TNFs-RII/Fc [recombinant mature human TNF-RII/Fc chimera is a disulfide-linked homodimeric protein (R&D Systems)], hymenialdisine (generous gift from Suntory, Tokyo, Japan), or NAC (N-acetyl-L-cysteine; Sigma). Moreover, total RNA was extracted from osteoclasts that were incubated with $1.25(OH)_2D_3$ (10^{-7} M) or TNF-α (10 ng/ml) in the presence of M-CSF in RA primary culture for 3 weeks.

RT-PCR analysis. After RNA extraction, cDNA was synthesized using the RNA LA PCR™ Kit (AMV), Version 1.1 (Takara, Osaka, Japan). As a control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected. The following primer sequences were used: RANKL, sense 5'-AGA CAC AAC TCT GGA GAG TCA AG-3' and antisense 5'-TAC GCG TGT TCT CTA CAA GGT C-3'; OPG, sense 5'-AAC CCC AGA GCG AAA TAC-3' and antisense 5'-AAG AAT GCC TCC TCA CAC-3'; carbonic anhydrase II (CAII), sense 5'-GAG GAT CCT CAA CAA TGG TCA-3' and antisense 5'- GAA GAG AGG GGT GGT CAG TG–3'; calcitonin receptor (CTR), sense 5'-CTG CTG GCT GAG TGT GGA AAC-3' and antisense 5'-AAG AAG CCC TGG AAA TGA ATC-3'; cathepsin K, sense 5'-ATA TGT GCA GAA GAA CCG GG-3' and antisense 5'-AGA GCA GGA TGG ATT TGG CT-3'. Product sizes corresponding to RANKL, OPG, CAII, CTR, and cathepsin K cDNA were 815 bp, 219 bp, 416 bp, 273 bp, and 458 bp, respectively.

Northern blot analysis. Total RNA from cells was separated via 1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, UK) followed by ultraviolet crosslinking. The blot was hybridized with cDNA labeled with [α-^{32}P] dCTP (NEN Life Science Products, Boston, MA, USA) using a random-primed DNA labeling kit (Roche Diagnostic, Mannheim, Germany). PerfectHyb hybridization solution (Toyobo, Osaka, Japan) was used according to the supplier's protocol. OPG cDNA probe (736 bp) was prepared by RT-PCR (Life Science Inc., Woodland Hills, CA, USA) using a sense primer 5'-GCC CTG ACC ACT ACT ACA CA-3' and an antisense primer 5'-TCT GCT CCC ACT TTC TTT CC-3'. ^{32}P-labeled human ß-actin cDNA probe was purchased from Wako Pure Chemicals.

Measurement of OPG concentration. Conditioned medium was harvested from cell culture and centrifuged at 5000 rpm for 5 min to remove cells and debris. Samples were stored at –20°C until used. Amounts of OPG were measured by ELISA. Briefly, a well in a 96 multi-well plate was coated with 100 µl of 2 µg/ml mouse anti-human OPG antibody (R&D Systems) overnight at room temperature. The well was then blocked with 300 µl of blocking solution consisting of 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% NaN_3 in PBS, for 1 h at room temperature. The plate was stored at 4°C until used. One hundred microliters of sample or authentic recombinant human OPG (R&D Systems) were added and incubated 2 h at room temperature. The bound protein was detected with 100 µl of biotinylated goat anti-human OPG antibody (R&D Systems). After 2 h incubation, streptavidin-horseradish peroxidase, using 1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine) in Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was added to the plate. The reaction was terminated with 1 M H_2SO_4, and the plate was read at 450 nm in a microplate reader. Between steps of the ELISA, the plate was washed 3 times with PBS containing 0.05% Tween 20. An ELISA for sample volumes of 100 µl with a range of 31.2–2000 pg/ml was obtained. Used in combination with the biotinylated anti-human OPG detection antibody in the ELISA, roughly 1% cross-reactivity with recombinant mouse OPG was observed and less than 0.06% cross-reactivity with recombinant human (rh) CD40, rhTNFs-RI, and TNFs-RII were observed.

DNA extraction. DNA from synovial fibroblasts was extracted by incubating the fibroblasts with 5% perchloric acid solution at 70°C for 30 min and centrifuging them at 10,000 rpm at 4°C for 5 min. The supernatant was reacted with diphenylamine solution using Burton's method[27] and optical density was measured at 600 nm in a spectrophotometer.

RESULTS

Effects of $1,25(OH)_2D_3$ and TNF-α on expression of OPG in RA synovial fibroblasts. First, we investigated the effect of $1,25(OH)_2D_3$ and TNF-α on the expression of OPG in RA synovial fibroblasts. Northern blot hybridization analysis showed that treatment with $1,25(OH)_2D_3$ (10^{-7} M) decreased the concentrations of OPG mRNA and that TNF-α (10 ng/ml) increased the levels of OPG mRNA in RA synovial fibroblasts (Figure 2A). ELISA results showed that the production was decreased by the addition of $1,25(OH)_2D_3$ (10^{-7} M) and increased by the addition of TNF-α (10 ng/ml) (Figure 2B). This is consistent with reports that $1,25(OH)_2D_3$ (10^{-7} M) decreased OPG production and stimulated osteoclastogenesis in the primary cultures of synovial cells and cocultures of RA synovial fibroblasts with peripheral blood mononuclear cells in the presence of M-CSF[1]. To confirm the upregulation of OPG in the presence of TNF-α, we investigated the levels of OPG in RA, OA, and noninflammatory synovial fibroblasts.

Expression of OPG mRNA in RA, OA, and noninflammatory synovial fibroblasts. To analyze the expression of OPG in various synovial fibroblasts, we compared concentrations of OPG mRNA among RA, OA, and noninflammatory synovial fibroblasts. Northern blotting hybridization analysis showed that RA synovial fibroblasts expressed OPG abundantly compared to OA and noninflammatory fibroblasts (Figure 1A, 1B). However, the level of OPG expression was varied among the patients with RA. While most RA synovial fibroblasts expressed higher levels of OPG (Figure 1, lanes 3–7), one of 6 RA samples (lane 8) expressed a lower level of OPG. TNF-α (10 ng/ml) increased the expression of OPG in all tested synovial fibroblasts, even those from a patient with OA and from noninflammatory subjects. The rate of TNF-α-induced OPG expression was varied among the patients, irrespective of disease status. ELISA results showed that the production of OPG was higher in RA than in OA and noninflammatory synovial fibroblasts, with the exception of lane 8. TNF-α (10 ng/ml) increased the levels of OPG in all synovial fibroblasts except those in lanes 6 and 7 (Figure 1C). In the samples in lanes 6 and 7 (patients with Larsen grade V disease) OPG may be easily degraded by any proteases or inhibition of translation induced by TNF-α. As a result, they might cause production of insufficient OPG in RA synovial fibroblasts, which could not prevent severe bone destruction.

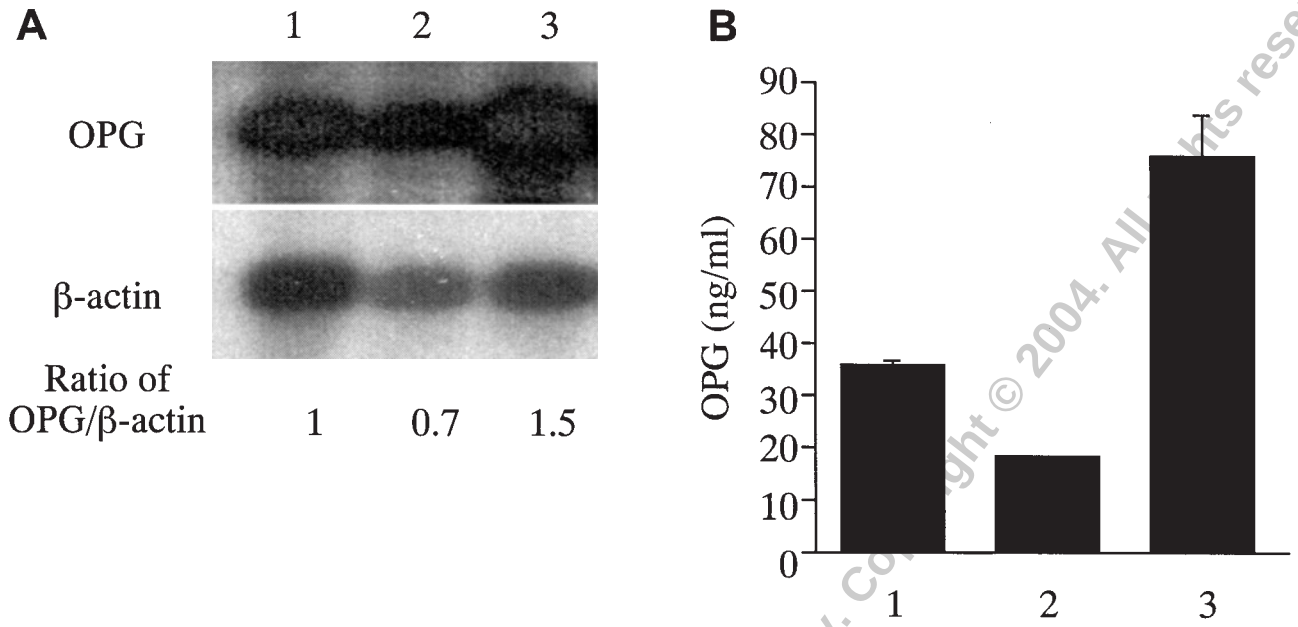

Figure 2. Expression of OPG mRNA and production of OPG in RA. Total RNA was extracted from cultured rheumatoid synovial fibroblasts (RASF, lane 5 in Figure 1) treated with $1,25(OH)_2D_3$ (10^{-7} M) and TNF-α (10 ng/ml) for 24 h. Total RNA (10 μg) was analyzed by Northern blot hybridization (A). Levels of OPG in conditioned medium were measured by ELISA (B). Lane 1, control; lane 2, $1,25(OH)_2D_3$ (10^{-7} M); lane 3, TNF-α (10 ng/ml).

Another experiment also showed that TNF-α-induced OPG expression and OPG production were higher in synovial fibroblasts from 3 RA patients than in 2 noninflammatory subjects and 3 OA patients (data not shown).

Next, we investigated the effect of increasing the concentration of TNF-α on the expression of OPG mRNA after 24 h of treatment in cultures of RA synovial fibroblasts. TNF-α increased the levels of OPG mRNA in a dose-dependent manner (Figure 3A, 3B). The effects of TNF-α on the expression of OPG were induced at 0.1 ng/ml, and a 460% increase of OPG mRNA expression was detected at a concentration of 1 ng/ml. ELISA results showed that the levels of OPG were increased at 0.1 ng/ml and the level reached a plateau (at an increase of about 250%) at 1 ng/ml (Figure 3C). An increased level of OPG in this treatment represented as ng/μg DNA was confirmed in RA synovial fibroblasts (data not shown). We investigated the time course of the effect of TNF-α on both mRNA and protein levels of OPG in RA synovial fibroblasts. On Northern blot hybridization, these stimulatory effects were clearly detected at 6 h and became more prominent at 12 h (Figure 4A, 4B). TNF-α resulted in a 250% increase over control levels. The levels of OPG production, represented as ng/mg DNA, showed that the effect was significant after 24 h. TNF-α increased the levels of OPG production by roughly 80% compared to the untreated control at 24 h (Figure 4C).

Regulation of TNF-α-induced OPG expression in RA synovial fibroblasts. We investigated the effect of hymenialdisine, which specifically inhibits NF-κB activity[28], on both mRNA and protein levels of OPG in RA synovial fibroblasts. Treatment with 5 μM and 10 μM hymenialdisine resulted in 43% and 58% decrease of OPG production (TNF-α 10 ng/ml; 24 h incubation), respectively (Figure 5A). In addition, we investigated the effects of NAC, a well known inhibitor of NF-κB activity[29], on the production of OPG in RA synovial fibroblasts. Fibroblasts were pretreated with NAC for 2 h prior to TNF-α (10 ng/ml) stimulation. After incubation for 24 h, the conditioned medium was harvested. Treatment with 1, 10, and 30 mM NAC resulted in 36%, 64%, and 96% decrease of OPG production, respectively (Figure 5B).

TNF-RI and TNF-RII differentially regulate OPG and RANKL expression in RA synovial fibroblasts. To determine whether TNF-RI or TNF-RII dominates the regulation of TNF-α-induced OPG expression in RA synovial fibroblasts, we investigated TNF-α-induced OPG expression and production in the presence of human soluble TNF receptor I/Fc chimera (TNFs-RI/Fc chimera) or TNFs-RII/Fc chimera. Cells were incubated with these agents for 24 h. By Northern blot hybridization, TNF-α-induced OPG expression was inhibited by TNFs-RI/Fc dose-dependently, whereas that was not the case with TNFs-RII/Fc. TNFs-RI/Fc (100 ng/ml) resulted in a 50% decrease of TNF-α-induced OPG mRNA levels, while TNFs-RII/Fc (100 ng/ml) had little effect on TNF-α-induced OPG mRNA levels. In other words, TNFs-RI/Fc reduced TNF-α-induced OPG expression to the untreated control level. By contrast, human IgG-Fc, which was used to investigate nonspecificity of fusion protein, had little effect on the level of TNF-α-induced OPG expression. Addition of TNFs-RI/Fc or TNFs-

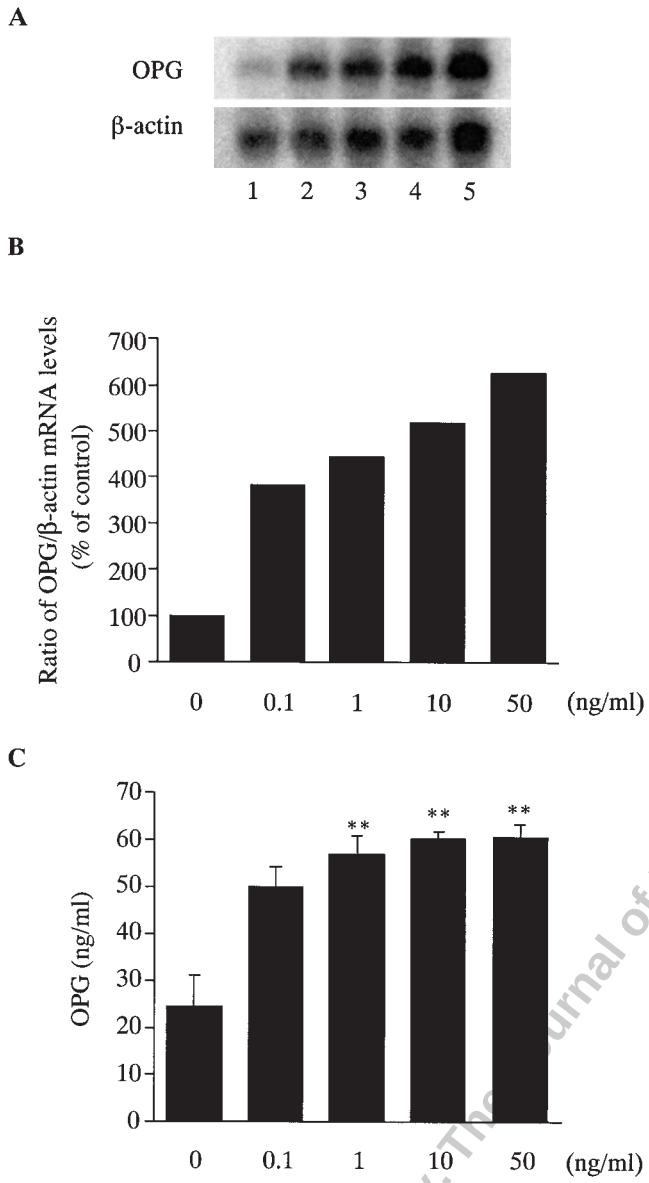

Figure 3. Dose-dependent effects of TNF-α on levels of OPG mRNA and production in RA synovial fibroblasts. Total RNA was extracted from cultured RA fibroblasts treated with various concentrations of TNF-α for 24 h. Total RNA (6 µg) was analyzed by Northern blot hybridization (A). Level of OPG mRNA was compared to the level of ß-actin mRNA by densitometry. The ratio of OPG mRNA to ß-actin mRNA is presented as the percentages of untreated control (B). Levels of OPG in the conditioned media were measured by ELISA (C). OPG production in TNF-α-treated cells was increased in comparison with untreated control, with levels significantly different from that of untreated control. **p < 0.01 versus untreated control. Lane 1, 0 ng/ml; lane 2, 0.1 ng/ml; lane 3, 1 ng/ml; lane 4, 10 ng/ml; lane 5, 50 ng/ml TNF-α.

RII/Fc and the addition of TNFs-RI/Fc plus TNFs-RII/Fc in the absence of TNF-α did not affect the expression of OPG (Figure 6A, 6B).

To confirm the OPG production, conditioned medium was collected from cultured RA synovial fibroblasts and

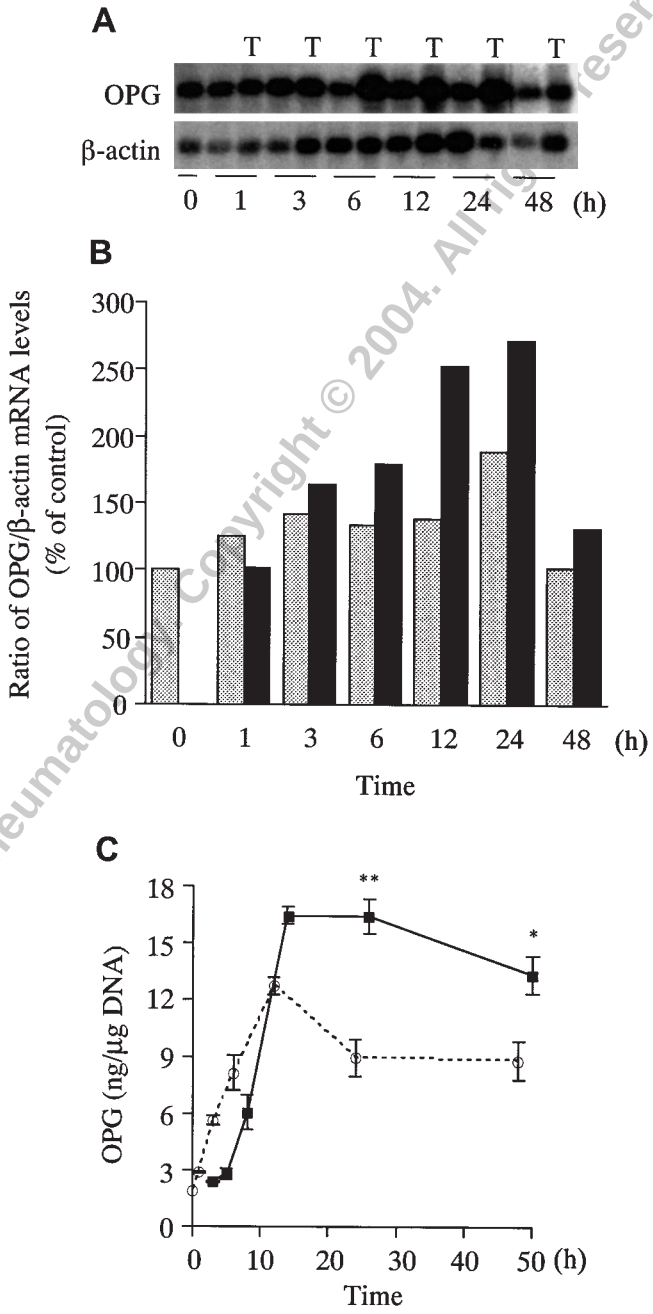

Figure 4. Time-dependent effects on the levels of OPG mRNA and the production of OPG in RA synovial fibroblasts. Both total RNA and conditioned medium were collected after the indicated times in cultures in the presence (black bar) or absence (shaded bar) of TNF-α. Total RNA (6 µg) was analyzed by Northern blot hybridization (A). Level of OPG mRNA was compared to the level of ß-actin mRNA by densitometry. The ratio of OPG mRNA to ß-actin mRNA is presented as percentages of untreated control (B). Time-dependent increase of OPG production in RA synovial fibroblasts treated with TNF-α (C). Cells were incubated in the absence (○) or presence (■) of TNF-α. Conditioned medium was collected at the indicated times and measured by ELISA. Data are expressed as ng/mg DNA. ***p < 0.001 versus untreated control.

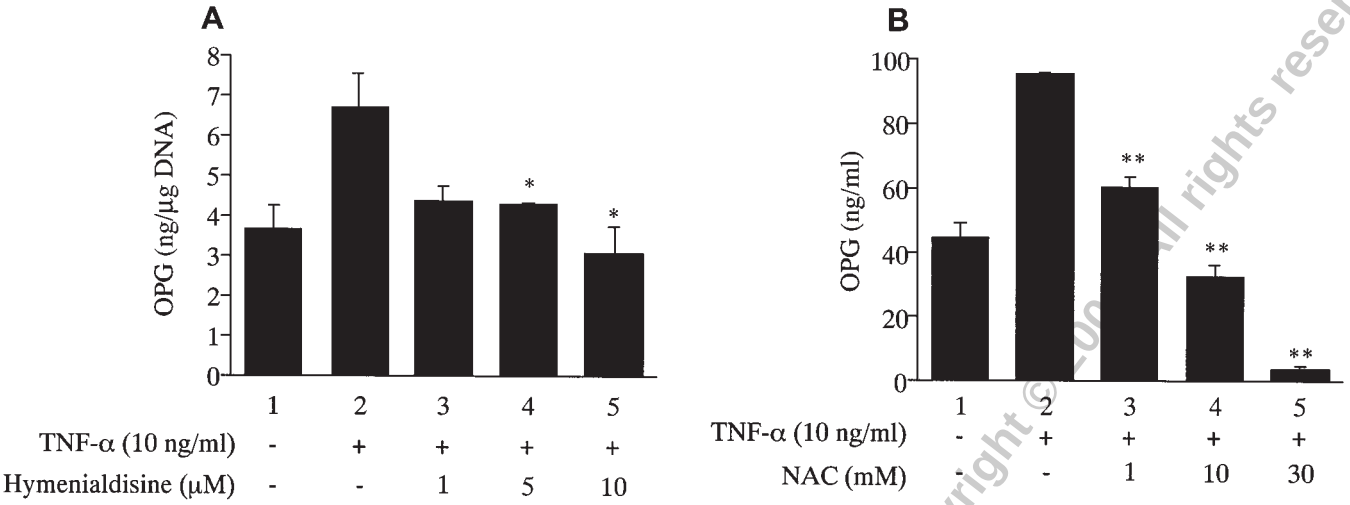

Figure 5. Inhibitory effect of NF-κB inhibitors on the production and mRNA expression of OPG. RA synovial fibroblasts were incubated with TNF-α (10 ng/ml) in the presence of various concentrations of hymenialdisine or NAC for 24 h. NAC was added 2 h before addition of TNF-α. OPG level in conditioned medium was measured by ELISA. Hymenialdisine significantly inhibited TNF-α-induced OPG production. Data are expressed as ng/μg DNA (A). *$p < 0.05$ versus lane 2. NAC significantly inhibited TNF-α-induced OPG production (B). **$p < 0.01$ versus lane 2.

analyzed by ELISA. TNFs-RI/Fc (100 ng/ml) resulted in a 50% decrease of TNF-α-induced OPG production when cells were incubated with TNF-α (10 ng/ml) for 24 h, whereas TNFs-RII/Fc had little effect on TNF-α-induced OPG production compared to TNF-α alone (Figure 6C). In contrast, RT-PCR revealed that the expression of RANKL was upregulated by TNF-α (10 ng/ml) and that 10 and 100 ng/ml of TNFs-RI/Fc resulted in 13.5% and 85.3% inhibition of RANKL mRNA levels, respectively, whereas TNFs-RII/Fc had little effect on RANKL mRNA levels compared to TNF-α (Figure 7).

TNF-α-induced osteoclastogenesis in RA synovial cells. We investigated the formation of TRAP-positive multinucleated cells (MNC) in primary culture of RA synovial cells. The formation of TRAP-positive MNC with more than 3 nuclei was seen in the presence of TNF-α and M-CSF (2 ng/ml) in primary culture of RA synovial cells. But the number of MNC was decreased by the addition of TNF-α in a dose dependent maner, except at 1 ng/ml TNF-α (Figure 8). The reason why the stimulation effect of 1 ng/ml TNF-α is lower than that of 10 ng/ml TNF-α is not known. To investigate the effect of OPG on TNF-α-induced MNC formation, OPG was added to a primary culture of RA synovial cells. In the presence of OPG (20 ng/ml), the formation of TNF-α-induced MNC was significantly inhibited (Figure 8).

We investigated the morphological features of TNF-α-induced MNC in primary culture of RA synovial cells. The formation of TRAP-positive MNC was observed in cultures treated with both $1,25(OH)_2D_3$ and TNF-α. TRAP-positive cells with a large number of nuclei were observed in TNF-α-induced MNC (Figure 9B). The band of F-actin containing podosomes was recognized on the periphery of TNF-α-induced MNC. TNF-α-induced MNC were larger than cells treated with $1,25(OH)_2D_3$ (Figure 9C, 9D). We examined the bone resorption activity of TNF-α-induced MNC. Synovial cells in RA primary culture were incubated in the presence of M-CSF and TNF-α or $1,25(OH)_2D_3$ on osteologic discs for 3 weeks, and we observed pit formation on both TNF-α- and $1,25(OH)_2D_3$-induced MNC. But the area of each resorption pit in cultures treated with TNF-α was smaller than that of cells treated with $1,25(OH)_2D_3$ (Figure 9E, 9F). The percentages of total resorption pit per area were 15.9 ± 0.75% for $1,25(OH)_2D_3$ and 8.41 ± 0.46% for TNF-α, when untreated control cells showed 1.45 ± 0.86% (n = 3). The resorption activity of TNF-α-treated cells was almost 2-fold weaker than that of $1,25(OH)_2D_3$-treated cells. The ratio of OPG to RANKL is critical in determining whether effects of TNF-α are bone resorptive and protective. As shown in Table 1, the ratio of OPG to RANKL mRNA in TNF-α-treated cells increased almost 2-fold compared with that in $1,25(OH)_2D_3$-treated cells.

To investigate the characteristics of TNF-α-induced MNC, we performed RT-PCR to analyze the expression of CAII, CTR, and cathepsin K, which are regarded as the characteristic features of osteoclasts. We detected expression of CAII, CTR, and cathepsin K in TNF-α-induced MNC as well as that of $1,25(OH)_2D_3$-induced MNC (Figure 10), but the stromal cell/synovial fibroblast-rich fraction showed little of this expression. From these observations, we concluded that TNF-α-induced MNC satisfied the criteria for osteoclasts.

DISCUSSION

We observed that OPG was spontaneously expressed abundantly in RA synovial fibroblasts compared to OA and noninflammatory synovial fibroblasts. TNF-α-induced OPG

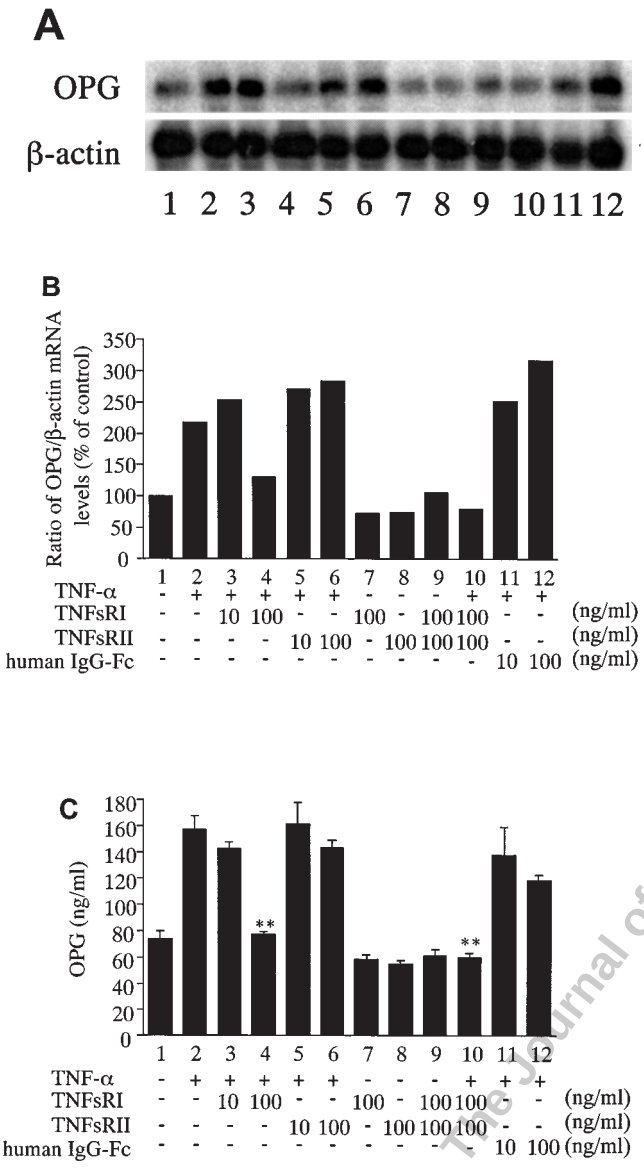

Figure 6. Effects of TNF-soluble receptors/Fc on the levels of OPG mRNA and OPG production in RA synovial fibroblasts. Total RNA was extracted from fibroblasts treated with TNF-α (10 ng/ml) for 24 h in the presence of TNF-soluble receptor/Fc fusion protein (TNFs-RI and TNFs-RII). Total RNA (6 µg) was analyzed by Northern blot hybridization (A). Level of OPG mRNA was compared to the level of ß-actin mRNA by densitometry. The ratio of OPG mRNA to ß-actin mRNA is presented as percentages of untreated control (B). Levels of OPG in conditioned medium were measured by ELISA (C). Addition of TNFs-RI/Fc significantly inhibited TNF-α-induced (10 ng/ml) OPG production. **p < 0.01 versus TNF-α (10 ng/ml).

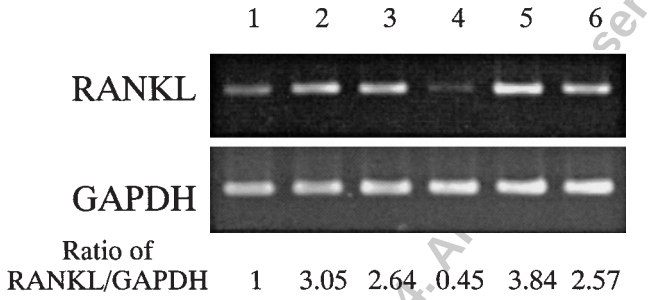

Figure 7. Effects of TNFs-RI/Fc and TNFs-RII/Fc on expression of mRNA of RANKL in RA synovial fibroblasts, the same sample used in Figure 5, by RT-PCR. Level of RANKL mRNA was compared to the level of GAPDH mRNA by densitometry. Lane 1, control; lane 2, TNF-α (10 ng/ml); lane 3, TNF-α + TNFs-RI/Fc (10 ng/ml); lane 4, TNF-α + TNFs-RI/Fc (100 ng/ml); lane 5, TNF-α + TNFs-RII/Fc (10 ng/ml); lane 6, TNF-α + TNFs-RII/Fc (100 ng/ml).

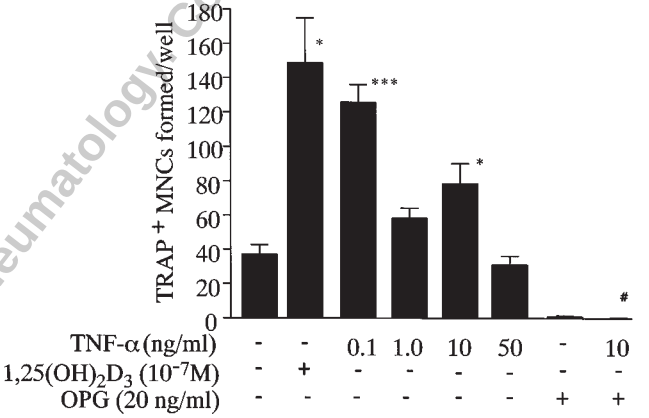

Figure 8. TRAP-positive multinucleated cell (MNC) formation induced by TNF-α in primary cultures of rheumatoid synovial cells. RA synovial cells were treated with various concentrations of TNF-α or OPG (20 ng/ml) in the presence of M-CSF (2 ng/ml). TRAP-positive cells induced by TNF-α were fewer than those of $1,25(OH)_2D_3$ (10^{-7} M), and formation was completely inhibited by the addition of OPG. *p < 0.05 and ***p < 0.001 versus untreated control cells. #p < 0.001 versus addition of TNF-α (10 ng/ml).

expression in RA was higher than that in OA and noninflammatory subjects, with the exception of one subject. In addition, the level of OPG mRNA was varied among the patients with RA. In this study, TNF-α induced a higher level of OPG mRNA in lanes 6 and 7 of Figure 1, but it did not induce production of OPG in lanes 6 and 7, in spite of induction of their mRNA. In this case, one possibility is that OPG may be easily degraded by any proteases or inhibition of translation induced by TNF-α. As a result, there is insufficient OPG production in RA synovial fibroblasts for protection against severe bone destruction. Accordingly, the regulatory mechanism of OPG expression at a post-transcriptional level is important as well as at the transcriptional level. In addition, we did not investigate the soluble RANKL in the conditioned medium; we cannot exclude the possibility that OPG secreted by RA synovial fibroblasts was bonded to the soluble RANKL. Since noninflammatory and OA fibroblasts did not produce OPG abundantly, we concluded that RA fibroblasts have an inherent potential to express OPG and upregulate it under inflammatory condi-

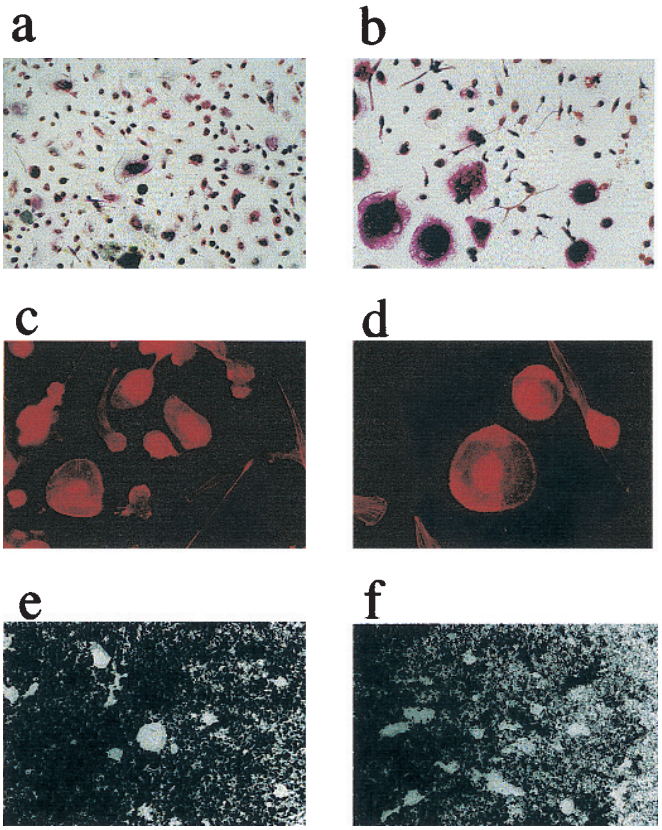

Figure 9. Characteristics of the formation of TNF-α-induced TRAP-positive MNC in primary cultures of RA synovial cells. RA synovial cells were cultured with TNF-α (10 ng/ml) or $1,25(OH)_2D_3$ (10^{-7} M) in the presence of M-CSF (2 ng/ml) for 3 weeks. TRAP staining was performed (a, b), formation of an actin ring was evaluated by rhodamine-conjugated phalloidin staining (c, d), and the resorbing activity of TRAP-positive MNC was evaluated. RA synovial cells were cultured with TNF-α (10 ng/ml) or $1,25(OH)_2D_3$ (10^{-7} M) on osteologic discs in the presence of M-CSF (2 ng/ml) for 3 weeks (e, f). Panels a, c, e: $1,25(OH)_2D_3$ (10^{-7} M); b, d, f: TNF-α (10 ng/ml).

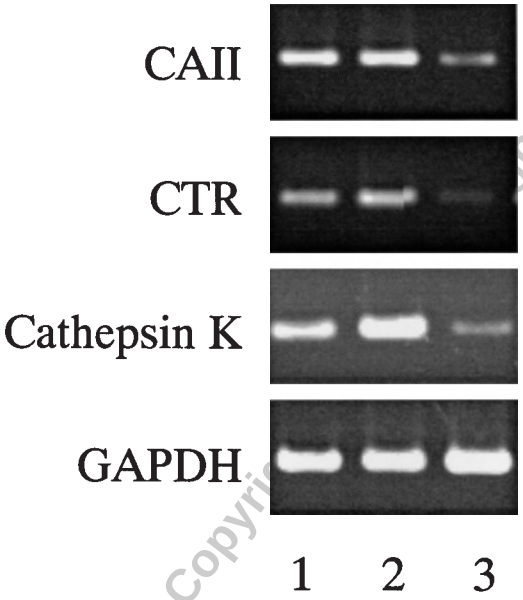

Figure 10. mRNA expression of CAII, CTR, and cathepsin K in TNF-α-induced osteoclasts. Expression of mRNA of CAII, CTR, cathepsin K, and GAPDH of TNF-α (10 ng/ml) or $1,25(OH)_2D_3$ (10^{-7} M)-induced osteoclasts by RT-PCR. Total RNA was extracted from purified osteoclasts in primary rheumatoid synovial cell cultures. Lane 1, $1,25(OH)_2D_3$ (10^{-7} M)-induced osteoclasts; lane 2, TNF-α (10 ng/ml)-induced osteoclasts; lane 3, stromal cell/synovial fibroblast-rich fraction in the presence of $1,25(OH)_2D_3$ (10^{-7} M).

Table 1. The ratio of OPG/RANKL mRNA in TNF-α-treated RA synovial fibroblasts. Total RNA was extracted from cultured fibroblasts treated with 1,25 $(OH)_2D_3$ (10^{-7}M) and TNF-α (10 or 50 ng/ml) for 24 h. OPG, RANKL, and ß-actin mRNA were analyzed by Northern blot hybridization and levels of mRNA were determined by densitometry (n = 3). The levels of OPG and RANKL mRNA were compared to the level of ß-actin mRNA. The ratio of OPG to RANKL was calculated from the average value of each mRNA.

Treatment	mRNA Level (mean ± SE) OPG/ß-actin	RANKL/ß-actin	Ratio of OPG/RANKL
Untreated	1.00 ± 0.10	0.38 ± 0.06	2.63
1,25 $(OH)_2D_3$	0.88 ± 0.10	1.13 ± 0.13	0.78
TNF-α (10 ng/ml)	1.58 ± 0.07	1.57 ± 0.14	1.01
TNF-α (50 ng/ml)	1.43 ± 0.23	0.93 ± 0.09	1.53

tions. TNF-α may contribute to synovitis and bone destruction by inducing proinflammatory cytokine[30] and collagenase release from synovial cells[31]. Accordingly, even overproduction of OPG may not be sufficient to protect a person from progressive bone destruction.

We also demonstrated that TNF-α increases the expression of OPG mRNA levels in RA synovial fibroblasts in a time and dose- dependent manner, and predominantly via TNF-RI more than TNF-RII. Since addition of high concentration of TNF-RII/Fc (1000 ng/ml) reduced to 55% of the expression of OPG mRNA, TNF-RII/Fc has the potential to inhibit cell activity. It was recently reported that TNF-α may mediate its diverse biological effects via the 2 receptors TNF-RI and TNF-RII, and the function of TNF-α may vary over the time course of disease, from initiation to a later stage[32]. TNF-α acts as a proinflammatory force during the course of disease initiation, whereas it plays a disease-suppressive role at a later stage. It was reported that the systemic blockade of TNF-RI in autoimmune demyelination might inhibit the proinflammatory activities of TNF-α without compromising its immunosuppressive properties against T cells in patients with multiple sclerosis[32].

Synovial cells are divided into 3 major populations, synovial macrophages (type A), synovial fibroblasts (type B), dendritic cells (type D), and the other T cells, according to the morphology and expression of surface antigen[33]. As

we used synovial cells cultured for 5–8 passages in this study, the effects of T cells, macrophages, and dendritic cells were excluded. To our knowledge, this is the first study to demonstrate that TNF-α may contribute to protection from bone destruction by upregulating the expression of OPG, which is regulated differentially by TNF-RI and TNF-RII in rheumatoid synovial fibroblasts. In addition we investigated the levels of OPG in noninflammatory synovial fibroblasts. Both the level and production of OPG mRNA were induced by TNF-α and inhibited by TNF-RI/Fc in a dose-dependent manner; however, the level of OPG was lower than that of RA synovial fibroblasts at any concentration. These results suggest that every synovial fibroblast has the potential to respond to TNF-α and to express OPG to protect from bone destruction.

On the other hand, we observed TNF-α-induced osteoclastogenesis in the presence of M-CSF in primary RA cultures, which was inhibited by the addition of OPG. Figure 8 shows that formation of TNF-α-induced MNC was increased at 10 ng/ml of TNF-α, but there was a reduction of the number of MNC treated with 50 ng/ml compared with 10 ng/ml. These results suggest that OPG expression was more stimulated in higher concentrations of TNF-α and the balance between RANKL and OPG declined to inhibit the osteoclastogenesis. Indeed, the ratio of OPG to RANKL mRNA expression in the presence of TNF-α (50 ng/ml) increased almost 2-fold compared to use of $1,25(OH)_2D_3$ (Table 1).

In mouse bone marrow culture, TNF-α increased osteoclastogenesis, at least up to a concentration of 200 ng/ml, in a dose-dependent manner. The discrepancy in the effects of TNF-α on osteoclastogenesis between the human RA synovial cell primary culture and mouse bone marrow culture systems might be caused by the difference of OPG production; that is, TNF-α increases OPG production in human RA synovial cells, but decreases that in mouse stromal cells.

It has been reported that TNF-α-induced osteoclastogenesis in the absence of supporting cells, such as stromal cells, osteoblasts, or synovial cells, was not inhibited by the addition of OPG[34,35]. In contrast, TNF-α and RANKL synergistically induce osteoclastogenesis[36]. Since rheumatoid synovial tissue contains various cell types as described, the possibility is that TNF-α and RANKL, expressed by activated T cells[37] or synovial fibroblasts, synergistically induced osteoclastogenesis in this culture. Interestingly, however, TNF-α-induced osteoclastogenesis (Figure 8) and the size of the TRAP-positive MNC and actin ring were larger than that of $1,25(OH)_2D_3$ (Figure 9A, 9B), and the pit areas treated with TNF-α were smaller than those of $1,25(OH)_2D_3$. The percentage of total resorption pit per area of TNF-α was 2 times lower than that of $1,25(OH)_2D_3$. These results might be consistent with the findings that TNF-α induces differentiation and survival of osteoclasts, but not their function[35].

Osteoclastogenesis is assumed to be controlled by the balance between RANKL and OPG *in vivo*; therefore, it would be important to evaluate the expression of RANKL in RA. TNF-α also induced mRNA expression of RANKL in RA synovial fibroblasts (Figure 7), but in noninflammatory and OA synovial fibroblasts, RANKL expression was not detected, as reported previously[1]. RANKL has been found to be the essential factor for osteoclastogenesis *in vivo*, since targeted disruption of RANKL gene led to osteopetrosis in mice[38]. Accordingly, bone destruction in noninflammatory and OA was not severe compared with RA, as the expression of RANKL and OPG were lower than that of RA. In RA, when abundant inflammatory cytokine such as TNF-α was produced during inflammation, the balance between RANKL and OPG caused disruption of bone metabolism, which may be followed by bone destruction. Thus, OPG may be a candidate for therapy to prevent bone destruction in RA.

It is reported that blocking TNF-α in RA reduces disease activity and inhibits the progression of structural damage in patients who had failed multiple disease modifying antirheumatic drugs[39]. Interestingly, 2 TNF-α antagonists, infliximab (a chimeric monoclonal antibody) and etanercept (a p75 TNF receptor/Fc fusion protein), show similar clinical results in patients with RA[7,8], despite having different profiles in Crohn's disease[40], and these findings may reflect the different roles of the 2 receptors. Recently it was reported that TNFs-RI with OPG suppressed both inflammation and bone loss in rats with adjuvant arthritis[41]. Together with these observations, selective blockade of TNF-α-induced inflammation, without compromising the self-production of OPG via TNF-RI in RA synovial fibroblasts, may contribute to protection against bone destruction.

Our results suggest that TNF-α induces OPG that is predominantly mediated by TNF-RI, and that plays a role in protection from bone destruction in RA as well as osteoclastogenesis.

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