# Increase in RANKL: OPG Ratio in Synovia of Patients with Temporomandibular Joint Disorder

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# Increase in RANKL: OPG Ratio in Synovia of Patients with Temporomandibular Joint Disorder

## ABSTRACT

Although a recent study suggested the involvement of RANKL and osteoprotegerin (OPG) in the pathogenesis of bone-destructive disease, no study has focused on the RANKL:OPG ratio in the synovial fluid of patients with temporomandibular joint (TMJ) disorder. This communication reports on the concentrations of RANKL and OPG in synovial fluid from TMJ patients and healthy control individuals. In contrast to an unchanged concentration of RANKL, a strong decrease in the concentration of OPG was detected in the synovial fluid from patients with TMJ internal derangement. Treatment with the synovial fluid of osteoarthritis (OA) patients resulted in the high production of osteoclast-like cells from blood mononuclear cells *in vitro*, as well as in pit formation in dentin slices. The addition of anti-RANKL IgG or OPG attenuated OA-synovial fluid-induced osteoclast formation, suggesting that the increase in the RANKL:OPG ratio in the microenvironment of the joint has the potential to induce osteoclastogenesis in TMJ osteoarthritis.

**KEY WORDS:** osteoprotegerin, RANKL, synovial fluid, osteoarthritis, temporomandibular joint.

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## INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease that is characterized by articular cartilage degradation and concomitant reparative/adaptive osteogenesis. However, the molecular and cellular basis of the pathophysiology of OA is still unclear. Many lines of evidence indicate that variable degrees of inflammation exist in some temporomandibular joint (TMJ) disorders. Recent advances in biochemical analysis of the contents of synovial fluid have provided new insights into the pathophysiologic nature of TMJ disorder; that is, various inflammatory mediators—*e.g.,* interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), and prostaglandins—are considered to serve as possible markers of active TMJ disorder (Shafer *et al.*, 1994; Sandler *et al.*, 1998).

A recent study also suggested the involvement of the receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in the pathogenesis of bone-destructive disease, such as rheumatoid arthritis and periodontal disease (Kotake *et al.*, 2001; Romas *et al.*, 2002; Mogi *et al.*, 2004; Ohazama *et al.*, 2004). OPG, a secreted glycoprotein of the TNF receptor superfamily, is a decoy receptor for RANKL (Simonet *et al.*, 1997). When OPG is present to bind to RANKL, the cell-to-cell signaling between marrow stromal cells and osteoclast precursors is inhibited, and thus osteoclasts are not formed (Simonet *et al.*, 1997; Wong *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998; Kong *et al.*, 1999). Thus, the cytokines and decoy receptors expressed by bone-associated cells play important roles during osteoclast formation, by balancing induction and inhibition (Hofbauer and Heufelder, 2001; Ohazama *et al.*, 2004). Osteoblasts in culture hardly release RANKL, but activated T-cells in culture release a large amount of RANKL in its soluble form (Wong *et al.*, 1997; Kong *et al.*, 1999; Nakashima *et al.*, 2000). In addition to the increase in the level of RANKL protein in the inflamed synovium of rheumatoid arthritis patients (Romas *et al.*, 2002), we have demonstrated that OPG concentrations in the synovial fluid were lower in patients with rheumatoid arthritis (as compared with patients with other forms of arthritis), which resulted in an increased local and systemic RANKL:OPG ratio (Kotake *et al.*, 2001). A recent study using animal models suggested the involvement of RANKL and OPG in the pathogenesis of periodontal disease (Teng *et al.*, 2000). We have also demonstrated an elevation of RANKL and a decrease in OPG in gingival crevicular fluid of patients with periodontal disease (Mogi *et al.*, 2004), suggesting that RANKL and OPG are important factors involved in bone and joint destruction in human rheumatoid arthritis and periodontal disease, and that OPG has a protective role in bone-destructive diseases.

We hypothesize that RANKL and OPG are associated with the pathogenesis of TMJ internal derangement, especially in OA. To test this hypothesis, we conducted *in vivo* studies on humans to elucidate the role of RANKL and OPG in 3 groups of TMJ internal derangement (OA, joints with disk displacement with reduction [DDwR], and joints with disk displacement without reduction [DDw/oR]).

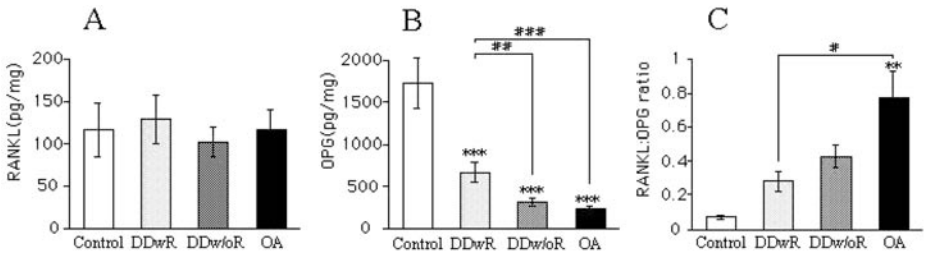


**Figure 1.** Plots depicting RANKL (**A**, pg/mg protein) and OPG (**B,** pg/mg protein) concentrations, and the RANKL:OPG ratio (**C**) in the synovial fluid of joints with disk displacement with reduction (DDwR), joints with disk displacement without reduction (DDw/oR), and osteoarthritis (OA) in patients and control participants. The content of RANKL in the synovial fluid was determined by an ELISA, and that of OPG, by a commercially available ELISA. Data are expressed as the mean ± SEM. Data from control (Cont; n = 13), DDwR (n = 25), DDw/oR (n = 39), and osteoarthritis (OA; n = 53) participants were analyzed. Fisher's protected least-significant-difference *post hoc* test was used when multiple groups were compared with a single control group. $^{**}P < 0.01$, $^{***}P < 0.005$ (compared with the control), and $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.005$, as indicated by the brackets.

## MATERIALS & METHODS

### Participant and Site Selection

One hundred and 17 patients with moderate or severe TMJ dysfunction were selected from those patients referred to the Temporomandibular Disorder Clinic of Aichi-Gakuin University Hospital. Informed consent was obtained from all participants at the first visit, and the protocol for human participants was reviewed and approved by our institutional panel. Patients were given no medication for at least 2 wks before synovial fluid sampling was performed. Clinical examination was performed, and signs and symptoms were recorded by two independent examiners. The patients were divided into 3 groups according to their principal clinical signs and symptoms and MR imaging modalities: disk displacement with reduction (DDwR, 25 patients; six males + 19 females, mean age, 32.4 ± 2.2 yrs), disk displacement without reduction (DDw/oR, 39 patients; four males + 35 females, mean age, 37.4 ± 2.5 yrs), and osteoarthritis (OA, 53 patients; six males + 47 females, mean age, 42.4 ± 2.6 yrs) of the TMJ. The following clinical criteria were used: DDwR, reciprocal clicking in the joint with joint pain and intermittent locking of short duration; DDw/oR, locking of the TMJ, pronounced impairment of joint mobility, joint pain, and a history of clicking and intermittent locking; and OA of the TMJ, impaired joint mobility, joint pain, and degenerative changes in osseous joint surfaces, as evaluated by tomography and magnetic resonance imaging. Individuals in the control group (Cont, 13 persons; seven males + six females; mean age, 34.2 ± 4.0 yrs) were healthy.

### Sample Collection

Synovial fluid samples were obtained after local anesthesia with lidocaine, which was injected into the extracapsular region of the TMJ. A total of 2 mL of saline solution was injected into the superior compartment of the joint. The mixture of synovial fluid and saline solution was aspirated. The sample was centrifuged for the removal of cells and was stored at -80°C for later processing. The protein concentration of the synovial fluid was estimated by the method of Bradford (1976), with bovine serum albumin as the standard.

### RANKL and OPG Determination

A free soluble form of RANKL was measured by a two-site ELISA (Kinpara *et al.*, 2000). OPG was also determined by use of a commercially available two-site sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA). All samples and standards were assayed twice. Data are reported as the concentrations of ligand or decoy-receptor (pg/mg of total protein), and were presented as the means ± SEM.

### Functional Assay for Osteoclast Differentiation

Human studies were approved by the Aichi-Gakuin University Institutional Review Board. Informed consent was obtained in all cases before blood aspiration. Formation of osteoclast-like cells (OCLs) was determined as previously reported (Kotake *et al.*, 2001). Human peripheral blood mononuclear cells (PBMC, 2 x $10^5$ cells/well) were cultured for 6 days with recombinant human macrophage-colony-stimulating factor (M-CSF, 100 ng/mL; R&D Systems) in 0.3 mL of α-MEM containing 10% fetal calf serum in 24-well plates. After having been cultured for the desired periods, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells appeared as red cells. TRAP-positive multinucleate cells containing more than 3 nuclei were counted as osteoclasts. The results obtained from 1 experiment, which were typical of those of at least 3 independent experiments, were expressed as the mean ± SEM of 3 cultures. The significance of the differences was determined by one- or two-way ANOVA.

Pit-forming activity of OCLs formed in the cultures was assayed according to the procedure described previously (Kotake *et al.*, 2001). PBMC were cultured on dentin slices (diameter, 10 mm) in the presence of M-CSF (100 ng/mL) in 24-well plates, and the resorption pits on the slices were then stained with Mayer's hematoxylin. The total area of resorption pits (reflecting bone-resorbing activity) was quantified by densitometric analysis of images of the whole area of dentin slices (circular, 10 mm in diameter), with the use of image analysis (NIH imaging, Bethesda, MD, USA).

### Statistical Analysis

The results are expressed as the means ± SEM of quadruplicate cultures. Statistical analysis was carried out by one- or two-way ANOVA. Fisher's protected least-significant-difference *post hoc* test was used when multiple groups were compared with a single control group.

## RESULTS

Concentrations of RANKL in TMJ synovial fluid samples are shown in Fig. 1A. Although the synovial fluid contained a low amount of RANKL (around 100-130 pg/mg protein) in the healthy participants, RANKL levels in the synovial fluid of patients with each degree of TMJ internal derangement showed no statistical difference from those in the controls. In contrast, the concentrations of OPG in the synovial fluid were significantly lower in patients with TMJ internal derangement than in the healthy controls ($p < 0.005$; Fig. 1B). The OPG

value for the OA group was only 37% of the level for the DDwR group ($p < 0.005$). Interestingly, there was also a significant difference in the OPG level between DDwR and DDw/oR groups ($p < 0.01$; Fig. 1B). When we calculated the ratio of the concentration of RANKL to that of OPG, the ratio was significantly higher in OA patients than in the control participants ($p < 0.01$; Fig. 1C).

To test the significance of the RANKL:OPG ratio in osteoclastogenesis in the TMJ, we performed further experiments. We confirmed that the co-presence of M-CSF (100 ng/mL) and RANKL (30 ng/mL) could induce potent multinucleate TRAP-positive cells from PBMC and pit formation in dentin slices (data not shown). Next, we tested whether an artificial sample containing RANKL and OPG in the same proportions as those in the synovial fluid would have the potential to induce osteoclast formation from human monocytes in the presence of M-CSF. In general, the volume of synovial fluid is around 20 μL (Aghabeigi *et al.*, 1993). Since we collected the synovial fluid mixed with 2 mL of saline, the real concentration of RANKL and OPG must be around 100-fold higher than the estimated values in the samples. According to our data, the RANKL levels in the synovial fluid of patients with TMJ internal derangement were 100-300 pg/mL. Therefore, the real concentration of RANKL was calculated as 10-30 ng/mL of synovial fluid in each participant. According to the estimated mean value of the RANKL:OPG ratio in control (0.07), DDwR (0.30), DDw/oR (0.43), and OA (0.77), we prepared the respective mixtures of RANKL and OPG in α-MEM. We then tested the mixtures in the osteoclast formation assay, using PBMC, and in the pit-formation assay. Surprisingly, we found that the OA-artificial sample containing RANKL and OPG could potently induce multinucleate OCLs, in comparison with the weak induction by the treatment with the control or DDwR artificial sample (Fig. 2). The DDw/oR artificial sample containing RANKL and OPG could also induce multinucleate OCL formation. When human PBMCs were cultured on dentin slices with OA artificial samples, many resorptive pits were formed on the slices, at a level that was statistically significant compared with that for any of the other groups (Fig. 2), suggesting that the synovial fluid with a high RANKL:OPG ratio has the potential to cause PBMCs to differentiate into active osteoclasts.

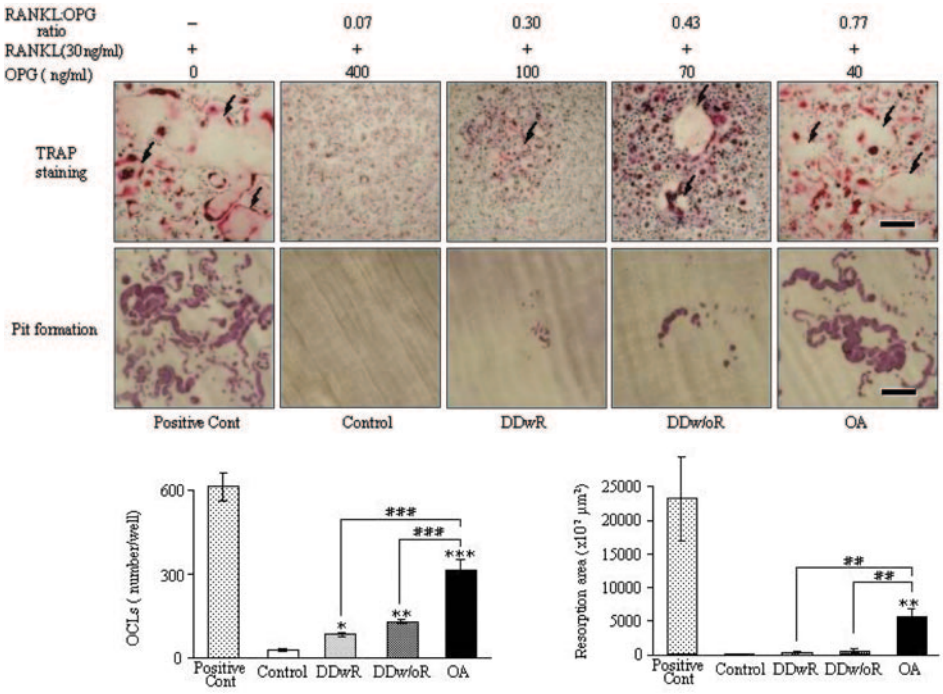


**Figure 2.** RANKL+OPG induces osteoclast formation from human peripheral monocytes in the presence of M-CSF. Human peripheral blood mononuclear cells were isolated, washed, and cultured for 6 days with M-CSF (100 ng/mL) in 24-well plates. According to the estimated value of the RANKL:OPG ratio in the control (0.07), DDwR (0.30), DDw/oR (0.43), and OA (0.77) participants, we prepared the respective mixtures of recombinant human RANKL (PeproTeck, London, UK) and OPG (OPG/Fc chimera, R&D Systems, Minneapolis, MN, USA) in α-MEM. With RANKL fixed at 30 ng/mL in the sample, OPG was added to the sample at a final concentration of 400 ng/mL (Cont), 100 ng/mL (DDwR), 70 ng/mL (DDw/oR), or 40 ng/mL (OA). Then the mixture was used in the osteoclast formation assay by PBMC (TRAP staining), and to the pit-formation assay, in the presence of M-CSF (100 ng/mL). TRAP-positive cells containing 3 or more nuclei were counted as osteoclasts.

Values are expressed as the mean ± SEM (number/well) of 3 cultures. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ (compared with the control), and $\#\#\#P < 0.005$ as indicated by the brackets. Experiments were repeated 3 times, and similar results were obtained. Bar = 200 μm.

Pit-forming activity of OCLs formed in the cultures was assayed on dentin slices (diameter, 10 mm) in 24-well plates. After having been cultured for 21 days, the adherent cells were removed from the slices, and the resorption pits on the slices were then stained with Mayer's hematoxylin. Osteoclastic bone-resorption activity was measured in terms of the pit area formed by osteoclasts. Values are mean ± SEM of 3 cultures. $**P < 0.01$ compared with control groups. $\#\#P < 0.01$ as indicated by the brackets. Bar = 500 μm. Each experiment was repeated 3 times, and the data shown are representative of 3 independent experiments. Arrows point to osteoclasts. Original magnification, 40x for TRAP staining, and 100x for pit-formation assay.

Next, we examined whether the synovial fluid of the patients with OA had the potential to induce OCL formation from human monocytes in the presence of M-CSF. As described above, we collected the synovial fluid mixed with 2 mL of saline. Therefore, the synovial fluid samples were de-salted by dialysis, lyophilized, and concentrated up to original concentration levels (around 100-fold-concentrated). We demonstrated that the addition of synovial fluid from patients with OA resulted in the potent formation of OCLs from human monocytes and resorption pits on dentin slices, and the difference between OA samples and any of the others was statistically significant (Fig. 3). The synovial fluid of the patients with DDw/oR had a small,

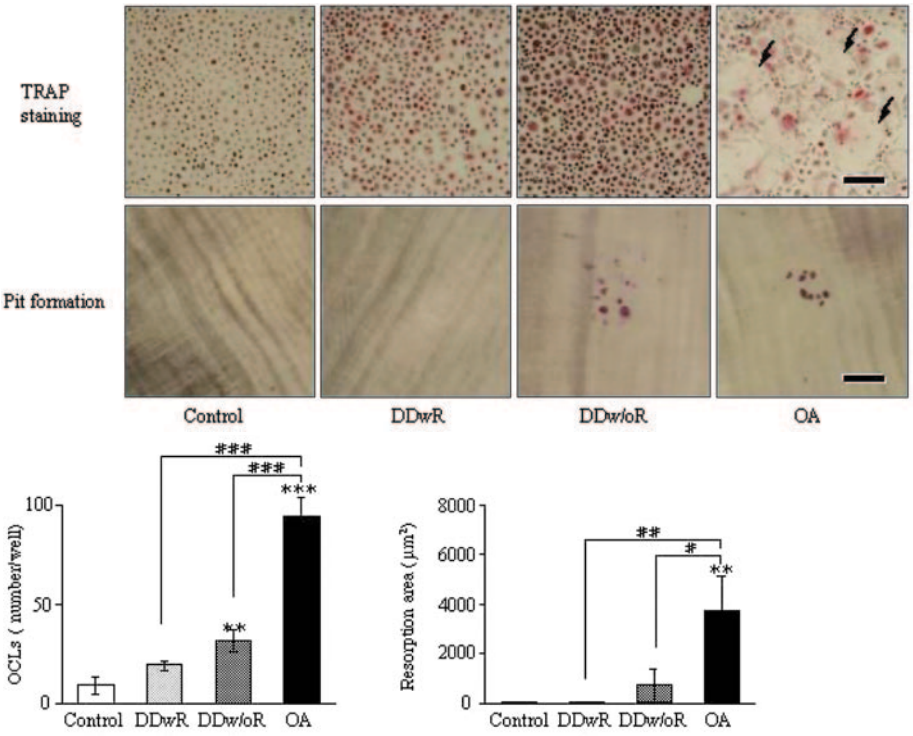


**Figure 3.** Synovial fluid induces osteoclast formation from human peripheral monocytes in the presence of M-CSF. The synovial fluids were de-salted by dialysis, lyophilized, and concentrated up to the original concentration (around 100-fold). Then, the reconstituted samples were applied to the osteoclast formation assay by PBMC, and to the pit-formation assay, in the presence of M-CSF (100 ng/mL). $^{**}P < 0.01$, $^{***}P < 0.005$ compared with control groups. $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.005$, as indicated by the brackets. Experiments were repeated 3 times with similar results. Arrows point to osteoclasts. Original magnification, 40x for TRAP staining (bar = 200 μm), and 100x for pit-formation assay (bar = 500 μm).

but substantial, potential for activating osteoclasts, which also led to the formation of resorption pits (Fig. 3).

Finally, we tested whether the high RANKL:OPG ratio in OA synovial fluid mainly contributed to the induction of osteoclastogenesis *in vitro*. The addition of anti-RANKL IgG (10 μg/mL) or OPG (200 ng/mL) could similarly block the formation of OCLs ($p < 0.005$, Fig. 4), thus suggesting that the increase in the RANKL:OPG ratio in the synovial fluid was the main contributor to osteoclastogenesis *in vitro*.

## DISCUSSION

The synovial fluid of patients with TMJ disorder contains many kinds of molecules that can induce osteoclastogenesis, *e.g.*, IL-1, IL-6, TNF-α, and prostaglandin-$E_2$ (Shafer *et al.*, 1994; Sandler *et al.*, 1998). In addition to these molecules, our current results clearly demonstrated a constant level of RANKL, but a potent decrease in OPG levels in the synovial fluid of patients with TMJ internal derangement, as well as the significance of the RANKL:OPG ratio in osteoclastogenesis *in vitro*. We and other researchers previously demonstrated that bone-destructive diseases, such as rheumatoid arthritis, periodontitis, and OA of TMJ disorder, similarly show a specific decrease in OPG at the site of the disease (Kotake *et al.*, 2001; Romas *et al.*, 2002; Kaneyama *et al.*, 2003a,b; Mogi *et al.*, 2004). Kaneyama *et al.* (2003b) reported that OPG was expressed in the endothelial cells, synovial lining cells, and fibroblast cells in TMJ. They also demonstrated a significant negative correlation between the expression of OPG in endothelial cells and the degree of articular cartilage degeneration. Although the deregulation of OPG production remains to be elucidated, our current study clearly demonstrates that OPG is involved in the pathogenesis of bone-destructive diseases, especially in OA.

Regarding TMJ internal derangement, what is the biological significance of the RANKL:OPG ratio? As described previously (Nakamura *et al.*, 2003; Mogi *et al.*, 2004), OPG is locally present in an excess amount over RANKL under physiological conditions. Since OPG traps RANKL at local sites, all the RANKL complexed with OPG may be in its inactive form. Therefore, an increase in the local or systemic RANKL:OPG ratio is important for RANKL action, especially in osteoclastogenesis *in vivo*. We previously demonstrated an increase in the RANKL:OPG ratio in the synovial fluid in patients with rheumatoid arthritis and periodontitis (Kotake *et al.*, 2001; Mogi *et al.*, 2004). The RANKL:OPG ratio was also increased in multiple myeloma, and this increase correlated positively with markers of bone resorption, osteolytic lesions, and markers of disease activity in this disorder (Terpos *et al.*, 2003). Our current study clearly demonstrated, for the first time, that the artificial combination of RANKL + OPG and synovial fluid of OA patients has the potential to cause OCL formation *in vitro* (Figs. 2, 3). The addition of anti-RANKL IgG or OPG could block synovial-fluid-induced osteoclastogenesis (Fig. 4), suggesting that the high RANKL:OPG ratio, especially due to the decrease in OPG in the microenvironment, practically contributed to osteoclastic bone resorption there, and led to the progression of the pathophysiology of OA.

As to why the RANKL level was constant in the synovial fluid of the different groups, we have no definite idea at this time. Since the production of RANKL and the RANKL:OPG ratio are not as high in the synovial fluid of OA patients, this might explain why OA in TMJ internal derangement is a relatively mild bone-destructive disease in comparison with other rheumatoid and periodontal diseases (Kotake *et al.*, 2001; Romas *et al.*, 2002; Mogi *et al.*, 2004). When we examined whether an increase in the RANKL:OPG ratio was directly related to cartilage/bone degradation in diseased TMJs, there was no association between the ratio and cartilage/bone degradation, especially in OA. These findings are perhaps not surprising, because OA is a slowly evolving condition that is characterized by continuous remodeling with

a failure of the reparative events to counteract the degeneration process effectively.

Analysis of previous data, taken together with our current findings, suggests that treatment with OPG and/or anti-RANKL antibody for patients with TMJ internal derangement may be a promising new therapeutic strategy for the inhibition of bone destruction.

In conclusion, anlysis of our present data clearly demonstrates the significance of the RANKL:OPG ratio in osteoclastogenesis *in vitro*, and suggests that an increase in this ratio in the microenvironment has the potential to induce osteoclastogenesis in TMJ osteoarthritis.

## ACKNOWLEDGMENTS

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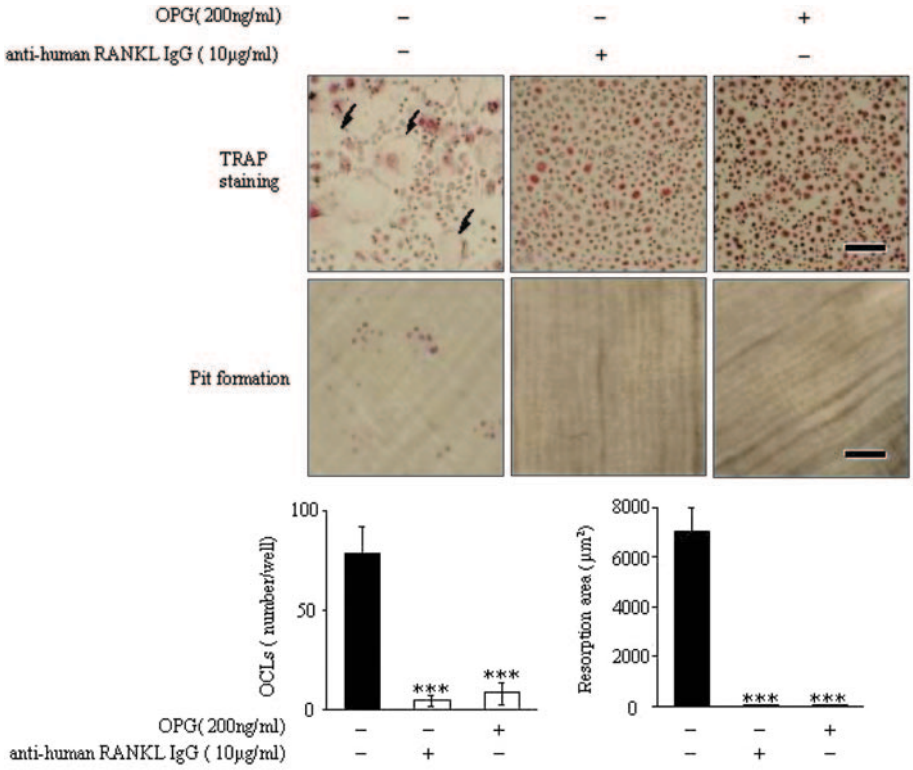


**Figure 4.** Anti-RANKL IgG or OPG blocks OA-synovial fluid-induced osteoclast formation. The reconstituted synovial fluid (OA) was applied to the osteoclast formation assay using PBMC, and to the pit-formation assay, in the presence of M-CSF (100 ng/mL), along with anti-human RANKL IgG (10 μg/mL, PeproTeck, London, UK) or human recombinant OPG (200 ng/mL, R&D Systems). ***$P < 0.005$ compared with the data obtained in the absence of either additive. Experiments were repeated 3 times with similar results. Arrows point to osteoclasts. Original magnification, 40x for TRAP staining (bar = 200 μm), and 100x for pit-formation assay (Bar = 500 μm).

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