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B and T Lymphocytes Are the Primary Sources of RANKL in the Bone Resorptive Lesion of Periodontal Disease

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Receptor activator of nuclear factor-κB (RANKL)-mediated osteoclastogenesis plays a pivotal role in inflammatory bone resorption. The aim of this study was to identify the cellular source of RANKL in the bone resorptive lesions of periodontal disease. The concentrations of soluble RANKL, but not its decoy receptor osteoprotegerin, measured in diseased tissue homogenates were significantly higher in diseased gingival tissues than in healthy tissues. Double-color confocal microscopic analyses demonstrated less than 20% of both B cells and T cells expressing RANKL in healthy gingival tissues. By contrast, in the abundant mononuclear cells composed of 45% T cells, 50% B cells, and 5% monocytes in diseased gingival tissues, more than 50 and 90% of T cells and B cells, respectively, expressed RANKL. RANKL production by nonlymphoid cells was not distinctly identified. Lymphocytes isolated from gingival tissues of patients induced differentiation of mature osteoclast cells in a RANKL-dependent manner *in vitro*. However, similarly isolated peripheral blood B and T cells did not induce osteoclast differentiation, unless they were activated *in vitro* to express RANKL; emphasizing the osteoclastogenic potential of activated RANKL-expressing lymphocytes in periodontal disease tissue. These results suggest that activated T and B cells can be the cellular source of RANKL for bone resorption in periodontal diseased gingival tissue. ***(Am J Pathol 2006, 169:987–998; DOI: 10.2353/ajpath.2006.060180)***

Periodontal disease (or periodontitis) is an inflammatory lesion that is accompanied by soft tissue destruction and bone resorption in the tooth-supporting structures. A positive correlation between the occurrence of disease and elevated serum antibody response to the oral bacteria colonizing the gingival crevice[1–3] suggests the involvement of an immune response to the multiple bacteria in the onset and development of periodontal disease. In general, immune responses to bacteria are considered to be a host protective mechanism to pathogenic bacteria. However, despite the elevated IgG antibody response to certain disease-associated bacteria colonizing the periodontal crevice, inflammation and/or bone resorption proceed in the periodontitis lesions. The question is posed as to whether immune response to periodontal bacteria is protective, or otherwise pathogenic, in the context of periodontal disease.

The fundamental cytokine system that underlies bone resorption processes is dependent on the osteoclast differentiation, activation, and survival factor, receptor activator of nuclear factor-κB (RANKL), and its soluble decoy receptor osteoprotegerin (OPG).[4–6] Involvement of immune cells in the course of bone resorption has been demonstrated by the expression of RANKL on activated T cells.[7] RANKL expressed by T cells, as well as by osteoblasts and bone marrow stromal cells, triggers signaling in osteoclast precursor cells that elicits the differentiation into their mature form.[8] OPG is expressed ubiquitously by many types of cells and tissues, and it counterregulates

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the excessive bone loss by antagonizing the RANKL-binding to its receptor RANK.[9] The paradigm of osteoclast differentiation regulation is based on the RANKL/OPG ratio expressed in the microenvironment surrounding osteoclast precursor cells.[10]

An active periodontal lesion is characterized by the prominent infiltration of B cells[11,12] and T cells.[13,14] Although the T cells infiltrating the inflamed gingival tissues express activation markers such as CD45RO[15] or CD29,[16] functional roles of these activated T cells are not completely clear. We demonstrated that adoptive transfer of RANKL$^+$, antigen-specific T cells can induce bone loss in rat periodontal tissue that received local injection of the T-cell antigen.[17,18] Teng and colleagues[19] reported that adoptive transfer of an *Actinobacillus actinomycetemcomitans*-specific human T cell line isolated from patients with aggressive (juvenile) periodontal disease could induce significant periodontal bone loss in NOD/SCID mice orally inoculated with *A. actinomycetemcomitans* every 3 days.[19] Although the latter report showed that systemic administration of OPG-Fc could reduce periodontal bone loss, it is not clear whether the transferred human T cells or bystander cells that might be secondarily stimulated by the transferred human T cells are the source of RANKL. It has been reported that RANKL produced by B cells is responsible for the devastating bone resorption in multiple myeloma.[20] Activation of B cells *in vitro* can induce expression of RANKL, but these cells are deficient in production of OPG.[21] Recently, we reported that antigen-specific activated B cells can induce periodontal bone resorption in a rat model.[22] However, it is unclear if B cells accumulating in periodontal diseased tissue express RANKL.

To determine the cellular source of RANKL in bone resorptive periodontitis, enzyme-linked immunosorbent assay (ELISA) and double-color confocal microscopic analyses were used. Results of ELISA demonstrated that soluble RANKL (sRANKL) production was significantly elevated in gingival tissues with periodontal disease compared to healthy gingival tissues. Confocal microscopic analyses showed that both T cells and B cells, but not monocytes or fibroblasts, are the cellular source of RANKL in the bone resorptive lesion of periodontal disease. Importantly, RANKL expressed by periodontal T cells and B cells appeared to be the osteoclastogenic functional component, as determined by *in vitro* RANKL-dependent osteoclast differentiation assays.

Materials and Methods

Patients

Patients diagnosed with chronic periodontitis [n = 32 (including three smokers), 12 males and 21 females; average age, 46.9 years; range of ages, 33 to 67 years] were otherwise systemically healthy patients. These patients had periodontal bone resorption diagnosed by X-ray examination, bleeding on probing, and clinical gingival crevice probe depths of greater than 3 mm at the diseased site. Informed consents from all patients were obtained before sample collection. The diseased gingival tissue lesions and healthy tissues were sampled during surgical treatment. Healthy gingival tissues were collected from patients with gingival crevice depth of equal to or less than 3 mm and with no X-ray indication of bone loss, at surgery for tooth restorative purposes including crown lengthening [n = 12 (including one smoker), five males and seven females; average age, 43.4 years; range of ages, 25 to 72 years].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from gingival tissues and RT-PCR was performed as previously described.[23,24] Primer pairs for human RANKL and OPG were as follows: RANKL forward primer, 5′-TCAGAAGATGGCACT-CACTG-3′ and RANKL reverse primer, 5′-AACATCTC-CCACTGGCTGTA-3′ (PCR product size 879 bp),[25] OPG forward primer, 5′-GCCCTGACCACTACTACACA-3′ and OPG reverse primer, 5′-TCTGCTCCCACTTTCTTTCC-3′ (PCR product size 736 bp). Total RNA isolated from gingivae (1 μg) was synthesized to cDNA. The resulting cDNA was subject to PCR by amplifying 30 cycles for RANKL or for OPG along with β-actin[23] as an internal control (94°C for 30 second, 60°C for 1 minute, 72°C for 1 minute, and final elongation at 72°C for 10 minutes). PCR products were separated in 1.7% agarose gels and stained with ethidium bromide.

Preparation of Gingival Tissue Homogenates

Gingival tissues were homogenized with a Dounce glass homogenizer in phosphate-buffered saline supplemented with 0.05% Tween 20, phenylmethyl sulfonyl fluoride (1 mmol/L; Sigma, St. Louis, MO), and protease inhibitor cocktail (Sigma), as published with slight modification.[26] For ELISA, the concentration of sRANKL, interleukin (IL)-1β, and OPG in the tissue homogenates or culture supernatants were measured with ELISA kits for human soluble RANKL (sRANKL) (Peprotech, Rocky Hill, NJ) and DuoSet ELISA for human IL-1β, OPG, IL-10, IL-12 p70, and GM-CSF (R&D Systems, Minneapolis, MN). The method for detection of IgG response to oral bacteria has been previously published.[27,28] To detect anti-bacterial IgG levels in the gingival homogenates, the following formalin-fixed bacteria were used as antigen in ELISA: *A. actinomycetemcomitans* strain Y4 (American Type Culture Collection no. 43718), *Fusobacterium nucleatum* str. 25586, *Eikenella corrodens* str. 23834, *Porphyromonas gingivalis* str. W83, and *Prevotella intermedia* str. 25611.

Culture of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were collected from healthy patients under informed consent agreement (n = 4; 28- to 40-year-old males; average age, 34.5 years). The mononuclear cell

(MC) fraction was separated from blood by gradient centrifugation using Histopaque-1077 (Sigma) and incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 2-mercaptoethanol, L-glutamine, and penicillin and streptomycin. T cells were enriched from PBMCs by glass-wool and nylon-wool column purification.[24] B cells were enriched from PBMCs by negative selection using magnetic beads coated with a mixture of anti-CD3 monoclonal antibody (mAb) (OKT3: American Type Culture Collection, Rockville, MD; UCTH1: R&D Systems), CD4 (OKT4), CD8 (OKT8), and CD28 (CD28.2; BD Pharmingen, San Diego, CA). The enriched T cells or B cells were stimulated with immobilized antibodies on a 96-well culture plate with mouse mAb to CD3 (OKT3) and CD28 (CD28.2) or mAb to CD40 (5C3, BD Pharmingen) and goat polyclonal anti-human IgM (Serotec, Oxford, UK), respectively.

Immunofluorescent Laser-Scanning Confocal Microscopy

Basic fluorescent staining technique was performed as previously reported.[24] Sectioned tissues mounted on glass slides were fixed with 2% paraformaldehyde. T cells, B cells, and monocytes were stained with the mouse monoclonal antibodies to CD3 (UCTH1), CD20 (H1; BD Pharmingen), and CD14 (3C10; American Type Culture Collection), respectively, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) as a secondary reagent. RANKL expression was monitored by biotinylated-OPG-Fc followed by Texas Red-Avidin (Invitrogen, Carlsbad, CA). To block FcRn and FcγRII, which can bind to monomeric γ chain of human IgG-Fc, human IgG was prereacted with some of the gingival tissue sections. OPG-Fc was generously provided by Dr. Colin Dunston (Amgen, Thousand Oaks, CA) under mutual material transfer agreement and was conjugated with biotin using EZ-Link sulfo-NHS-biotin (Pierce, Rockford, IL). A human IgG-Fc fusion protein (L6) conjugated with biotin was used as a negative control. After washing, the cover glass was set on the sample with Fluoromount-G mounting medium (Southern Biotechnology, Birmingham, AL). The staining pattern was analyzed by 0.3 μm sequential optical sectioning at $\times$400 or $\times$1000 magnification with a Leica TCS/SP-2 laser-scanning confocal microscope (Leica, Wetzlar, Germany).

Osteoclast Differentiation Assay

MCs were isolated from patient gingival tissue biopsies as previously described.[23] Patient gingival MCs, fresh PBMCs, stimulated PB T cells, or stimulated PB B cells *in vitro* were fixed with formalin. Human PB CD14$^+$ monocytes were isolated from PBMCs using a magnetic bead-based monocyte-negative isolation kit (Dynal Biotech, Oslo, Norway). In some experiments, patient gingival MCs were incubated in 96-well plates for 3 hours, and nonadherent cells were separated from the adherent cells on the culture plate. The adherent gingival MCs were ~80% CD14$^+$ monocytes, whereas nonadherent gingival MCs contained less than 2% CD14$^+$ cells by immunostaining. The resulting adherent cells in the wells and nonadherent gingival MCs were fixed with formalin and were used in the following co-culture system. The fixed human lymphocytes were co-cultured in 96-well plates with the mouse osteoclast precursor cell line MOCP-5[29] or PB CD14$^+$ monocytes in the presence of M-CSF (10 ng/ml; Peprotech). Recombinant human RANKL (50 ng/ml; R&D Systems) was added to the MOCP-5 culture, as a positive control. To assess the involvement of RANKL in the osteoclast differentiation, OPG-Fc (10 μg/ml) was added to antagonize RANKL in some cultures. The culture medium was changed every 3 days by replacing half of the volume. After 6 to 8 days of culture for MOCP-5, or 14 to 16 days of culture for PB CD14$^+$ monocytes, when multinuclear osteoclast-like cells are observed by phase-contrast microscopy, cells were fixed with 5% formalin-saline. Differentiated osteoclasts were identified as tartrate-resistant acid phosphatase (TRAP)-positive cells with three or more nuclei as described previously.[30] The TRAP$^+$ cells with more than three nuclei were counted as osteoclasts using phase contrast microscopy and expressed as cell number/well of 96-well plates. The bone resorption activity of osteoclasts was evaluated by a pit formation assay using a calcium phosphate-coated tissue culture vessel system (Biocoat Osteologic System; BD Biosciences, San Jose, CA) or dentin disks (Alpco Diagnostics, Windham, NH) according to the manufacturers' instructions.

Results

Detection of sRANKL and OPG Proteins in Gingival Tissue Homogenates

The concentration of sRANKL protein in gingival tissue was significantly elevated ($P < 0.02$, *t*-test) in diseased tissues as compared to healthy gingival tissues (Figure 1A). In addition, there was a positive correlation between the level of sRANKL in tissues and the depth of the gingival crevice where the tissues were sampled (Figure 1B). Differences in OPG concentration between diseased and healthy tissues were not statistically significant (Figure 1C). The proinflammatory cytokine IL-1β was monitored in the same groups of gingival tissues (Figure 1D), as an indicator of the degree of inflammation in each tissue. All gingival tissues from diseased lesions expressed significantly higher IL-1β amounts than the healthy gingival tissues (Student's *t*-test, $P < 0.05$). We also evaluated the concentrations of the osteoclastogenesis inhibitory cytokines, IL-10, IL-12 p70, and GM-CSF and the osteoclast precursor chemoattractant factor, MIP-1α, compared to healthy tissues. However, although the diseased gingival tissues had higher mean concentrations of each of the four factors, none of the differences were statistically significant. Compared to healthy patients' antibody levels, the tissue homogenates of patient gingival tissues also demonstrated significantly higher IgG antibody levels to three of five periodontal disease-

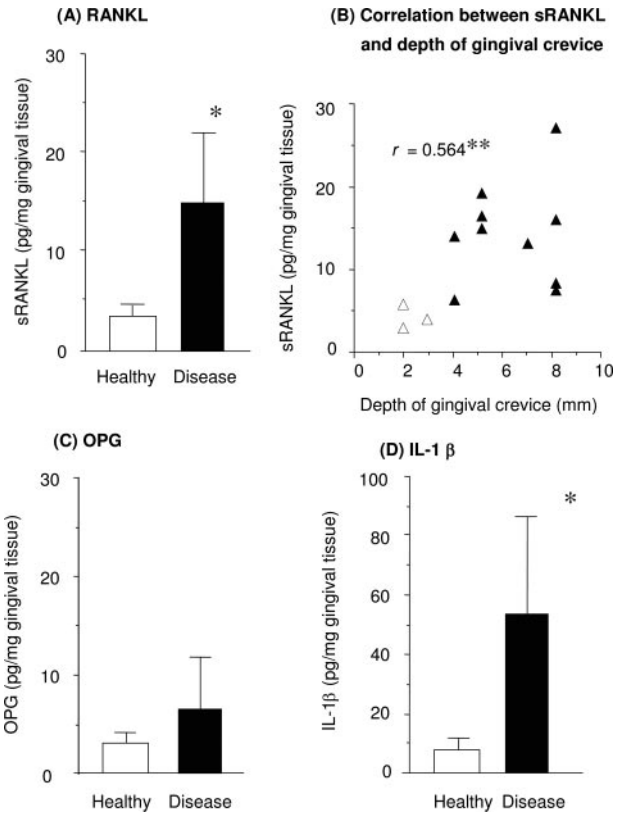


Figure 1. Protein concentrations of RANKL, OPG, and IL-1β in gingival tissues. Protein concentrations of RANKL and OPG in gingival tissues were measured by ELISA. Gingival tissue samples were homogenized in the presence of proteinase inhibitors (healthy, $n = 3$; disease, $n = 11$). The concentration of each protein, RANKL (**A** and **B**), OPG (**C**), and IL-1β (**D**), is expressed as pg per mg of gingival tissue (pg/mg). *Significantly higher than healthy control by Student's *t*-test ($P < 0.05$). **B:** The concentrations of RANKL were positively correlated with the gingival pocket depth where the biopsy was sampled. **Positive correlation ($n = 14$, $P < 0.01$).

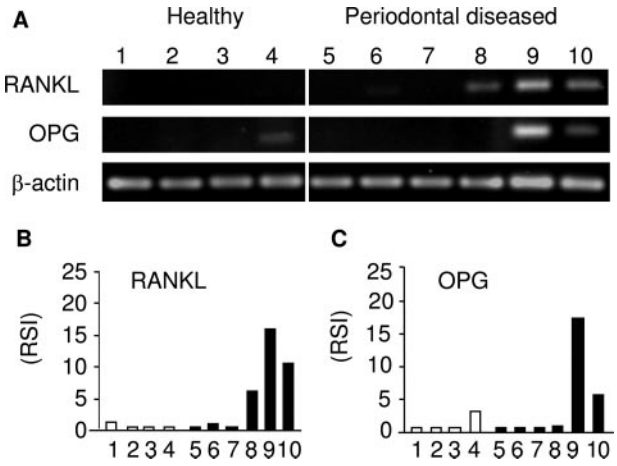


Figure 2. RANKL and OPG mRNA expression in gingival tissues. RT-PCR was performed to detect mRNA expression of RANKL and OPG in the total RNA extracted from whole gingival tissue samples. Expression of RANKL mRNA was detected in 67% of diseased periodontal tissues (solid bars, four of six), whereas no expression of RANKL mRNA was observed in the healthy gingival tissues tested (open bars, zero of four), whereas OPG mRNA message was observed from healthy patients (one of four) and from periodontal disease tissues (two of six). The relative intensity of each mRNA message in the acrylamide gel was scanned by densitometer using the AlphaImage analysis system and expressed as relative signal intensity (RSI) in the histograms (**B**, RANKL; **C**, OPG).

associated bacteria examined in this study (*F. nucleatum*, *P. gingivalis*, and *P. intermedia*; data not shown).

Expression of RANKL and OPG mRNA in Gingival Tissue

RT-PCR was performed to detect mRNA expression of RANKL and OPG in the gingival tissue RNA samples (Figure 2). RANKL mRNA expression was detected from 67% of the diseased gingival tissues (four of six) examined, whereas no expression of RANKL mRNA was observed from the healthy gingival tissues tested (zero of four). OPG mRNA message was observed in tissue from periodontally diseased patients (two of six) and one healthy subject (one of four).

RANKL Expression by T and B Lymphocytes in Gingival Tissue

To identify the cell types that express RANKL in inflamed gingival tissue, double-color confocal microscopy (Figure 3) was used after staining for RANKL (red) and lymphocyte-specific CD makers (green). Images of RANKL and CD marker-positive cells were merged in the computer system, and double-positive cells were displayed as yellow staining. Gingival tissues from periodontitis lesions demonstrated marked expression of RANKL (Figure 3, A–C). The cellular infiltrates in the diseased gingival tissue were predominantly T (Figure 3A) and B lymphocytes (Figure 3B), with few $CD14^+$ monocytes (Figure 3C). RANKL was expressed in these cellular infiltrates, especially by T cells and by B cells, and to a much lesser extent by monocytes. Although most B cells were positive for RANKL staining, not all T cells expressed RANKL. Very few lymphocytes expressing RANKL were present in healthy gingival tissues (Figure 3D). RANKL expression by other nonlymphoid cell types, such as fibroblasts, was not distinctly observed.

The numbers of $CD3^+$ and $CD20^+$ lymphocytes infiltrating the gingival tissues were significantly higher than those in the healthy gingival tissues, whereas the number of $CD14^+$ cells did not show a remarkable increase (Figure 4). Importantly, the percentage of $CD14^+$ cells to the number of total lymphocytes remained low irrespective of the gingival condition of disease/healthy (19% in healthy versus 5% in disease gingival tissue; Figure 4). These data strongly indicate that T cells and B cells are a much more significant source of RANKL than $CD14^+$ cells in the diseased gingival tissues.

The percentage of $RANKL^+$ cells in the population of T cells or B cells was also determined (Figure 5) based on counts of the images obtained in the confocal microscopic analyses (Figure 3). The percentage of RANKL-expressing T cells (Figure 5A) or B cells (Figure 5B) demonstrated a positive correlation with the depth of the gingival crevice. Importantly, in the diseased gingival tissues, $RANKL^+$ cells were greatly elevated compared to the healthy tissues, and a significantly higher percent-

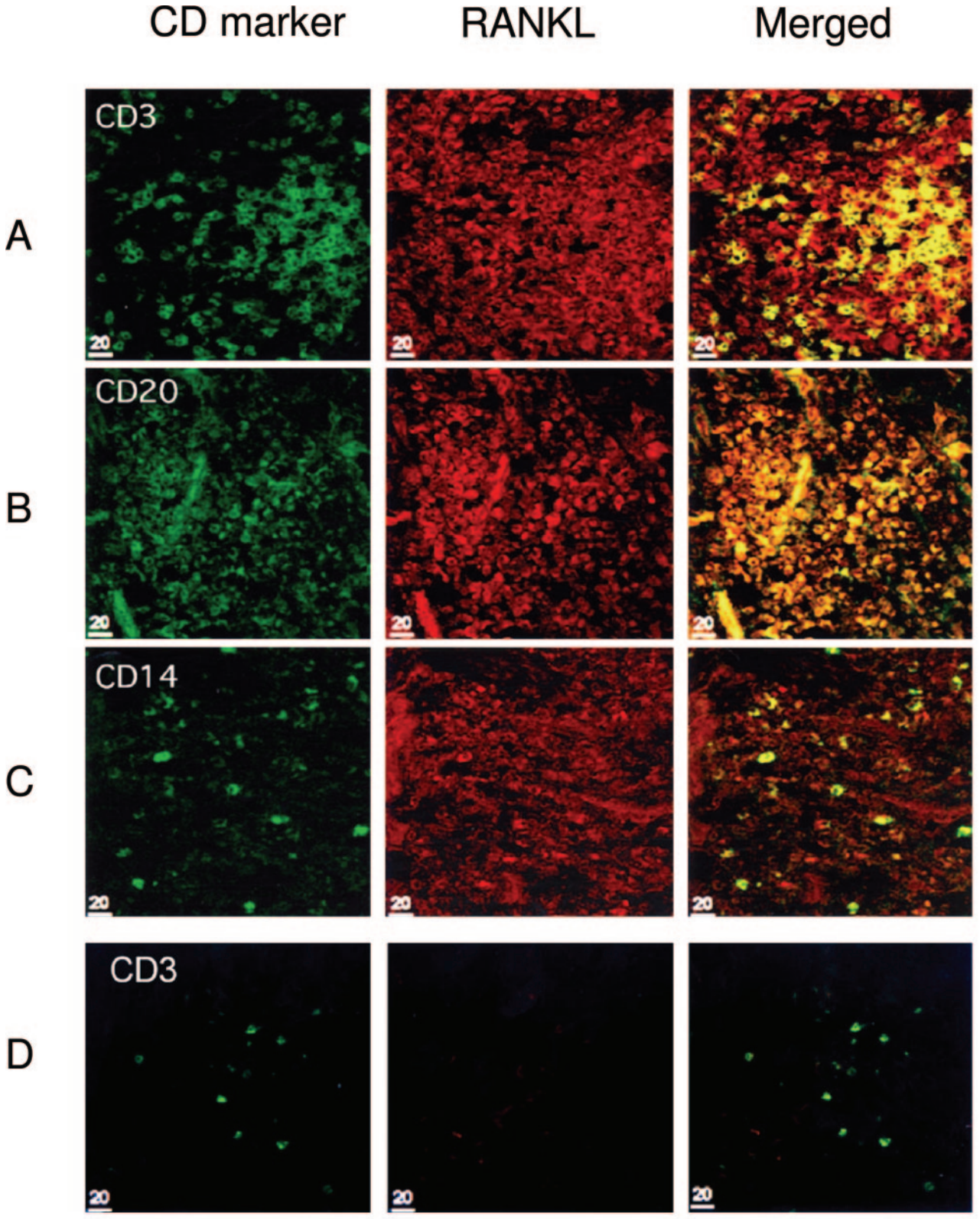


Figure 3. Confocal microscopic analyses of expression of RANKL in gingival tissues. Using double-color confocal microscopy, RANKL-expressing cells were identified in the gingival tissue of a periodontal disease patient (**A–C**) and in healthy gingival tissue (**D**). CD3 (**A** and **D**), CD20 (**B**), or CD14 (**C**) expression was indicated by staining with various specific mouse mAbs followed by fluorescein isothiocyanate anti-mouse IgG (left column). RANKL expression was detected by OPG-biotin followed by streptavidin-Texas Red (middle column). Both specific CD marker-stained cells and $RANKL^+$-stained cells were doubly exposed and expressed as yellow (merged; right column). $CD20^+$ and $CD14^+$ cells in healthy tissue (not shown) were as few as $CD3^+$ cells in healthy gingival tissue (**D**). Scale bars = 20 μm.

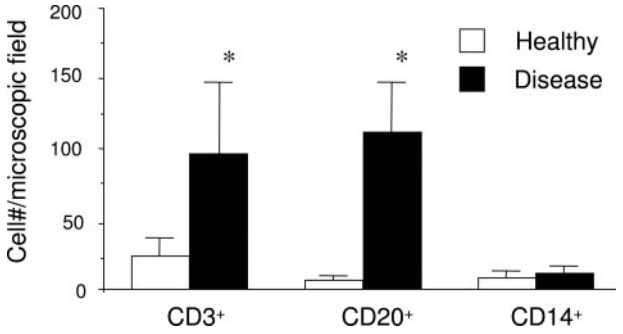


Figure 4. Numbers of lymphocytes in the gingival tissues. The total number of $CD3^+$, $CD20^+$, and $CD14^+$ cells in a confocal microscopic field of each tissue section was counted (×400, average cell number of at least three sections was enumerated as the number of cells per sample) and compared between healthy gingival tissues (open column) and disease gingival tissues (filled column). Mean cell number ± SD is shown. *Significantly higher than healthy gingival tissue by Student's *t*-test ($P < 0.01$).

age of B cells expressed RANKL (94 ± 5% SD) than T cells (51 ± 18% SD) (Figure 5C).

Activation of Peripheral Blood T Cells and B Cells Induces sRANKL Production

To investigate whether naïve or activated lymphocytes express RANKL, peripheral blood T cells or B cells from healthy patients were stimulated with immobilized anti-CD3 and anti-CD28 mAbs or with immobilized anti-CD40 and/or anti-IgM, respectively (Figure 6). The stimulation via CD3/CD28 or CD40/BCR (B-cell receptor; anti-IgM) induced proliferation and sRANKL expression by the purified T cells or B cells [Figure 6, A and C (T cells) and B and D (B cells)]. Although production of OPG by the activated T cells or B cells was also monitored (by ELISA), the level of OPG production was lower than the detection limit (4 pg/ml). An oral bacterial stimulus (fixed *A. actinomycetemcomitans*) also activated peripheral blood lymphocytes to produce both sRANKL and cellular RANKL from T cells and B cells (examined by confocal microscopy, not shown). The activated T cells and B cells isolated from peripheral blood were able to induce osteoclast ($TRAP^+$, multinucleated) cell differentiation in a RANKL-dependent manner (Figure 6E), whereas activated $CD14^+$ monocytes did not induce such osteoclastogenesis (Figure 6E). Of note, most published literature also supports the finding that $CD14^+$ cells express of RANK but not RANKL.[31,32] These data suggested that T cells and B cells that express RANKL in gingival tissues are probably in the activated form and that these activated T and B cells are the major source of RANKL.

Evaluation of the Osteoclast Differentiation Function of RANKL Expressed by Gingival Lymphocytes

To evaluate the functional aspects of RANKL expressed by MCs isolated from the diseased gingival tissue, an *in vitro* differentiation assay was outperformed using the osteoclast precursor cell line MOCP-5 (Figure 7A) or

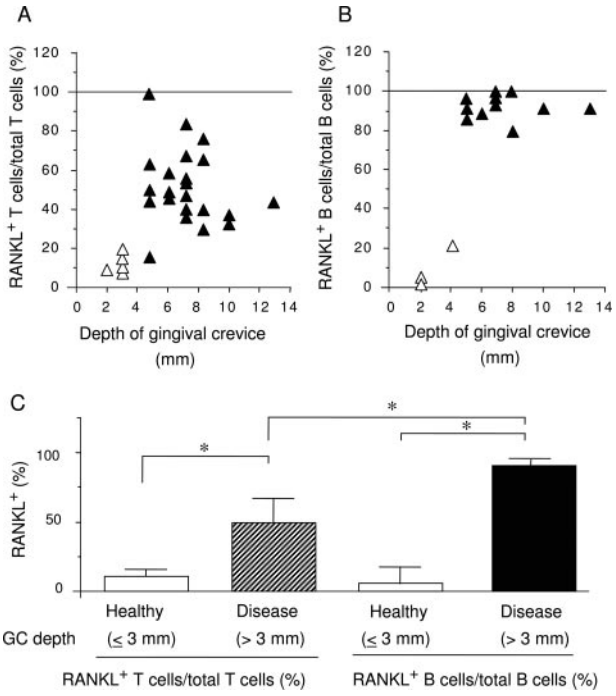


Figure 5. Expression of percentage of $RANKL^+$ T cells or B cells in the gingival tissues. After computer capture of the confocal microscopic image of each gingival section (×400), the percentage of yellow cells (double positive for CD3/RANKL or CD20/RANKL) of the total number of green cells ($CD3^+$ or $CD20^+$) were calculated and are shown on scatter plots (**A**, $CD3^+$ T cells; **B**, $CD20^+$ B cells), according to the depth of gingival crevice from which the samples were collected (open triangles, healthy patients; filled triangles, periodontal disease patients). Both $RANKL^+$ T cells and $RANKL^+$ B cells showed positive correlation with the depth of gingival crevice (T cells: $n = 34$, $r = 0.507$, $P < 0.01$; B cells: $n = 15$, $r = 0.702$, $P < 0.01$). **C:** Percentage of $RANKL^+$ T cells or B cells of the total T cells or B cells in the gingival tissues classified by the depth of the gingival crevice (GC depth) is shown. The data shown in **A** and **B** were converted to histograms, presented as mean ± SD of the percentage of $RANKL^+$ T cells or B cells. *Statistically significant by Student's *t*-test ($P < 0.0001$). Note: the percentage of $RANKL^+$ $CD14^+$ monocytes/total $CD14^+$ monocytes (not shown) was 15.3 ± 16.8 and 59.5 ± 39.9 in healthy and disease gingival tissues, respectively, showing no statistical differences by *t*-test.

peripheral blood $CD14^+$ monocytes (Figure 7B). Both MOCP-5 cells and $CD14^+$ monocytes differentiated into $TRAP^+$ multinucleated cells in response to recombinant human sRANKL in the presence of M-CSF. The isolated diseased gingival MCs as well as recombinant RANKL induced $TRAP^+$ multinucleated cells (Figure 7A). However, only nonadherent diseased gingival MCs, but not adherent gingival MC cells, induced RANKL-dependent osteoclastogenesis (Figure 7B), indicating that any RANKL expression that might have been detected on $CD14^+$ cells (Figure 3C) is not functionally active. The morphological appearance of the differentiated MOCP-5 cell line is shown [Figure 8, A (sRANKL) and C (patient gingival MC)]. Induction of $TRAP^+$ multinucleated cells was abolished by the addition of OPG-Fc into the culture [Figure 7 and Figure 8, B (sRANKL + OPG-Fc) and D (patient gingival MC + OPG-Fc)], demonstrating that RANKL expressed by MCs from diseased tissues can induce RANKL-dependent osteoclast differentiation. The pit formation assay was performed to assess the functional capabilities of differentiated multinucleated cells (Figure 8, E–J). Resorption pits shown as positively

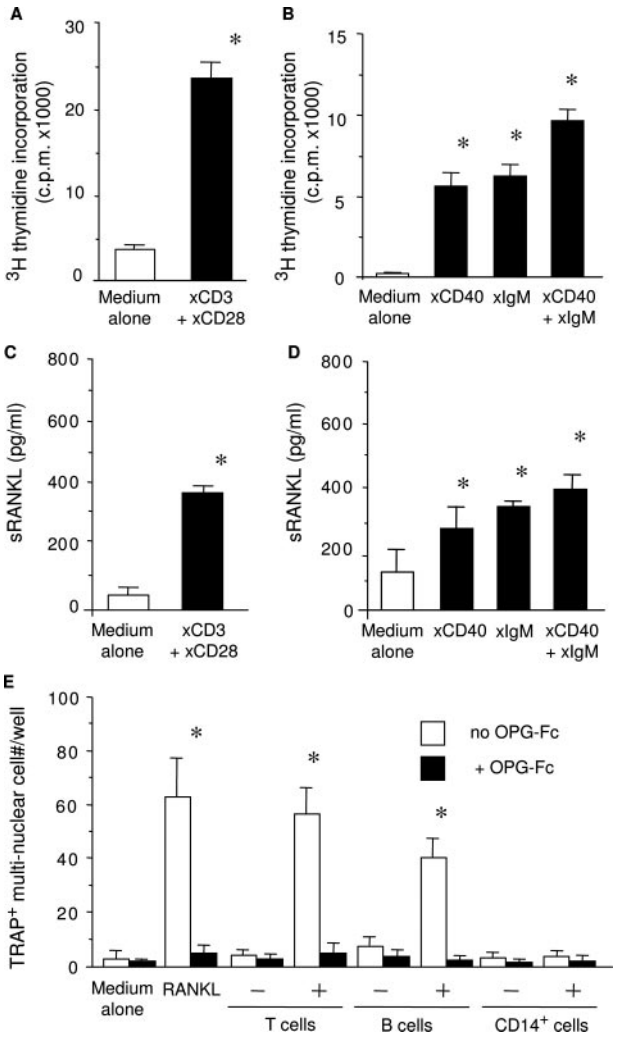


Figure 6. Activation of peripheral blood T cells or B cells induces sRANKL expression. Purified T cells isolated from a healthy subject PBMCs (2×10^5/well) were activated for 7 days with immobilized anti-CD3 (×CD3) and anti-CD28 (×CD28) mAbs, and proliferation (**A**) and sRANKL production (**C**) were measured by [^{3}H]thymidine incorporation assay and by ELISA, respectively. Purified B cells isolated from the PBMCs (2×10^5/well) were also incubated in a 96-well plate for 7 days with immobilized anti-CD40 (×CD40), anti-IgM (×IgM), and anti-CD40 and anti-IgM antibodies, and proliferation (**B**) and sRANKL production (**D**) were measured by the same method as for T cells. **E:** Osteoclastogenesis induced by the activated peripheral blood T cells, B cells, and CD14$^+$ monocytes is shown. The cultured T cells or B cells under the conditions shown in **A** or **B** were harvested and fixed on day 7. After activation of these CD14$^+$ monocytes with fixed *A. actinomycetemcomitans* for 4 days, CD14$^+$ monocytes produced IL-12 (143 + 23 pg/ml), but not RANKL or OPG. The fixed T cells, B cells, or CD14$^+$ monocytes were co-cultured with MOCP-5 (10^4/well) in the presence of M-CSF (10 ng/ml) as described under Materials and Methods. The number of TRAP-positive multinuclear cells induced in each well was counted and expressed as mean ± SD of triplicate determinants. *Significantly higher than medium alone by Student's *t*-test ($P < 0.05$). Other PBMC samples isolated from one periodontal diseased and two orally healthy patients showed similar results.

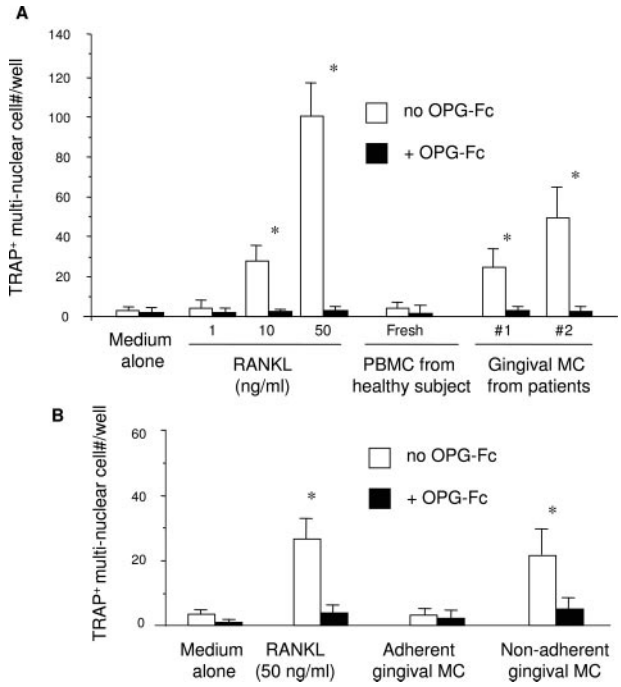
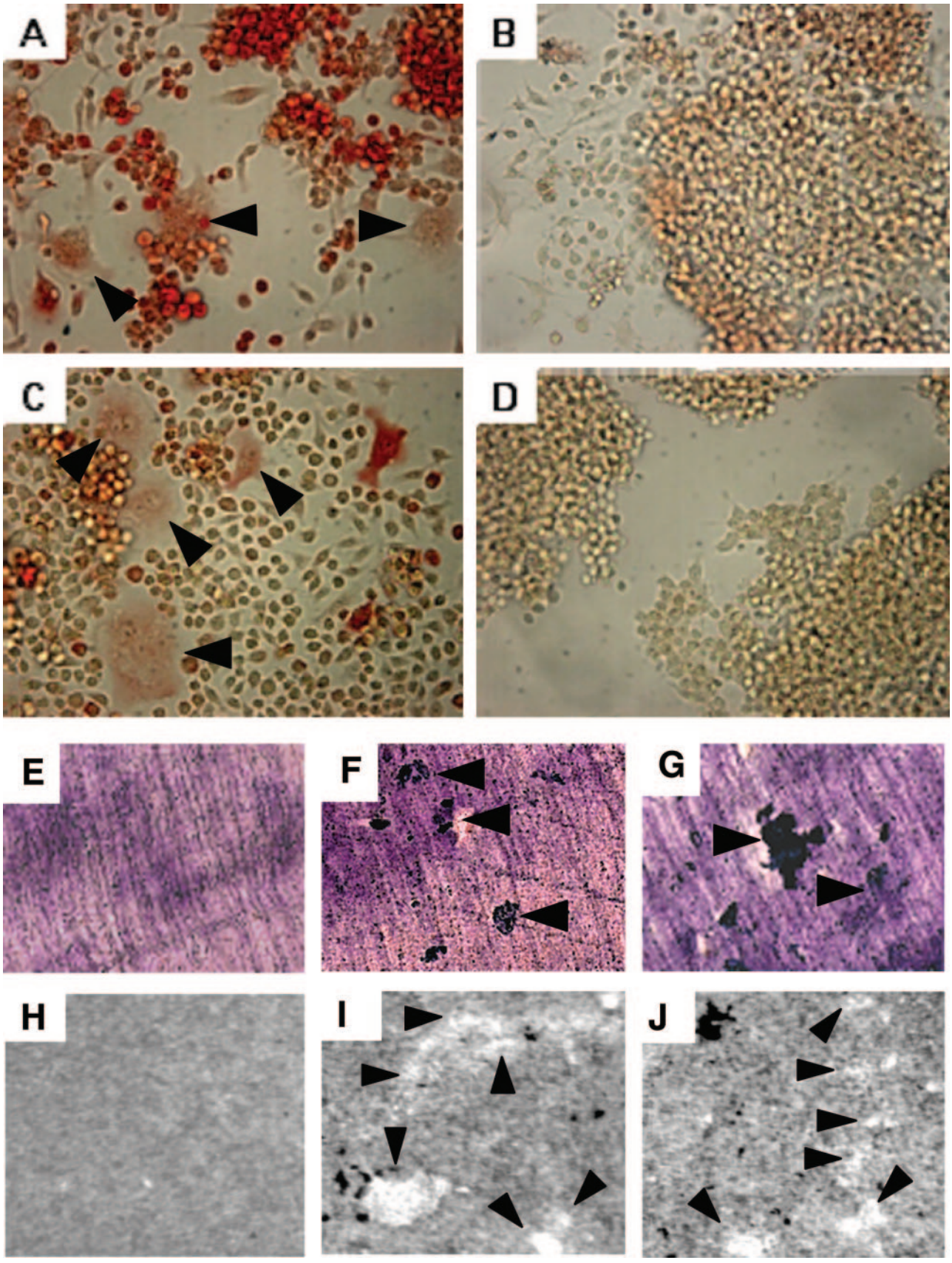


Figure 7. Osteoclast differentiation assay using the MOCP-5 osteoclast precursor cell line or human peripheral blood CD14$^+$ monocytes. **A:** MOCP-5 osteoclast precursor cells were co-cultured with human recombinant RANKL, fresh nonstimulated PBMCs, or gingival MCs isolated from periodontal disease patients. PBMCs and gingival MCs were prefixed with formalin before co-culture with MOCP-5 (10^4/well). **B:** CD14$^+$ monocytes isolated from PBMCs of a healthy subject (2×10^4/well) were co-cultured with human recombinant RANKL or with fixed adherent or nonadherent gingival MCs isolated from periodontal disease patient. All culture medium contained recombinant human M-CSF (10 ng/ml) in the presence or absence of OPG-Fc (10 μg/ml) (both **A** and **B**). Half of the total culture medium was exchanged every 3 days. On day 8 (**A**) or on day 16 (**B**), the cultures were fixed with formalin, and TRAP staining was outperformed. The numbers of cells with more than three nuclei demonstrating TRAP$^+$ staining were counted in each well under phase contrast microscopy. Similar results of TRAP$^+$ cell induction were obtained using the MCs isolated from two different patients' gingival tissues. *Significantly elevated compared to the control MOCP-5 (**A**); significantly elevated compared to human CD14$^+$ monocytes cultured in medium alone without OPG-Fc (**B**) by Student's *t*-test ($P < 0.01$).

stained by toluidine blue (indicated by arrows) were observed in the disks from wells in which MOCP-5 cells were co-cultured with sRANKL or MCs from diseased gingival tissue (Figure 8, F and G, respectively). The formation of resorption pits induced by MOCP-5 co-cultured with MCs from the diseased tissue was inhibited by the presence of OPG (not shown). The pit formation assay using a calcium-phosphate-coated tissue culture vessel system (Figure 8, H–J) showed similar results to the assay using the dentin disks (Figure 8, E–G). The MOCP-5 co-cultures with MCs from diseased tissue showed resorption pits (Figure 8J, white clear area, indicated by arrows) as well as MOCP-5 incubated with sRANKL (Figure 8I, arrows), compared to the negative control of MOCP-5 cultured in medium alone, which did not show such resorption pits (Figure 8H).

Discussion

The present study of the bone resorptive lesions of periodontal disease tissues demonstrated that, not only T cells, but also B cells, are major sources of RANKL. The concentrations of sRANKL and IL-1β examined in the gingival tissue homogenates were significantly elevated in the diseased gingival tissues compared to healthy tissues, whereas OPG protein production was not significantly higher in diseased tissues than healthy tissues. Overexpression of OPG in transgenic mice and RANK gene knockout mice develop severe osteopetrosis[33,34]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and OPG gene knockout mice demonstrate osteopenia.[35] The ratio of RANKL to OPG is normally increased in a bone resorptive lesion characterized by extensive osteoclastic activity.[36] Therefore nearly exclusive production of RANKL and the lack of production of OPG by lymphocytes seem to contribute to the increased RANKL to OPG ratio in the bone resorptive periodontitis lesions. Key studies showed that RANKL gene knockout mice not only develop osteopetrosis but are also deficient in both T and B lymphocytes,[10,37] implying a RANKL-associated linkage between T and B cells and bone resorption. Another study using a SCID-Hu mouse model indicated that human T cells can express RANKL in response to certain oral bacteria.[19] RANKL protein expression has been demonstrated by T cells isolated from periodontitis gingival tissue by flow cytometry.[38] Consistent with our findings that diseased gingival tissue contains much more sRANKL than healthy tissue (Figure 1A) and that the concentration of sRANKL is greater in deeper gingival crevices (Figure 1B) is the recent report that the ratio of sRANKL to OPG in gingival crevice fluid (GCF) is elevated in periodontitis patients compared to healthy patients.[39] Therefore, the findings presented herein that RANKL is expressed by T cells and also B cells and that a higher percentage of B cells express RANKL than T cells in periodontitis tissue provides information quite relevant to the elucidation of bone resorptive factors in periodontal disease.

We have recently demonstrated that activated antigen-specific B cells can induce periodontal bone resorption in a RANKL-dependent manner using a rat periodontitis model.[22] B cells primed *in vivo* with specific antigen (*A. actinomycetemcomitans*) up-regulated RANKL expression in response to *in vitro* stimulation with antigen. The adoptive transfer of these donor antigen-primed B cells to congenitally athymic recipient rats that received simultaneous gingival injection with antigen resulted in periodontal bone resorption in conjunction with elevated IgG antibody response to *A. actinomycetemcomitans.*[22] This periodontal bone resorption was abrogated by OPG-Fc local injection.[22] Accumulation of plasma cells and B cells in the disease lesion[11,12] and elevated IgG antibodies to periodontal bacteria in the serum[1,2,28] and gingival crevice fluid[40] are distinct features of periodontitis. However, the nature of B cells in the context of periodontal bone resorption has not been clearly elucidated until our previous study.[22] Herein, we revealed that B cells, in addition to T cells, are primary cellular sources of bone destructive factor by immunohistological studies of human periodontal disease lesions.

In our previous studies of the rat periodontal disease model using adoptive transfer of T-clone cells, antigen-specific stimulation of the T-clone cells enhanced expression of RANKL mRNA to a greater degree than expression of OPG mRNA as determined by RT-PCR, and resulted in local periodontal bone resorption.[17] Sakata and colleagues[41] reported that dental mesenchymal cells produce OPG and enhance OPG production in response to proinflammatory factors such as IL-1β or tumor necrosis factor (TNF)-α. Nagasawa and colleagues[38] showed that lipopolysaccharide stimulation of gingival fibroblasts induced OPG expression and inhibited differentiation of monocytes to osteoclast cells. Although the ELISA system in the present study detected OPG protein production in both healthy and diseased gingival tissues, mRNA message for OPG was not detected in healthy gingival tissue as measured by RT-PCR. Because the same RT-PCR system demonstrated the mRNA message of β-actin and RANKL, nonspecific mRNA degradation by RNases during the sampling process was excluded as the cause of such discrepancy between OPG mRNA and OPG protein expression. Furthermore, the difference between *in vivo* (present study) and *in vitro* cultures of dental mesenchymal cells[41] may cause the discrepancy in OPG mRNA detection. In the present study, stimulation of peripheral blood T cells and B cells *in vitro* did not produce detectable levels of OPG irrespective of stimulation (data not shown). Therefore, the OPG production in both healthy and diseased gingival tissues may be attributed, at least, to fibroblasts in periodontal tissue that are capable of producing physiologically relevant amounts of OPG, maintaining the homeostasis of healthy periodontal bone remodeling.

Although few studies investigated the mechanism underlying osteoclast recruitment to the bone resorption lesion, MIP-1α and other CCR1 β-chemokines seem to be associated with the osteoclast migration as well as osteoclast formation and bone resorption.[42,43] With respect to the chemotaxis of osteoclast precursors, the homing mechanism of such cells to gingival tissues remains unclear. In the present study, MIP-1α concentration in diseased gingival tissues was not significantly different from healthy tissues.

In metastatic bone disease, the α-chemokine IL-8 has been implicated as a stimulator of osteoclastogenesis and bone resorption.[44] However, IL-8 is an important chemoattractant factor to recruit neutrophils to gingival tissues because an innate immune reaction and defect in neutrophil function is thought to be a pathogenic mechanism in periodontal disease.[45] It is important to clarify

Figure 8. $TRAP^+$ multinuclear cells induced from MOCP-5 osteoclast precursors by recombinant RANKL and patient's gingival MCs. MOCP-5 osteoclast precursor cells were co-cultured with human recombinant RANKL or with gingival MCs isolated from the patient's periodontal disease lesion. Patient's gingival MCs fixed with formalin (10^4/well) were applied to the MOCP-5 culture. MOCP-5 cells were cultured on the oval-shape coverslip in a 96-well plate. Medium that contained M-CSF (10 ng/ml) was exchanged (50% of total culture volume) every 3 days. On day 8, the cultures were stopped, and the cells were fixed with formalin. The MOCP-5 cells adherent on the coverslip were reacted with TRAP reagent and mounted on glass slides. The figures demonstrate the appearance of TRAP staining of MOCP-5 co-cultured for 8 days with recombinant human RANKL (50 ng/ml) (**A**), recombinant human RANKL plus OPG-Fc (10 μg/ml) (**B**), fixed patient's gingival MCs isolated from a diseased lesion (**C**), and fixed patient's gingival MCs isolated from a diseased lesion plus OPG-Fc (10 μg/ml) (**D**). The bone-resorptive activities of $TRAP^+$ multinuclear cells were examined using dentin disks (**E–G**) and the Osteologic system (**H–J**). The MOCP-5 was cultured in a medium supplemented with M-CSF (10 ng/ml) for 8 days in the following different conditions; medium alone (**E**, **H**), in the presence of recombinant RANKL (50 ng/ml) (**F**, **I**), in the presence of patient's fixed gingival MCs (**G**, **J**). The **arrows** in **A** and **C** indicate the $TRAP^+$ multinuclear cells. The **arrows** in **E–J** indicate the resorption pits formed by $TRAP^+$ multinuclear cells. Of note, toluidine blue stains resportion pits dark brown (**F**, **G**), while the resorption pits in the Osteologic system are demonstrated as white clear areas (**I**, **J**).

the role of IL-8 in periodontal bone resorption because T cells and B cells, which are found to be the major sources of RANKL, are attracted by β-chemokines including MIP-1α but not by IL-8.

In addition to RANKL and OPG, other cytokines including M-CSF, IL-1, IL-6, IL-11, IL-17, and TNF-α have been reported to support osteoclast formation by up-regulating RANKL expression, promoting osteoclast differentiation in a RANKL-dependent or perhaps in a RANKL-independent manner.[5,46–49] By contrast to these osteoclastogenesis-promoting cytokines, IL-10 is reported to inhibit osteoclast differentiation.[50] Although the role of GM-CSF is controversial, this cytokine seems to decrease the osteoclastogenesis induced by bone marrow stromal cells.[51] IL-12 and IL-18 are indicated as osteoclast inhibitory cytokines through mechanisms dependent on GM-CSF[52] or IFN-γ.[53] In the present study, an inflammatory cytokine, IL-1β, measured in gingival tissues was significantly elevated in the diseased tissues as was RANKL (Figure 1, A and E), whereas IL-12, IL-10, and GM-CSF levels were not significantly different in the diseased gingival tissues compared to the healthy tissues. Because IL-1 is downstream of TNF-α-induced osteoclastogenesis,[54,55] it is conceivable that elevated IL-1β may reflect the TNF-α produced in periodontal tissues. The involvement of TNF-α in the periodontal bone loss was demonstrated in a primate model of periodontal disease; ie, soluble receptors to IL-1 or to TNF-α, respectively, can significantly inhibit the inflammatory response and bone loss.[56] TNF-α can cause osteoclastogenesis in a RANKL-independent manner,[46] and also synergistically increase RANKL-dependent osteoclast differentiation.[54] However, OPG-Fc mediated nearly complete inhibition of *in vitro* osteoclastogenesis from MOCP-5 and from human peripheral blood $CD14^+$ monocytes (Figure 7, A and B). These findings seemed to exclude the possible involvement of membrane-bound TNF-α and other secondary osteoclast-stimulatory components expressed on activated T cells and/or B cells.

It is well documented that generic inflammation in the synovial cavity can lead to the secondary expression of RANKL and other osteoclast differentiation factors.[55] We, however, hypothesize that the mechanism underlying periodontal bone resorption is different from the mechanism for rheumatoid arthritis, because the healthy oral cavity is constantly exposed to bacterial inflammatory mediators, such as lipopolysaccharide or peptidoglycan,[57] whereas the normal synovial cavity is free from bacterial components. Nonetheless, healthy gingival tissue is free from bone resorption, whereas mitogenic stimulation associated with bacteria may cause inflammation in synovial tissues. Our hypothesis is that generic inflammation, per se, is not the cause of bone resorption in the periodontal tissues. This hypothesis is supported by gingivitis, another form of periodontal inflammation,[58] that does not exhibit bone resorption. Our rat periodontal disease model also illustrated that antigen-specific T-cell and B-cell activation in the inflamed gingival tissues is necessary for the induction of periodontal bone loss, and simple inflammation induced by lipopolysaccharide alone does not cause periodontal bone resorption.[22,30] Therefore RANKL expression by activated lymphocytes may be a prerequisite to develop osteoclast differentiation in the gingival tissues having pre-existing inflammation.

It is conceivable that not only lymphocytes but also osteoblasts may be involved in increases of the RANKL to OPG ratio in the inflamed gingival tissue of periodontitis. The present study did not evaluate osteoblasts, which are also a potential RANKL source in periodontal disease tissue. IL-1 and TNF-α appear to be present in the inflamed gingival tissues[59] and are able to induce RANKL expression by osteoblast cells. Despite the potential involvement of other factors in the bone destruction process, prominent expression of RANKL by B and T cells in the periodontal disease lesion seems to play a primary role in the augmentation of bone resorption processes in this disease.

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