RANKL in human periapical granuloma: possible involvement in periapical bone destruction

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OBJECTIVES: The cytokine receptor activator of nuclear factor κB-ligand (RANKL) has been involved in both the physiological and pathological regulation of osteoclast life span and bone metabolism. Periapical granuloma is a periradicular lesion characterized by periapical bone destruction. The aims of this study were to associate the RANKL mRNA levels to periapical granulomas using the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) technique and to determine the specific cell involved in RANKL synthesis.

METHODS: In eight periapical granuloma and eight periodontal ligament samples from periodontally healthy volunteers, RANKL mRNA was detected by real-time RT-PCR. Expression of RANKL on infiltrate leukocytes was further investigated by flow cytometry in six periapical granulomas.

RESULTS: Receptor activator of nuclear factor κB-ligand mRNA levels were higher in periapical granulomas than in healthy periodontal ligament as its RANKL mRNA cycle threshold (Ct) and ΔCt were significantly lower than that of controls (33.07 ± 1.24 vs 36.96 ± 1.69 and 11.58 ± 3.02 vs 15.60 ± 3.31, respectively). A 16.2-fold (2.0–131.6) higher RANKL gene expression was detected in the granulomas compared with the control tissues. We determined by flow cytometry that lymphocytes were the predominant leukocyte cells (53.31%), and monocytes and dendritic cells were the main RANKL synthesizers in granuloma lesions.

CONCLUSIONS: These data indicate that monocytes synthesized RANKL in periapical granulomas and suggest that RANKL is involved in bone loss associated with periapical lesions.

Keywords: granuloma; immunology; infectious diseases; monocytes; RANKL

Introduction

Periapical lesions are destructive inflammatory pathologies that affect the periapical periodontium. They are characterized by periradicular periodontal ligament and bone destruction as a consequence of bacterial infection of the dental pulp (Wang and Stashenko, 1991; Stashenko et al, 1998; Marton and Kiss, 2000). Diverse inflammatory mediators such as interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, tumor necrosis factor (TNF)α, granulocyte-macrophage colony stimulating factor (GM-CSF), nitric oxide (NO), interferon (IFN)γ, prostaglandins, and metalloproteinases have been associated with periradicular lesions (Stashenko et al, 1998; Kawashima and Stashenko, 1999; Shimauchi et al, 2001; Ataoglu et al, 2002; Shin et al, 2002; Radics et al, 2003). In spite of the increasing advance in the knowledge of the pathogenesis of periradicular lesions, the precise bone-resorptive and regulatory cytokines, and the underlying pathologic mechanism associated with periapical bone destruction during its development remain unknown.

Receptor activator of nuclear factor κB-ligand (RANKL), also known as the osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), TNF-related activation-induced cytokine (TRANCE) and TNF-superfamily member 11 (TNFSF11), has recently been identified as a key regulator of bone metabolism (Lacey et al, 1998; Blaque and James, 2003). RANKL is involved in both the physiological and pathological regulation of osteoclastogenesis and osteoclastoactivation (Lacey et al, 1998; Takahashi et al, 1999; Hofbauer and Heufelder, 2001; Blaque and James, 2003). It has been associated with diverse osteodestructive pathologies, such as rheumatoid arthritis (Kong et al, 1999; Theill et al, 2002), bone tumors (Huang et al, 2000), osteoporosis (Hofbauer and Heufelder, 2001), osteolytic lesions of the facial skeleton (Tay et al, 2004), and periodontitis (Teng et al, 2002).
et al., 2000; Taubman and Kawai, 2001; Crotti et al., 2003; Liu et al., 2003; Mogi et al., 2004; Vernal et al., 2004). All these data strongly indicate that RANKL is an essential component for osteoclast activation in pathological bone resorption, and thus, could play a role in periapical bone destruction during periapical lesion development. The aims of our study were to determine the RANKL mRNA levels in periapical granuloma samples, a periradicular lesion characterized by periapical bone destruction, using the real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique and to investigate the infiltrate leukocyte cells responsible for the local RANKL synthesis, by flow cytometry.

Materials and methods

Patients
The patients for this study were selected from the Postgraduate Center of Diagnostics and Treatment (Faculty of Dentistry, Universidad Complutense, Madrid, Spain). Sixteen patients aged 16–27 years took part in the study. Periapical lesion samples with clinical and radiographic diagnosis of periapical granuloma (PG) were obtained from teeth of eight patients with surgical indication of extraction. As controls, apical periodontal ligament (PL) samples from eight periodontal healthy volunteers were obtained from teeth with orthodontic indication of extraction. For flow cytometry study, five patients, aged 22–25 years, were selected from the Dental Service of Barros Luco Hospital (Southern Metropolitan Health Service, Santiago, Chile). Six PG samples were obtained from teeth with surgical indication of extraction. Subjects did not suffer from systemic illness and had not received antibiotics or non-steroid anti-inflammatory therapy in the 6-month period prior to the study. The protocol was explained to all patients, the Institutional Review Board approved the study and informed consent was obtained from all patients.

Granuloma and PL samples
Both PG lesions and PL tissue were obtained by surgical separation from mineralized tissue of the tooth with curettes (Hu Friedy, Chicago, IL, USA) immediately posterior to teeth extractions. Samples for real-time PCR analysis were submerged in RNAlater (Ambion, Austin, TX, USA) and stored at 4°C for RNA extraction. Samples for flow cytometry analyses were washed extensively in phosphate-buffered saline (PBS) and immediately placed in a vial containing transporting media RPMI1640 supplemented with 50 UI ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin, and 200 mM l-glutamine (Sigma Chemical Co, St Louis, MO, USA). Vials were transported to the Immunobiochemistry Laboratory (Faculty of Chemistry and Pharmacology Sciences, University of Chile) and processed immediately.

Total RNA extraction
Tissue samples were minced as approximately 1 mm³ fragments and homogenized in 1 ml of TRIzol reagent (Invitrogen Corp., Barcelona, Spain); after incubation for 5 min at room temperature in an RNase-free tube, 0.2 ml of chloroform was added and incubated for 3 min at room temperature. After centrifugation at 12 000 g for 15 min at 4°C, the aqueous phase was transferred to a fresh RNase-free tube and RNA was precipitated by mixing it with 0.5 ml of isopropyl alcohol incubated for 10 min at room temperature and centrifuged at 12 000 g for 10 min at 4°C. Then, the RNA precipitate was washed once with 1 ml 75% ethanol and centrifuged at 7000 g for 5 min at 4°C. Finally, the sample was resuspended in 50 µl of RNase-free water diethylpyrocarbonate (DEPC) 0.1% (Sigma Chemical Co) for 10 min at 60°C. Previous quantification in spectrophotometer, total RNA samples were stored at −80°C in a 250 ng µl⁻¹ concentration until further analysis.

Reverse transcription
First-strand cDNA was synthesized using 500 ng of total RNA with TaqMan® Reverse Transcription Reagents (Roche Molecular Systems Inc., Belleville, NJ, USA) according to manufacturer’s instruction. Briefly, 30 µl volume reaction containing 3 µl of 10x RT buffer, 6.6 µl of MgCl₂, 6 µl of dNTP mixture, 1.5 µl of Oligo d(T), 0.6 µl of RNase inhibitor, 0.75 µl of Multi Scribe™ reverse transcriptase, 9.55 µl of RNase-free water and 2 µl of total RNA, were set up in a Primus 96 plus Thermal Cycler (MWG AG Biotech, Ebersberg, Germany) under the following conditions: 10 min at 25°C, 30 min at 48°C and 5 min at 95°C.

Real-time PCR
Real-time quantitative PCR was performed using a specific Assay-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA, USA), that contained a forward and a reverse primer at non-limiting concentrations and a TaqMan® MGB probe 6-FAM™ dye-labeled, specifically designed to detect and quantify cDNA sequences from multi-exon RANKL gene, without amplifying genomic DNA. Duplicated of 1× and 0.1× dilutions of 83.3 ng cDNA were amplified in a 20 µl volume reaction, that contained 10 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl of 20x Assay-on-Demand and 9 µl of cDNA diluted in RNase-free water, under the following conditions: a first step of 2 min at 50°C, a second step of 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C, in a ABI PRISM™ 7700 Sequence Detector System (Applied Biosystems). As in the endogenous control assay, 33.3 ng cDNA was amplified to determine the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression, using a TaqMan® Pre-Developed Assay Reagent Human GAPDH (Applied Biosystems), in the same dilutions and conditions described for RANKL.

Tissue enzymatic digestion
Periapical granulomas were processed by enzymatic digestion to obtain a total cell suspension (Vernal et al., 2005). Briefly, samples were minced into approximately 1 mm³ pieces and incubated in tissue digestion medium at 37°C for 90 min. The tissue digestion medium
Flow cytometry analysis of RANKL expression

Fifty microliters of PBS containing 200,000 total cells of each sample was incubated separately with 10 μl of phycoerythrin (PE)- and fluorescein isothiocyanato (FITC)-conjugated anti-CD monoclonal antibody (CD4 + T cells), anti-CD8 (CD8+ T cells), anti-CD14 (monocytes), anti-CD19 (B cells), anti-CD16 and anti-CD56 (natural killer cells), anti-CD62L (neutrophils) and anti-CD84 (dendritic cells) for 20 min at room temperature in the dark (Becton Dickinson Immunocytochemistry Systems, San José, CA, USA), and with 20 μg ml⁻¹ of mouse anti-human RANKL monoclonal antibody (R&D Systems Inc., Minneapolis, MN, USA) at 4°C overnight, then incubated with 1:64 diluted FITC-conjugated rabbit anti-mouse IgG (R&D Systems Inc.) for 1 h at room temperature. Cells were washed once in PBS and resuspended in 30 μl of PBS to be analyzed by flow cytometry (FACSort, Becton Dickinson Immunocytochemistry Systems). Cells were gated according to their forward- and side-scatter characteristics and their specific CD marker. FITC- and PE-conjugated isotype-matched control monoclonal antibodies were used to determine the positive and negative population. Gates of each specific cell were evaluated for RANKL expression.

Data analysis

Flow cytometry data were analyzed using software WinMDi 2.8 and represented as histograms. RANKL-positive cells were expressed in percentages. WinMDi 2.8 and represented as histograms. RANKL expression.

Gates of each specific cell were evaluated for RANKL expression. Isotype-matched control monoclonal antibodies were used in RANKL flow cytometry data were analyzed using software Data analysis.

Expression of RANKL was determined by the 2⁻ΔΔCt method (Livak and Schmittgen, 2001).

Results

The clinical characteristics of the patients included in the real-time PCR study are summarized in Table 1. Two males and six females were studied for each group. Mean ages were 21.00 ± 2.62 and 20.38 ± 3.25 years for the granuloma and control group, respectively, and no statistical difference between both groups was found. All PG samples had detectable levels of RANKL (8/8), whereas only 87.5% (7/8) had detectable levels of RANKL in PL samples. The undetectable level is represented by a Ct of 40.000 (Table 1).

Table 1 shows the mean, standard deviation and data range of Ct and ΔCt of RANKL and GAPDH of the granuloma and control group. The GAPDH and RANKL amplification plot of the smaller of each duplicate of granuloma and control group are shown in Figure 1. GAPDH Ct was similar (21.493 ± 2.56 vs 21.360 ± 1.94) but RANKL Ct and the ΔCt was significantly lower in granuloma group than in control group (33.071 ± 1.24 vs 36.959 ± 1.69 and 11.597 ± 3.02 vs 15.599 ± 3.31, P = 0.0001 and P = 0.02, respectively). The ΔCt variation with 100-fold range dilutions of GAPDH and RANKL (granuloma n = 3) shows a slope of 0.0966 (Figure 2); thus, the amplification efficiencies of both amplicons are similar and the fold change in RANKL gene expression was evaluated analyzing the ΔCt variation with template dilutions within a 100-fold range. A plot of ΔCt vs log CDNA dilution was made and the data were fit using least-squares linear regression analysis. The fold change in RANKL relative to the GAPDH endogenous control was determined by the 2⁻ΔΔCt method (Livak and Schmittgen, 2001).

Table 1 Clinical characteristics and Ct RANKL and GAPDH data of periodontal ligament controls and periapical granuloma lesion patients groups

<table>
<thead>
<tr>
<th>Patients Gender Age</th>
<th>RANKL Ct 1</th>
<th>RANKL Ct 2</th>
<th>GAPDH Ct 1</th>
<th>GAPDH Ct 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periapical granuloma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 F</td>
<td>21</td>
<td>33.721</td>
<td>33.377</td>
<td>17.180</td>
</tr>
<tr>
<td>2 F</td>
<td>20</td>
<td>34.706</td>
<td>34.671</td>
<td>20.350</td>
</tr>
<tr>
<td>3 F</td>
<td>27</td>
<td>33.268</td>
<td>32.779</td>
<td>19.590</td>
</tr>
<tr>
<td>4 F</td>
<td>20</td>
<td>30.875</td>
<td>31.114</td>
<td>22.740</td>
</tr>
<tr>
<td>5 F</td>
<td>19</td>
<td>33.073</td>
<td>33.321</td>
<td>21.060</td>
</tr>
<tr>
<td>7 F</td>
<td>27</td>
<td>33.268</td>
<td>32.779</td>
<td>19.590</td>
</tr>
<tr>
<td>8 F</td>
<td>19</td>
<td>34.131</td>
<td>34.101</td>
<td>25.020</td>
</tr>
</tbody>
</table>

Gender (M, male; F, female), age (years); Ct, threshold cycle (duplicates).
may be represented by the $2^{-\Delta Ct}$ method. The relative quantification of RANKL was determined using the $2^{-\Delta Ct}$ method, in PG samples there was a 16.2-fold increase in the RANKL expression compared with PL samples, with an exponential range of estimated error of 2.0–131.6 (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Granuloma group (n = 8)</th>
<th>Control group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct GAPDH</td>
<td>21.493 ± 2.56</td>
</tr>
<tr>
<td>range</td>
<td>17.205–25.075</td>
</tr>
<tr>
<td>Ct RANKL</td>
<td>33.071 ± 1.24*</td>
</tr>
<tr>
<td>range</td>
<td>30.995–34.689</td>
</tr>
<tr>
<td>$\Delta Ct$</td>
<td>11.579 ± 3.02**</td>
</tr>
<tr>
<td>range</td>
<td>8.265–19.543</td>
</tr>
</tbody>
</table>

Fold change of RANKL from granuloma vs control group using $2^{-\Delta Ct}$ method (fold change and estimated error).

*Granuloma vs control groups, $P = 0.0001$.

**Granuloma vs control groups, $P = 0.02$.

Figure 3 features side- and forward-scatter density plot and histograms of CD marker and RANKL expression of dendritic cells (CD83+) and monocytes (CD14+). The cells were gated and the results of monoparametric analyses of gated cells with the different antibodies are shown. The complete results of flow cytometry analysis are summarized in Table 3; 53.31%
of the analyzed cells were lymphocytes. Among lymphocytes, 26.83% were T cells, 11.57% were B cells (CD19) and 14.91% were natural killer cells. Among T lymphocytes, 5.81% and 21.02% were CD4 and CD8 cells, respectively. Among natural killer cells, 6.49% were cytotoxic (CD16) and 8.42% were secretory cells (CD56). Monocytes (CD14) were 13.33%, neutrophils (CD62L) were 2.75% and dendritic cells (CD83) were 18.62% of the isolated cells; 26.66% of the total isolated cells expressed RANKL; 13.11% of this expression was associated with monocytes and 13.55 with dendritic cells.

**Discussion**

We have examined the RANKL mRNA levels in periapical granuloma lesions. Using specific probes for RANKL and a real-time quantitative RT-PCR technique, we have demonstrated that the levels of RANKL mRNA were significantly higher in PG than in healthy PL control samples.

The periapical lesion represents an inflammatory and immune response against microorganisms that invade and destroy the dental pulp (Wang and Stashenko, 1991; Stashenko et al., 1998; Mártón and Kiss, 2000). The pathologic response appears to be similar to that which occurs in response to bacterial infections elsewhere in the body, with the additional feature that alveolar bone surrounding the root apex is resorbed (Kawashima and Stashenko, 1999).

Using small tissue biopsies it is possible to analyze the levels of specific cytokines being produced during the inflammatory process associated with tissue destruction and optimizes the measure of its transcriptional activity. Using quantitative RT-PCR with specific primers and TaqMan® probes for RANKL, we have determined that the Ct and ΔCt of RANKL in PG samples were lower than those observed in PL samples. Because of the fact that Ct values decrease linearly with increasing input target quantity (Giulietti et al., 2001), RANKL mRNA levels were higher in granuloma group than in the control group. This finding is in correspondence with our previous studies in periodontal disease, where we clearly demonstrated a relationship between RANKL and the marginal alveolar bone destruction observed in chronic periodontitis (Vernal et al., 2004).

Periodontitis and periapical lesions are characterized by alveolar bone destruction as a consequence of bacterial infection, and in both pathologies it has been proposed that inflammatory bone resorption may be upregulated in vivo by T-helper (Th)1-type mediators.
and downregulated by Th2-type mediators (Kawashima and Stashenko, 1999).

The periradicular cellular infiltrate in periapical lesions is mainly characterized by macrophages, and T and B cells (Matsuo et al., 1992). CD4+ T cells are the predominant lymphocyte cells (Sol et al., 1998) and they have been shown to be the principal cells observed in the acute state of pathologic development (Leonardi et al., 2000).

In other bone-destructive diseases, it has been shown that activated CD4+ T cells participate directly in pathologic bone destruction through RANKL expression (Kong et al., 1999; Taubman and Kawai, 2001; Theill et al., 2002).

We characterized the cells subpopulations in six periapical PGs by flow cytometry. In agreement with a previous flow cytometry study (Sol et al., 1998) the predominant isolated cells in our work were lymphocytes. Unlike them, we observed monocytes after the enzymatic digestion phase performed before the flow cytometric analysis. Monoocytes–macrophages have been established as important constituents of PGs and macrophage activity has been associated with the development of periapical lesions and bone destruction through secretion of bone-resorbing cytokines (Metzger, 2000). The presence of macrophages in human periapical inflammatory lesions has been a commonly reported finding (Stern et al., 1982; Piattelli et al., 1991). Macrophages may also serve as antigen-presenting cells as the dendritic cells. In our study, both of them were isolated in high levels. They process the antigen and present it to the antigen-specific clones of CD4+ T cells by a process involving major histocompatibility complex (MHC) II molecules. On the contrary, macrophages are considered a main source of cytokines (IL-1α, IL-1β, and TNFα), matrix metalloproteinases (MMPs) and prostaglandins, that contribute to the inflammatory process and to the destructive outcome of the periapical lesions (Metzger, 2000). Our results clearly associate the higher RANKL levels observed in the PG lesions to the monocyte activity, thus, activated macrophage would participate in the formation as well as the perpetuation of the periapical lesions.

Receptor activator of nuclear factor κB-ligand is an important regulator of the interactions between T cells and dendritic cells during the antigen presentation process. RANKL is also expressed on the surface of the dendritic cells and the interaction with its receptor can induce cluster formation and activation of T cells, dendritic cell survival, regulate the dendritic cell functions, and T cell–dendritic cell communication (Theill et al., 2002).

Receptor activator of nuclear factor κB-ligand is a bone-resorptive cytokine that has been established as an essential molecule in all phases of the osteoclast’s life span, and it has been catalogued as a key regulator of the physiological and pathological control of bone metabolism (Lacey et al., 1998; Takahashi et al., 1999; Hofbauer and Heufelder, 2001; Blaque and James, 2003). RANKL has been associated with diverse pathologies characterized by bone destruction, such as rheumatoid arthritis, osteoporosis, Paget’s disease, bone tumors, facial osteolytic lesions and periodontitis (Kong et al., 1999; Huang et al., 2000; Hofbauer and Heufelder, 2001; Taubman and Kawai, 2001; Theill et al., 2002; Crotti et al., 2003; Liu et al., 2003; Mogi et al., 2004; Tay et al., 2004; Vernal et al., 2004). It has been proposed as the common final pathway of the bone-resorptive and regulatory cytokines, in osteoclast differentiation and activation and in bone resorption.

A complex network of cytokines is secreted in the periradix in response to the pulp infection by fibroblasts and infiltrates macrophage–monocytes and lymphocytes (Kawashima et al., 1996). This cytokine network stimulates osteoclast activity and the periradicular bone resorption through a not yet completely known mechanism. The RANKL participation in the periradicular bone destruction is an interesting proposed pathway through which it is possible to explain the participation of the diverse cytokines associated to its pathogenesis.

Using the 2−ΔΔCT method (Livak and Schmittgen, 2001), our data show that in PG samples the RANKL expression was increased 16.2-fold in comparison with the control group, with an exponential range of estimated error of 2.0–131.6, indicating that RANKL gene is highly over-expressed in granuloma lesions associated with periradicular bone destruction.

These results demonstrate a clear relation between high RANKL levels and monocyte activity during periapical bone destruction in periapical granuloma. Given the central role of RANKL in the pathogenesis of other bone-destructive diseases, our findings indicate that RANKL plays a key role in the pathologic events associated with periradicular bone destruction, and that methods for controlling RANKL activity would be reasonable as a means of treating these disorders.

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