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ORIGINAL ARTICLE

Immunohistochemical expression of RANKL, RANK and OPG in gingival tissue of patients with periodontitis

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Abstract

Objectives. To evaluate the expression of the receptor activator of NF-κB (RANK), the receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), in the gingival tissue of patients with periodontitis. ***Materials and methods.*** Gingival tissue was obtained from 14 systemically healthy subjects with chronic periodontitis during conventional periodontal surgery. Immunohistochemistry was used to detect the expression of RANK, RANKL and OPG in the oral and periodontal pocket epithelium as well as in the connective tissue cells. ***Results.*** RANKL was negatively expressed in both oral and periodontal pocket epithelium. OPG was also negative or weakly positive in the whole epithelium. RANK showed moderate/strong positive staining mainly in the basal and suprabasal layer of oral and periodontal pocket epithelium. In most of the cases, more than 60% of the inflammatory cell infiltrate stained for RANK and RANKL. In these cases the intensity of the stained cells ranged from moderate-to-strong. In less than half of the cases, OPG was positive in more than 60% of the stained cells of the inflammatory cell infiltrate. ***Conclusion.*** The RANK, RANKL and OPG proteins are differentially expressed in periodontal tissues and may play a major role in the bone loss occurring in periodontitis.

Key Words: *RANKL, RANK, OPG, chronic periodontitis, immunohistochemistry*

Introduction

Periodontitis is an infection-induced inflammatory disease, characterized by loss of tooth supporting structures, such as connective tissue attachment and alveolar bone. Alveolar bone resorption is the result of excessive osteoclastic activity that leads to an imbalance in bone remodeling. Osteoprotegerin (OPG) and the receptor activator of NF-κB ligand (RANKL) have been recently identified as members of a ligand-receptor system that directly regulates osteoclast differentiation and bone resorption. The receptor activator of NF-κB (RANK), the RANKL and the OPG, known together as the RANK-RANKL-OPG system, has been demonstrated to effectively control the balance between osteoblasts and osteoclasts activity. RANK is the cognate receptor of RANKL and is expressed at high levels on osteoclast precursors [1]. The binding of RANK and RANKL activates the formation and maturation of osteoclasts, thus resulting in bone destruction [2]. OPG functions as a decoy receptor of RANKL and blocks the activation of RANK by preferentially binding itself to RANKL, thus protecting against bone destruction [3-5].

The RANK-RANKL-OPG system has been shown to play an essential role in the development and progression of ameloblastoma [6], in odontogenic tumors [7] and in human oral squamous cell carcinoma [8]. An imbalance in the RANK-RANKL-OPG expression has been observed in osteoporosis, rheumatoid arthritis, multiple myelomas and altered tooth eruption [5,9]. In general, up-regulation of RANKL and down-regulation of OPG have been observed in all these situations. The same mechanism seems to be involved in tissue destruction in periodontitis. The level of RANKL mRNA determined using semi-quantitative reverse transcription-polymerase chain

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reaction was found to be higher in advanced periodontitis as compared to the moderate periodontitis or periodontally healthy situations [10].

RANKL and OPG can be detected in the gingival crevicular fluid (GCF), with elevated levels of RANKL and decreased levels of OPG in periodontitis patients [11,12]. Furthermore, these proteins are also involved in alveolar bone remodeling during orthodontic tooth movement [13-15]: higher expression of RANKL was found in the compression side of teeth undergoing orthodontic tooth movement, whereas higher expression of OPG was found in the tension side. These results suggested that the RANKL/RANK/OPG system plays an important role in the bone remodeling process that takes place during orthodontic tooth movement. Interestingly, Kawasaki et al. [15] found that the average amount of tooth movement for juveniles was larger than that for adults and that this difference was associated with a decrease in the RANKL/OPG ratio in GCF of patients undergoing orthodontic tooth movement.

The present study investigates the immunohistochemical expression of RANK, RANKL and OPG in the oral and periodontal pocket area of patients with periodontitis. Associations between the expression of these three proteins, the number of stained inflammatory cells and the intensity of staining are evaluated.

Materials and methods

Study population

Six male patients and eight female patients with chronic periodontitis were randomly provided from the Department of Periodontology of the School of Dentistry of the University of Geneva. The Ethical Committee of the University Hospitals of Geneva approved the protocol. Research was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. Patients were informed of the procedures, and signed a consent form in advance of their participation in the study. Gingival tissue samples were obtained during conventional periodontal surgery after completion of phase I therapy (scaling and root planing). The day of the surgery, clinical parameters including probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP) were recorded at six sites on each tooth. The tissues used in this study would have been discarded after surgery.

Tissue preparation

The tissue samples were initially fixed in 10% formalin, dehydrated in graded alcohol, cleared in xylene and then embedded in paraffin. Sections of 4 μm were cut and stained with hematoxylin-eosin, for routine histological assessment (e.g. histological features of chronic periodonitis). Unstained slides were used for RANK, RANK-L and OPG immunohistochemical analyses.

Immunohistochemistry

The slides were initially deparaffinized with xylene and rehydrated in alcohol. For antigen retrieval, the slides were immersed in a 10 mM sodium citrate buffer (pH6) and boiled three times, for 5 min, in a microwave oven (600 W). The endogenous peroxidase activity was then blocked by immersion in 3% hydrogen peroxide, for 30 min. The slides were washed in PBS and incubated with rabbit serum block for RANK and OPG (kit SC−2051; Santa Cruz, CA, Biotechnology) and goat serum block for RANKL (kit SC-2053; Santa Cruz Biotechnology), overnight, at 4°C. Following rinsing in PBS, the slides were incubated with the following primary antibodies for 2 h: IgG polyclonal rabbit anti-OPG (code 11383, Santa Cruz Biotechnology, dilution 1/200); IgG polyclonal rabbit anti-RANK (code sc-9072, Santa Cruz Biotechnology, dilution 1/200); IgG polyclonal goat anti-RANKL (code sc-7628, Santa Cruz Biotechnology, dilution 1/200). The sections were carefully rinsed with PBS, incubated with biotinylated secondary antibodies for RANK and OPG (kit sc-2051) and RANKL (kit sc-2053), for 30 min and then incubated with Streptadivin-HRP (kit sc-2051 for RANK and OPG and s-2053 for RANKL) for 30 min. The histological sections were developed by using Carpinteria, CA, DAB + Chromogen (k3468, DAKO), for 10 min and lightly counterstained with Harris hematoxylin, for 30 s. Slides were dehydrated in graded alcohol, cleared in xylene and mounted in Neo-Mount (MERCK). The negative control was used by omitting only the primary antibody and replacing it with normal IgG rabbit (kit sc-2051) for OPG and RANK and with normal IgG goat (kit sc-2053) for RANKL. Periapical cysts containing osteoclasts at the periphery of fragments were used as positive control. Healthy oral mucosa was used as control.

Evaluation of RANK, RANK-L and OPG expression

The oral and periodontal pocket epithelium, as well as the connective tissue inflammatory cell infiltrate of the pocket area, were semi-quantitatively examined in order to analyse the stained cell proportion and the intensity of staining for RANK, RANKL and OPG. Three different layers of oral stratified squamous epithelium were analyzed in the oral and periodontal pocket area: basal, suprabasal and superficial. Inflammation was classified according to the number of inflammatory cells as: weak inflammation and moderate–strong inflammation. The scores of percentage of positive immunostaining cell proportion were classified as: 0 (1–29%); 1 (30–59%); and 3 (60–100%), whereas the scores for intensity of staining were

Table I. Distribution of cases in the periodontal pocket area with respect to RANK, RANKL and OPG in relation to inflammation.

	Stained inflammatory cell proportion								
	RANK			RANKL			OPG		
Inflammation	≤29%	30–59%	≥60%	≤29%	30–59%	≥60%	≤29%	30–59%	≥60%
Weak	2 (14.3%)	1 (7.1%)	0	3 (21.4%)	0	0	3 (21.4%)	0	0
Moderate/strong	0	2 (14.3%)	9 (64.3%)	1 (7.1%)	2 (14.3%)	8 (57.1%)	4 (28.6%)	2 (14.3%)	5 (35.7%)

Inflammation is expressed as weak or moderate/strong.
The scores of percentage of positive immunostaining are classified as: 0–29%, 30–50% and 60–100%.

classified as: 0, no staining; 1, weak staining; 2, moderate/strong staining.

Since the average of staining cell proportion was higher than 29% (except for the RANKL that was negative in both oral and periodontal pocket epithelium), a score of <29% of stained cells was considered as negative.

Statistical analysis

Qualitative variables such as inflammation, tobacco, RANKL, RANK and OPG expressions, staining intensity of RANK, RANKL and OPG in the three different layers (basal, suprabasal and superficial) and stained cell population for RANK, RANKL and OPG in the periodontal pocket area were summarized using frequency and percentage. Inflammation and scores of percentage of positive immunostaining for RANK, RANKL and OPG were compared using a Chi-square test or a Fisher exact test (if expected frequency was less than 5) with a threshold of 5%. The same tests were used to compare staining intensity of expression in the three different layers (basal, suprabasal and superficial) for RANK and OPG. Data analyses were performed by using STATA 10.0 (StataCorp LP, TX).

Results

For immunochemistry, a total of 14 biopsies from patients with chronic periodontitis were used. Patients presented periodontal pocket depths ranging from 6–12 mm, whereas six out of 14 patients (42.9%) were smokers. Each specimen contained the oral and periodontal pocket area, except for one specimen that did not contain the oral area. Under microscopy, inflammation varied from weak-to-strong and, based on morphological characteristics, was mainly represented by lymphocytes, plasma cells and some macrophages. In healthy oral mucosa epithelium, RANKL expression was negative, whereas the RANK and

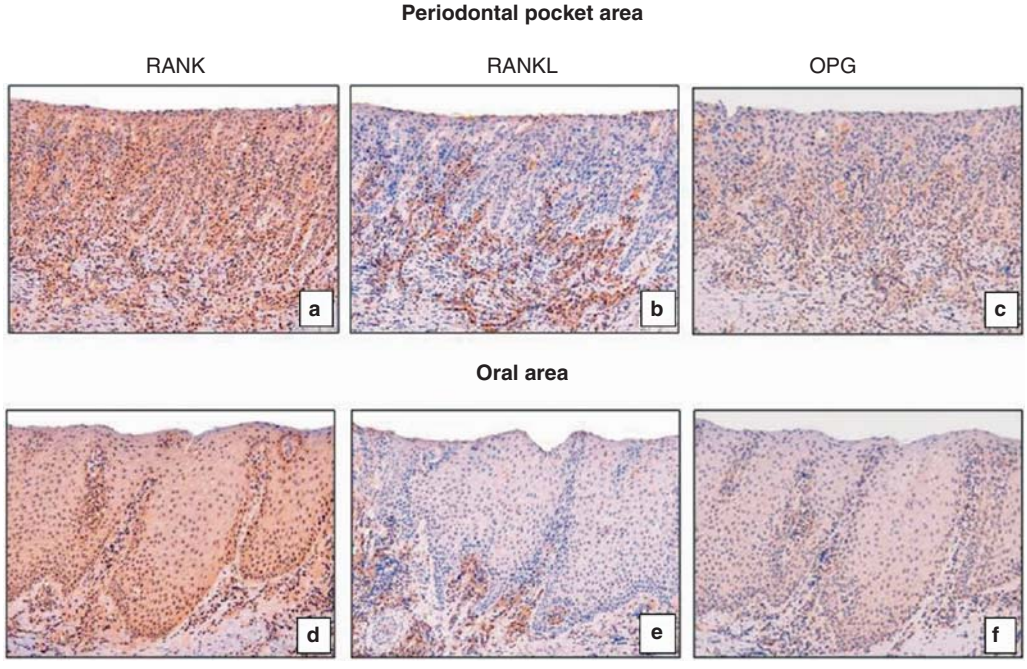

Figure 1. Immunohistochemical expression of RANK, RANKL and OPG in the periodontal pocket and oral area. (A, D) Strong expression of RANK in epithelium and inflammatory cell infiltrate; (B, E) RANKL is expressed only in the inflammatory cell infiltrate; (C, F) negative-to-weak expression of OPG in the epithelium and inflammatory cell infiltrate.

Table II. Distribution of cases in the periodontal pocket area in respect to the intensity of RANK, RANKL and OPG staining in the different layers.

	Staining intensity								
	Epithelium								
	RANK			OPG			Inflammatory cell infiltrate		
	Basal	Suprabasal	Superficial	Basal	Suprabasal	Superficial	RANK	RANKL	OPG
0	0	0	7 (50%)	4 (28.6%)	6 (42.9%)	9 (64.3%)	2 (14.3%)	1 (7.1%)	6 (42.9%)
1	1 (7.1%)	2 (14.3%)	3 (21.4%)	10 (71.4%)	8 (57.1%)	5 (35.7%)	1 (7.1%)	0	7 (50%)
2	13 (92.9%)	12 (84.7%)	4 (28.6%)	0	0	0	11 (78.6%)	13 (92.9)	1 (7.1%)

Intensity is expressed as 0, no staining; 1, weak staining; 2, moderate/strong staining.

OPG expression varied from negative to slightly positive in focal areas only of the basal layer (data not shown). In the diseased periodontal pocket area, plasma cells, macrophages, some lymphocytes, as well as fibroblasts and endothelial cells were positive to RANK, RANKL and OPG staining.

Table I shows the expression of RANK, RANKL and OPG in the periodontal pocket area in relation to inflammation. Inflammation (moderate or strong) was associated with higher RANK and RANKL expression in the inflammatory cells ($p = 0.07$ and 0.08, respectively). In fact, nine out of 14 cases (64.3%) and eight out of 14 cases (57.1%) showed more than 60% of stained cells in the periodontal pocket area for RANK and RANKL, respectively. OPG expression was not correlated with inflammation. As shown in Figure 1, immunostaining for RANK, RANKL and OPG was cytoplasmic. Only some cells showed a positive nuclear immunostaining for RANK.

RANKL was not expressed in the oral and periodontal pocket epithelium (Figures 1B and E). As shown in Table II, in periodontal pocket epithelium, RANK showed mainly moderate or strongly positive staining in the basal and suprabasal layer and negative or weakly positive staining in the superficial layer (Figures 1A and D). Only in four cases (28.6%) did RANK expression show a moderate-to-strong staining in the superficial layer. Conversely, OPG expression was mainly negative or weakly positive in the whole epithelium (Figures 1C and F). In the same table, the expression of RANK, RANKL and OPG in respect to the intensity of stained cells is presented: 11 out of 14 (78.6%) and 13 out of 14 (92.9%) of the specimens showed a moderate/strong expression for RANK and RANKL, respectively. As has been already shown in Table I, nine out of 14 (64.3%) and eight out of 14 (57.1%) of the cases presented more than 60% of the stained cells in the periodontal pocket for RANK and RANKL, respectively.

In the oral epithelium, the moderate/strong intensity for RANK was mainly confined to the basal layer (92%) and only 38.5% of the cases in the suprabasal layer. The superficial layer was negative for RANK. OPG staining intensity varied mainly between negative and weakly positive in basal and suprabasal layers. Similarly, in the superficial layer OPG was not expressed (results not shown).

With regard to the positive immunostaining cell proportion, RANK expression was significantly higher as compared to OPG expression in the periodontal pocket epithelium ($p = 0.004$): in 100% of the cases, more than 60% of the stained cells were positive for RANK, and only 42% of the cases for OPG (Table III).

In the inflammatory cell infiltrate of the periodontal pocket area, the expression of RANK and RANKL showed significant differences as compared to the expression of OPG ($p = 0.001$ and 0.006, respectively). The latter presented from 30–59% of labelled cells in two out of 14 (14.3%) cases and less than 29% of stained cells in seven out of 14 (50%) cases, whereas nine out of 14 (64.3%) cases for RANK expression and eight out of 14 (57.1%) cases for RANKL expression had more than 60% of labelled cells (Table III)

Table III. Percentage of RANK, RANKL and OPG stained cell population in the periodontal pocket area.

	Stained cell population				
	Epithelium		Inflammatory cell infiltrate		
	RANK	OPG	RANK	RANKL	OPG
0–29%	0	7 (50%)	2 (14.3%)	4 (28.6%)	7 (50%)
30–59%	0	1 (7.1%)	3 (21.4%)	2 (14.3%)	2 (14.3%)
60–100%	14 (100%)	6 (42.9%)	9 (64.3%)	8 (57.1%)	5 (37.7%)

The scores of percentage of positive immunostaining are classified as: 0–29%, 30–50% and 60–100%.

No significant effect of tobacco consumption on RANK, RANKL and OPG expression was found (data not shown).

Discussion

The present study examined the RANK, RANKL and OPG expression on gingival biopsies of patients with periodontitis. Immunohistochemical localization of the three proteins was performed in both oral and periodontal pocket epithelium as well as in the connective tissue cells.

RANKL, its receptor RANK and the decoy receptor OPG are the three key molecules that regulate osteoclast recruitment and function. RANKL-binding to its receptor RANK on osteoclast precursor cells elicits their differentiation and activation. On the other hand, OPG-binding to RANKL interrupts RANKL-RANK ligation; consequently, OPG inhibits the ability of RANKL to induce osteoclastogenesis. RANKL is involved not only in physiological osteoclastogenesis, but also in pathological bone loss.

In our study, immunohistochemical staining results showed that RANKL was not expressed in the oral and periodontal pocket epithelium. However, RANKL-positive cells were widely distributed in the inflammatory cell infiltrate of the connective tissue, showing a moderate/strong staining. This is in accordance with the study of Lu et al. [16], who reported a wide distribution of RANKL-positive cells in the gingival connective tissue of patients with chronic periodontitis. On the contrary, rare OPG-positive cells were observed in the zone of inflammation in the gingival connective tissue. However, in a recent study [17], the immunohistochemical evaluation of the RANKL and OPG expression performed in healthy and diseased human gingival tissue revealed that RANKL was expressed both in the oral epithelium and more intensively in the inflammatory cells in the healthy specimens (50%) as well as in the diseased samples (75% in the epithelial cells and 87% in the inflammatory cells). The expression of OPG was observed more frequently in the oral epithelium than in the inflammatory cells and in the diseased samples more than in the healthy specimens. Contrary to our study, the positive expression of RANKL in the oral epithelium may be due to methodology differences.

The up-regulation of RANKL and down-regulation of OPG is considered to be the master mechanism modulating local bone destruction in periodontitis. The cellular source of RANKL in the bone resorptive lesions of periodontal diseases is mainly the activated T- and B-cells, as more than 50 and 90% of these cells were found to express RANKL, respectively. In healthy gingival tissues, less than 20% of both B- and T-cells were found to express RANKL [18].

The level of RANKL mRNA has been reported to be highest in advanced periodontitis, whereas the level of OPG mRNA was lower in both advanced and moderate periodontitis as compared to the periodontally healthy group [10]. Similar results have been reported by Valverde et al. [19], with qualitative elevation of RANKL mRNA levels and decreased OPG mRNA levels in the gingival tissues of patients with chronic periodontitis. RANKL and OPG gene expressions are differentially regulated in gingival tissues depending on the form of periodontal disease and that the increase in the RANKL/OPG ratio in the tissues may indicate the occurrence of periodontitis [20].

In the gingival crevicular fluid (GCF) RANKL, but not OPG, was elevated in diseased sites of patients with periodontitis [11,16,20]. The RANKL and OPG found in the GCF originate mainly from lymphocytes and dental mesenchymal cells, respectively. It seems that the low concentration of OPG allows an increased rate of RANK binding to RANKL.

In conclusion, an enhanced immunohistochemical expression of RANK and RANKL and a weak expression of OPG in the inflammatory cell infiltrate of diseased gingival tissue has been demonstrated. This suggests a contribution of the RANK-RANKL-OPG system in tissue destruction in periodontitis. Prevention of the production of RANKL or blockage of the RANK/RANKL interaction may be a new therapeutic approach for periodontal disease [21]. In fact, various regimens aimed at inhibiting RANKL expression by activated T- and B-cells and the subsequent reduction of RANKL-dependent periodontal bone resorption are currently under intensive investigation in several laboratories.

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