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Receptor activator of nuclear factor-kappa B ligand antagonists inhibit tissue inflammation and bone loss in experimental periodontitis

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Abstract

Aim: The purpose of this study was to assess the role of anti-bone resorptive agents and an anti-inflammatory compound in murine *Porphyromonas gingivalis* (*P. gingivalis*)-induced periodontitis.

Material and Methods: Six randomly assigned groups were administered vehicle (saline, control) ($n = 6$), *P. gingivalis* infection only (untreated) ($n = 6$), human-Fc ($n = 4$), Kavain ($n = 6$), OPG-Fc ($n = 6$) and Receptor activator of nuclear factor-kappa B (RANK)-Fc ($n = 6$) intraperitoneally at day 0, 3 and 7. Animals were euthanized on day 10 and subjected to comprehensive histomorphometric analysis. To capture the progress of inflammation, serum samples were collected at days 0, 3, 7 and 10 for levels of pro-inflammatory cytokines.

Results: Compared with control group, OPG-Fc, RANK-Fc and Kavain treatment showed significant bone loss reduction with OPG-Fc performing better than RANK-Fc or Kavain. Epithelial down-growth showed significant reduction in treatment groups with OPG-Fc performing better than RANK-Fc or Kavain. Finally, Kavain, OPG-Fc and RANK-Fc-treated mice displayed reduced inflammatory cell counts and cytokine expression particularly at day 7 postinfection.

Conclusions: RANKL antagonists and Kavain effectively reduced alveolar bone loss in *P. gingivalis*-induced periodontitis in our mice model. Compared with RANK-Fc, Kavain-treated animals showed milder improvement of bone and connective tissue inflammation. Therapeutic implications in the prevention of periodontal bone loss are discussed.

Key words: bone loss; experimental periodontitis; inflammation; Kavain; osteoprotegerin-Fc; receptor activator of nuclear factor-kappa B ligand antagonist; receptor activator of nuclear factor-kappa B-Fc

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Conflict of interest and source of funding statement

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Chronic periodontal disease, the most common form of periodontitis estimated to afflict 70–80% of adults in the United States (Eke et al. 2010), is now thought of as a multifactorial disease initiated by dental plaque, a highly organized bacterial biofilm, resistant to both endogenous host mechanisms and exogenous antibacterial agents (Albandar 2005, Amar et al. 2007). Interestingly, a consensus is now emerging that the actual resultant tissue damage observed in periodontal disease is mostly from host-response inflammatory mechanisms that attempts to eradicate the biofilm from the oral cavity and less from pathogen-mediated mechanisms. Achieving a critical concentration of inflammatory

mediators that contributes to bone resorption depends upon the expression of pro-inflammatory cytokines such as interleukin (IL)-1, -6, -11, -17 and tumour necrosis factor-alpha (TNF-α). These pro-inflammatory cytokines are integral to the propagation of inflammatory response to regions proximal to bone (Assuma et al. 1998, Cochran 2008, Preshaw & Taylor 2011). Inflammation caused by bacterial infections has been known to increase bone destruction (Verdrengh et al. 2010), aggravate coronary artery disease and amplify periodontal damage (Zelkha et al. 2010).

Alveolar bone loss and inflammation are hallmarks of chronic periodontal disease. Bone and tooth loss appear to be the result of osteoclast-mediated bone resorption initiated through the activation of the inflammatory process via interactions of receptor activator of nuclear factor-kappa B (RANK) and the balance between RANK ligand (RANKL) and osteoprotegerin (OPG), the endogenous RANKL antagonist (Cochran 2008). These mediators are directly involved in osteoclastogenesis, activation, differentiation and survival of osteoclasts and their monocyte-derived precursors (Wong et al. 1997, Lacey et al. 1998, Yasuda et al. 1998). RANKL has been shown to mediate alveolar bone loss in vivo in periodontitis (Crotti et al. 2003, Jin et al. 2007). It is therefore suggested that RANK-RANKL/OPG signalling network plays a crucial role in the pathophysiology of periodontal disease, specifically in the mediation of alveolar bone loss.

It has been proposed that approaches preventing RANK-RANKL interactions or over-expressing OPG could potentially reduce bone destruction and prevent future tooth loss seen in periodontal disease. Denosumab, a fully human monoclonal antibody to RANKL that blocks the RANK-RANKL interaction, successfully reduced the incidence of new fractures in women with osteoporosis, presumably by the prevention of osteoclastogenesis and subsequent bone resorption (Cummings et al. 2009). We previously showed that soluble receptors to TNF (TNFR-Fc) or IL-1 (IL-1R-Fc) can be used successfully to reduce inflammation and bone loss in experimental periodontal disease and periodontal wound healing (Assuma et al. 1998, Zhang et al. 2004). Furthermore, RANK-Fc has been found effective in suppressing the bone resorption in tumour associated hypercalcaemia. This data indicates the potential anti-resorptive role of RANK-Fc in vivo, highlighting its possible function in osteolytic conditions (Oyajobi et al. 2001). Recently, the use of fusion protein OPG-Fc for blocking RANKL activity was found to significantly reduce bone loss in experimentally induced periodontitis (Jin et al. 2007). However, the role of RANK-Fc in periodontal disease has never been evaluated nor compared with OPG-Fc or to an anti-inflammatory agent.

Kavain, an extract from the pepper plant *Piper methysticum* has been commonly used as an anxiolytic, antiasthmatic and antiarthritic (Pittler and Edzard 2001, Bielory 2004, Sarris & Kavanagh 2009). It was shown to prevent the degradation of IB in response to TNF-α, thereby increasing the inhibition of NF-B and limiting the effect of pro-inflammatory mediator TNF-α (Folmer et al. 2006). Furthermore, our laboratory has demonstrated that Kavain inhibits lipopolysaccharide (LPS)-induced TNF-α factor-mediated TNF-α secretion in an in vitro and in vivo where Kavain-treated mice were immune to lethal doses of LPS (Pollastri et al. 2009). Taken together, Kavain appears to be a promising anti-inflammatory agent, which may help prevent alveolar bone loss observed in periodontitis.

Thus, the purpose of this study was to compare the effects of soluble RANKL receptors OPG-Fc, RANK-Fc and Kavain compound in *P. gingivalis*-soaked ligature-induced periodontitis murine model. Bone and connective tissues parameters were quantified and analysed to determine the role each of treatment in experimental periodontitis. To our knowledge, this is the first study addressing the role of soluble RANKL receptors OPG-Fc, RANK-Fc and Kavain compound on bone loss and inflammatory parameters.

Material and Methods

All measurements and assessments were performed by individuals blinded to all groups.

Reagents

OPG-Fc, RANK-Fc and human-Fc (Hu-Fc) portion for control were generously provided by Amgen Inc. (Thousand Oaks, CA, USA). Kavain compound was purchased from Avachem, Scientific LLC (San Antonio, TX, USA).

Bacteria

P. gingivalis (wild-type strain A7436) was cultured and maintained in Schaedler broth (Fischer Scientific, Pittsburgh, PA, USA), supplemented with 5 μg/ml hemin) and 1 μg/ml menadione (Sigma-Aldrich, St. Louis, MO, USA), in an anaerobic chamber with 85% N_2, 10% H_2 and 5% CO_2 at 37°C. For oral infection, the 5-0 silk ligatures were immersed in supplemented Schaedler broth containing *P. gingivalis* in an anaerobic chamber at 37°C for 2 days (Amar et al. 2007).

Animal and experimental periodontitis model

All the experimental procedures in this study were approved by the Institutional Animal Care and Use Committee at Boston University Medical Center. To minimize any potential oestrogen effects, 12-week old male C57BL/6 mice were used. A total of 34 mice were randomly assigned to one of the six groups: (i) vehicle, saline injected no *P. gingivalis* infection ($n = 6$), (ii) positive control, *P. gingivalis* infected no treatment ($n = 6$), (iii) Hu-Fc, *P. gingivalis* infected with Hu-Fc treatment ($n = 4$), (iv) Kavain, *P. gingivalis* infected with Kavain treatment ($n = 6$), (v) OPG-Fc, *P. gingivalis* infected with OPG-Fc treatment ($n = 6$) and (vi) RANK-Fc, *P. gingivalis* infected with RANK-Fc treatment ($n = 6$). This study was carried out in the specific pathogen-free unit of the animal facility. The mice were fed sterile food and distilled water ad libitum. After intraperitoneal injection of anaesthetic [mixture of Ketamine (80 mg/kg) and Xylazine (10 mg/kg)], the mice were induced for periodontitis by wrapping a ligature inoculated with *P. gingivalis* around both the left and right maxillary second molar, carefully pushing the ligature into the gingival sulcus and knotting mesiobuccally. The

P. gingivalis-soaked ligatures were replaced every other day to maintain a sufficient microbial load. Following ligature placement, Hu-Fc (5 mg/kg, twice/week), OPG-Fc (5 mg/kg, twice/week) or RANK-Fc (5 mg/kg, twice/week) were subcutaneously delivered to randomly assigned mouse groups while intraperitoneal injection of Kavain (40 mg/kg, twice/week) or vehicle (saline) was administered to other corresponding groups, respectively. Injections were performed at day 0, 3 and 7 of the study. Serum collections were carried out at 0, 3, 7 and day 10 prior to sacrifice.

Identification of *P. gingivalis*, total bacterial count, tissue specimen preparation, morphometric and histomorphometric analysis

Please refer to Appendix S1 information.

Pro-inflammatory cytokine level measurement

Serum cytokine (IL-6, MCP-1, TNF-α) quantifications were determined using the BioPlex Protein Array System with cytokine-specific antibody-coated beads (Bio-Rad, Hercules, CA, USA). The assay was performed according to the manufacturer's instructions. The three cytokine concentrations in a triplicate of 25 μl per well of serum from each individual experimental or control mice were automatically calculated with BioPlex Manager software using a standard curve derived from recombinant cytokine standards.

Statistical analysis

Statistical comparisons were made using Student's *t*-test analysis between two groups. Multiplicity of comparisons to the control group were performed using ANOVA.

All values were reported as the mean ± standard error of the mean (SEM). A *p*-value below 0.05 was considered statistically significant.

Results

P. gingivalis bacterial counts

Colony forming unit (CFU) data gathered from the cultures indicated no significant statistical difference regarding bacterial CFU counts in all groups at each individual time course, thus eliminating its influence on the results obtained (Fig. 1A).

Alveolar bone loss and epithelial down growth reduction with TRAP results

The vehicle group exhibited least amount of bone loss when compared with positive control. Interestingly, Hu-Fc group exhibited no significant difference with the positive control (Fig. 1B,C). Mice treated with Kavain, OPG-Fc and RANK-Fc exhibited significantly less bone loss both macroscopically (31.5%, 34% and 26%) and microscopically (23%, 55% and 33%) respectively, when compared with positive control. Both macroscopic and microscopic assessments showed similar trends with OPG-Fc performing better than RANK-Fc, while RANK-Fc presenting almost equal or better protection than Kavain. For epithelial down-growth, mice treated with OPG-Fc, RANK-Fc or Kavain exhibited significantly less down-growth compared with the positive

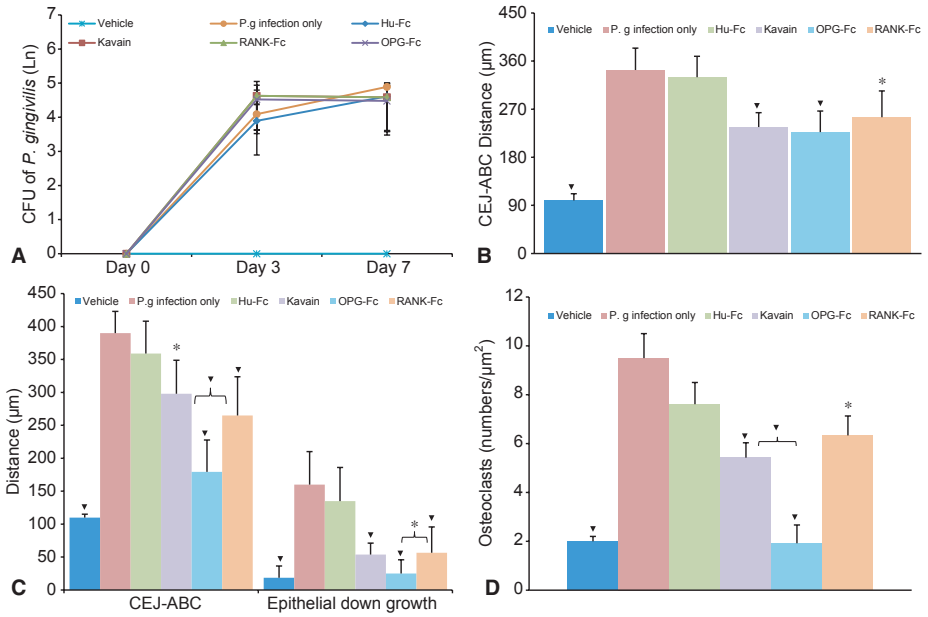

Fig. 1. Bacterial counts, alveolar bone resorption, epithelial down growth and TRAP positive osteoclasts distribution. (A) Bacterial samples isolated from the animals from day 0, 3 and 7 displayed no significant differences in all the groups at different time points. *P. gingivalis* was recovered from all the five groups which were infected, whereas no control mice harboured *P. gingivalis*. (B) Morphometric analysis of alveolar bone resorption. Bone loss was quantified as the distance between cemento-enamel junction (CEJ) and alveolar bone crest (ABC) (30× magnification) in methylene blue stained molar sections. (C) Histomorphometric evaluation of alveolar bone resorption (left) and epithelial down growth (right). The bone resorption was measured as the distance between the CEJ and the ABC. Epithelial down-growth was defined as the distance from the CEJ to the apical extent of the junctional epithelium in H&E stained molar sections (100× magnification). (D) The distribution of TRAP positive osteoclasts ($n = 6$ for all groups except $n = 4$ for Hu-Fc, 400× magnification). Statistical significance *p-value ≤ 0.05; ▼$p \leq 0.01$.

control group (83.8%, 64.5% and 66.5%) respectively (Fig.1C). Positive control showed maximum epithelial down-growth (87%) compared with negative control which had the least amount of epithelial down-growth (Fig. 1C). Trap positive multinucleated osteoclast counts for Kavain, OPG-Fc and RANK-Fc were reduced by 43.7%, 79% and 37.5% respectively, when compared with positive control. All treatment groups had statistical significance compared with the positive control group except Hu-Fc group (10.4%) (Fig. 1D).

Eect of treatments on inflammatory cell infiltrates and apoptosis in *P. gingivalis*-induced gingival infection

The number of polymorphonuclear leucocytes (PMNs), mononuclear leucocytes and connective tissue fibroblasts were counted in all three defined regions Box 1 (top), Box 2 (middle) and Box 3 (deep) (Fig. 2D). Box 1 cell counts showed significant reductions in PMN for Kavain (33%), OPG-Fc (64%) and RANK-Fc (68%) and in mononuclear leucocyte cells by 28%, 52% and 77% reduction respectively. Hu-Fc-treated animals did not show any significance when compared with positive control. No significant changes were observed for connective tissue fibroblast cells between the groups (Fig. 2A). Box 2 demonstrated significant reductions in the PMN population for Kavain, OPG-Fc and RANK-Fc-treated groups (36%, 52% and 60%), respectively, while mononuclear cells showed significant reduction for OPG-Fc and RANK-Fc (35% and 42%), respectively. Only Kavain showed significant reduction in connective tissue fibroblasts (26%), while OPG-Fc showed a significant increase (11%) (Fig. 2B). In Box 3, all PMN values showed significance in reductions for Kavain, OPG-Fc and RANK-Fc (26%, 33% and 53%) respectively. Mononuclear leucocytes also had similar significant reductions in Kavain, OPG-Fc and RANK-Fc treatment groups (33%, 50% and 48%) respectively. Although higher in counts, no statistical significance was observed in the number of connective tissue fibroblasts between all the groups and the positive control except for Kavain which was 16% lower (Fig. 2C).

Apoptosis cell counts normalized over the number inflammatory showed significant reduction compared with the positive control in all three boxes for OPG-Fc and RANK-Fc treatment groups (Box 1: 32% and 13%; Box 2: 37% and 28% and Box 3: 43% and 14% respectively) while Kavain treatment showed similar counts as the positive control or Hu-Fc (Fig 3).

Eect of treatments on pro-inflammatory cytokine levels in *P. gingivalis*-induced experimental periodontitis mice

Serum levels for pro-inflammatory cytokines (IL-6, MCP-1, TNF-α) were obtained for all animals at different time points (days 0, 3, 7 and 10). Cytokine levels in vehicle treated animals did not display any significant changes throughout the study. In the positive control group, the three cytokines started to increase expression from day 0 to 3 and reached their peak expressions at day 7 followed by a return to baseline levels at day 10 (Fig. 4). Hu-Fc-treated mice presented higher cytokine levels than Kavain, OPG-Fc and RANK-Fc groups. Compared with positive control, Kavain inhibited IL-6 expression by 60% at day 7 ($p < 0.05$) but showed increase on day 10 although not significant.

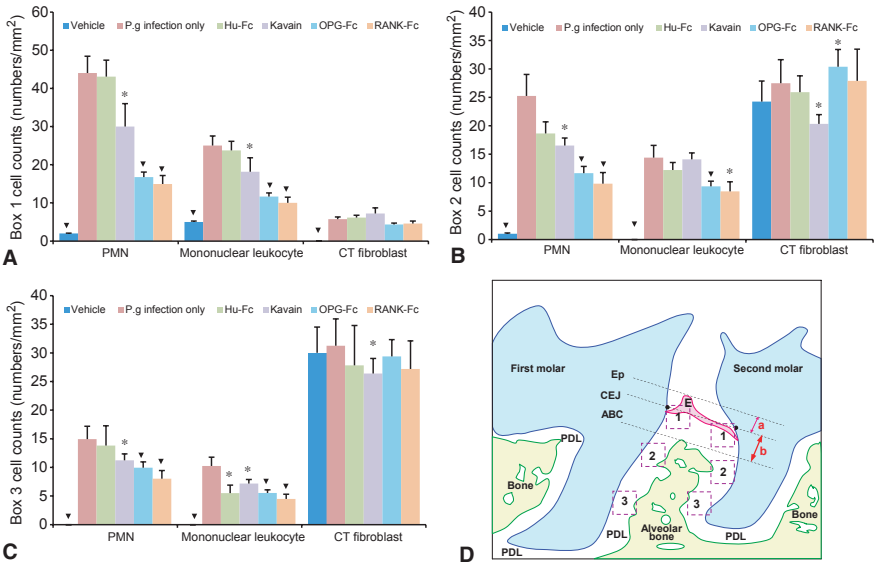

Fig. 2. Histomorphometric analysis of regional infiltration of inflammatory cells and diagrammatic representation. Polymorphonuclear cells (PMN), mononuclear leucocytes and gingival fibroblasts infiltrated in the gingival tissue were quantified in different regions of gingiva top (Box 1, panel A), middle (Box 2, panel B) and deep gingival areas (Box 3, panel C). The molar sections were H&E stained ($n = 6$ for all groups except $n = 4$ for Hu-Fc, 400× magnification). Statistical significance *p-value ≤ 0.05; ▼$p \leq 0.01$. D) Diagrammatic representation of landmarks and areas used for the histomorphometric analysis and cell apoptosis counts. Areas (in purple dashed line boxes) of interest are represented by Box 1, Box 2 and Box 3. Distance (in red arrow) of Epithelial down-growth is represented by "a", while the region of alveolar bone loss by "b". Ep = epithelial tissue; PDL = periodontal ligament.

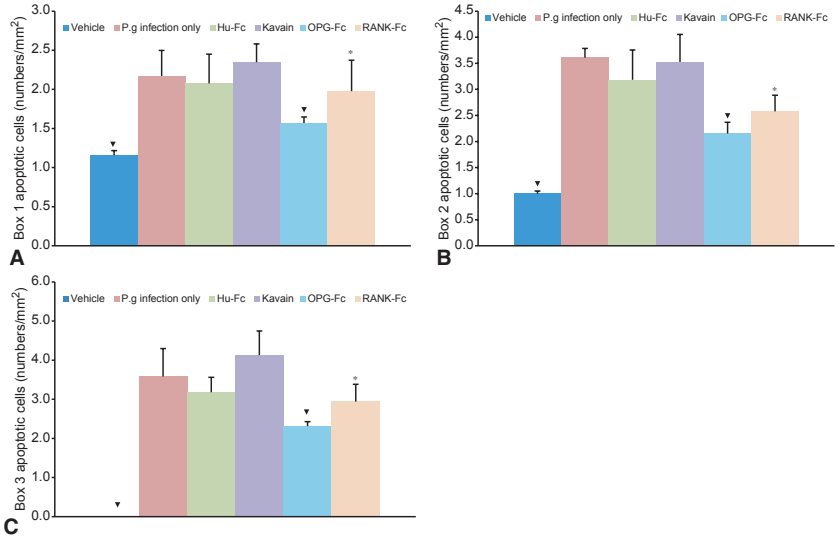

Fig. 3. Histomorphometric analysis of regional cell apoptosis. Using the diagrammatic representation as a template, the three regions (A) Box 1, (B) Box 2, (C) Box 3 for the six groups were analysed for apoptotic cell using TUNEL assay and normalized by total inflammatory cell ($n = 6$ for all groups except $n = 4$ for Hu-Fc). Statistical significance $*p < 0.05$; $\blacktriangledown p \leq 0.01$.

OPG-Fc-treated animals displayed higher levels of IL-6 than those in the Kavain or RANK-Fc-treated group on day 7, but when compared with positive control, expression was reduced by 47% ($p < 0.01$). Similarly, Kavain and RANK-Fc reduced expression by 80% and 86% respectively (Fig. 4A). MCP-1 expression was significantly reduced by Kavain (75%), OPG-Fc (25%) and RANK-Fc (80%) on day 7 (Fig. 4B, $p < 0.05$). TNF-α expression (Fig. 4C) mirrored IL-6 expression profile with OPG-Fc group (23%, $p < 0.05$) displaying higher expression than Kavain (81%) and RANK-Fc (90%) groups ($p < 0.01$). Surprisingly, TNF-α expression increased once again on day 10 in the Kavain-treated group (Fig. 4C). Hu-Fc-injected group always displayed increased pro-inflammatory cytokine expressions compared with Kavain, OPG-Fc and RANK-Fc-treated groups, except for TNF-α on day 7 for OPG-Fc. Vehicle-treated group constantly showed baseline expression throughout the experiment for all days. OPG-Fc and RANK-Fc groups demonstrated significance when compared with each other on day 7.

Discussion

P. gingivalis-induced murine experimental periodontitis offers a reliable model with site-specific, time-dependent alveolar bone loss, epithelial down growth, inflammatory cell infiltrates and cytokine expressions. The osteoclastic activity observed in

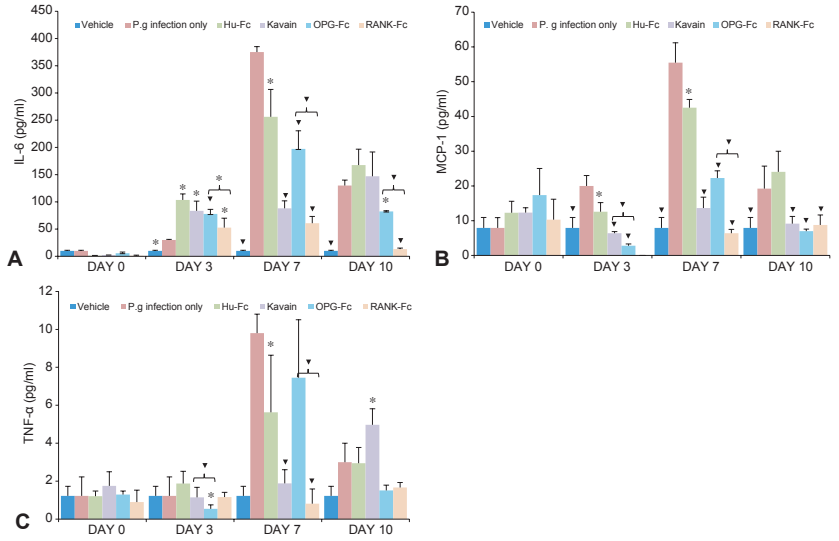

Fig. 4. Detection of serum inflammatory cytokine expressions at different treatment time points. The circulatory pro-inflammatory cytokines during the time course were analysed with Bioplex Luminex assay plates. IL-6 (panel A), MCP1 (panel B) and TNF-α (panel C) were detected at days 0, 3, 7 and 10 from the six groups (vehicle, *P. gingivalis* infection only, Hu-Fc, Kavain, OPG-Fc and RANK-Fc). ($n = 6$ for all groups except $n = 4$ for Hu-Fc). Statistical significance $*p \leq 0.05$; $\blacktriangledown p \leq 0.01$.

the model, which has been used by our laboratory with reasonable success is consistent with the progression of an inflammatory front comparable with human periodontal pathogenesis (Amar et al. 2007, Li & Amar 2007). The purpose of this study was to assess the role of anti-bone resorption agents and an anti-inflammatory compound in the course of murine *P. gingivalis*-induced experimental periodontitis.

Our data demonstrate that treatments with RANKL inhibitors or Kavain significantly reduced experimental periodontal bone loss with OPG-Fc performing better than RANK-Fc and Kavain. Indeed, both macroscopical (alveolar bone levels) and microscopical (TRAP, epithelial down-growth) data concurred with these findings. These observations are consistent with a previous report using OPG only, in experimental periodontitis (Jin et al. 2007). Regarding the use of a RANKL specific blocker, recent studies confirmed that RANK-Fc/OPG-Fc treatments in *Staphylococcus aureus*-induced arthritic model exhibited similar bone protection for trabecular bone and mineral density identical to that of naïve, uninfected animals (Verdrengh et al. 2010). Notably, the treatment improved the strength and density of the cortical bone and inhibition of RANKL signalling efficiently prevented bone loss in the mouse model of bacterial arthritis (Ominsky et al. 2008). All together, our data provide evidence regarding the benefit of RANKL blockers in the prevention of experimental periodontal bone loss and to our knowledge this is the first report on the role of Kavain in preventing bone loss.

An additional aim of our study was to evaluate the role of OPG, RANK and Kavain in the management of inflammatory lesions, given that an important feature of periodontitis is the inflammatory lesion preceding temporo-spatially bone loss. The use of well-defined boxes helped capture the progression of inflammatory cell counts which showed gradual decrease, while steady increase for fibroblasts from superficial to deeper structures respectively. In all three regions, OPG-Fc, RANK-Fc and Kavain treatments significantly reduced the inflammatory infiltrate equally affecting PMN and mononuclear cells. RANK-Fc displayed maximum effect, followed by OPG-Fc and Kavain. Furthermore, OPG-Fc treatment showed a significant increase in fibroblast counts for (11%) possibly due to a positive role of OPG on apoptosis as shown in the present study or on a cell proliferation. Altogether the present data support a beneficial role of these compounds in reduction of inflammation.

Previous studies have reported upregulated inflammatory cytokines in the serum of human periodontal disease patients as well as in experimental animal models (D'Aiuto et al. 2004, Ekuni et al. 2010, Passoja et al. 2010, Dias et al. 2011, Oz and Puleo 2011). Our data concord with previous studies and show maximum expression of pro-inflammatory cytokines of IL-6, TNF-α and MCP-1 on day 7 and a return to lower levels at day 10 mostly due to the fading bacterial stimulation. Congruent with our inflammatory cell infiltrate data, RANK-Fc treatment led to the most robust reduction of all cytokine levels advocating for RANK-Fc as an important anti-inflammatory agent compared with OPG-Fc and Kavain. All treatment groups showed a downregulation of inflammatory cytokines except for OPG-Fc. Indeed a previous report using OPG-Fc in a rat model to treat rheumatoid arthritis reported a lack of reduction of cytokines in these animals (Neumann et al. 2006). The present data warrant for further studies to substantiate this finding but in the meantime, caution must be exercised with the use of OPG-Fc if the goal is to reduce cytokine levels in addition to preventing bone loss. Kavain treatment had modest cytokine response and placed itself between RANK-Fc and OPG-Fc. Although day-10 cytokine levels were found elevated for Kavain, the shorter half life of Kavain (Mathews et al. 2005) and the lack of treatment on day 10 may partially explain this result.

Regarding apoptosis, maximum reduction was seen in OPG-Fc-treated animals. This is supported by the fact that OPG is a survival factor and exerts its effect by engaging tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and preventing it from binding to the apoptosis receptors DR4 and DR5 (Emery et al. 1998). Intermediate levels of apoptosis were observed with RANK-Fc-treated animals advocating for a balance between pro and anti-apoptosis action. For Kavain-treated animals, apoptosis counts were greater than the two other antagonists possibly to provide for a higher clearance of affected cells. Furthermore, Kavain treatment also showed a decrease in fibroblasts counts (26%) in contrast with OPG-Fc treatment which showed a significant increase (11%). Very little is known on Kavain effect on apoptosis or cell proliferation in connective tissue fibroblasts. A recent report demonstrated an effect of Kava extract and flavokavains on apoptosis and cell proliferation (Zi & Simoneau 2005) strongly supporting our findings.

It is important to notice that, although OPG-Fc emerged as the best option for prevention of bone loss, RANK-Fc treatment improved both alveolar bone loss and inflammation. Thus, if the goal of periodontal treatment is to prevent bone resorption with little consideration to the inflammatory process, the therapeutic choice would be OPG-Fc. However, our experimental data may not be capturing maintenance of alveolar bone levels over time and a consensus for the current literature strongly supports the requirement of a tight control of the inflammatory process to successfully maintain alveolar bone levels. Therefore our first therapeutic choice would be RANK-Fc followed by Kavain to reduce both bone resorption and inflammation.

It is important to notice that only a single dose of each therapeutic agent was tested. Although a significant improvement of all bone and inflammation parameters was achieved, future studies should include a dose-response arm to identify the optimal dosage and provide a deeper analysis of the effectiveness of Kavain versus OPG-Fc or RANKL-Fc.

Regarding the safety issue in using these blockers, the following observations can be made. RANK-RANKL inhibitors have shown little or no potential interference with the overall bone metabolism. Furthermore and somewhat incongruous with our findings, RANK-Fc use

was reported to have almost undetectable effect on innate or adaptive immune response when administered at concentrations effective in modulating bone metabolism (Miller et al. 2007). A possible explanation for this discrepancy may be that, in this report, a single dose of infection with a viral agent was used while our study employed multiple infections with a bacterial agent. OPG treatments in mice do not affect cell-mediated reactions or liver damage. However, OPG has modest immunoregulatory effects that seem to be confined to humoral response to specific antigen a finding supported by previous studies (Stolina et al. 2003 and Neumann et al. 2006) and confirmed by the present data. Safety reports on use of Kavain in infectious diseases are scarce, but case reports exist on its use in treatment of gonorrhoea, rheumatism, bronchitis, asthma, as well as stomach aches and headaches. Therefore, further safety studies using Kavain are warranted.

While our data do not show that the blockers have any effects on the impairment of infection and bacterial burden, caution must be exercised as to the excessive dampening of inflammatory/immune response that could generate the emergence of opportunistic superinfection as observed with the other cytokine blockers (Scheinfeld 2004).

In conclusion, treatment with RANKL inhibitors or Kavain effectively reduce alveolar bone loss and inflammation in *P. gingivalis*-induced periodontitis in mice model and offer a novel therapeutic indication in the prevention of periodontal bone loss.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Material and Methods.

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Clinical Relevance

Scientific rationale for the study: Periodontal disease presents itself by way of a wide range of clinical variability and severity, all showing two essential components: (i) inflammation, (ii) bone loss.

Principle findings: RANKL blockers and Kavain proved effective in reducing bone loss and inflammation in experimental periodontal model in mice.

Practical implications: This study aims to assess the role of anti-bone resorption agents and an anti-inflammatory compound for developing therapeutic treatment modalities in periodontal disease.