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Short communication

The gingipains from *Porphyromonas gingivalis* do not directly induce osteoclast differentiation in primary mouse bone marrow cultures

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Background and Objective: Porphyromonas gingivalis is a major aetiological agent in the development of periodontitis, the major clinical hallmark of which is bone resorption. The cysteine proteases (gingipains) produced by *P. gingivalis* have a critical role in the pathogenesis of the disease, and previous studies on whole bacteria have implicated these enzymes in osteoclastogenesis, a process which serves to upregulate bone resorption. The effects of the gingipains from *P. gingivalis* on osteoclast differentiation were investigated here to determine whether the enzymes directly contribute to osteoclastogenesis and thus to bone resorption.

Material and Methods: The effects of the gingipains on osteoclast differentiation were investigated in primary mouse bone marrow cultures. The cultures harvested from C57BL6/J mice were incubated in the presence of parathyroid hormone, a known osteoclastogenic factor, or active/inactivated forms of three gingipains. Osteoclast differentiation was quantified by counting the number of multinucleated cells positive for tartrate-resistant acid phosphatase, an enzyme marker for these cells.

Results: After 10 days of culture, the gingipains, either active or inactive, failed to stimulate osteoclast differentiation in comparison to the parathyroid hormone.

Conclusion: The data presented here demonstrate that the gingipains do not induce osteoclast differentiation in this system, indicating that the bacterium uses other mechanisms to induce bone loss.

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Periodontitis is a chronic inflammatory disease characterized by soft tissue destruction and bone resorption of the supporting structures of the teeth. Severe periodontal disease is most commonly associated with the presence of a 'red complex' of bacteria (*Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*) within the periodontium (1), with *P. gingivalis* being identified as a major causative agent (2). *Porphyromonas*

gingivalis has a variety of virulence factors, including the gingipain cysteine proteases (3–5), two of which are arginine specific (HRgpA and RgpB) and one lysine specific (Kgp; 3). Both HRgpA and Kgp are high molecular weight complexes, consisting of the catalytic domains non-covalently associated with adhesin subunits, while RgpB consists of a catalytic domain and a small immunoglobulin-like domain (6). The gingipains have a wide variety of pathogenic activities within the host (7).

Alveolar bone resorption, a major clinical characteristic of periodontal disease, is regulated by a system comprised of the receptor activator for nuclear factor κB ligand (RANKL), a tumour necrosis factor family molecule, its decoy receptor osteoprotegerin and the receptor activator for nuclear factor κB (RANK; 8,9). The RANKL is expressed on osteoblast/stromal cells (8), and interaction between RANKL and RANK, which is expressed on osteoclasts, is essential for induction of osteoclastogenesis and the differentiation of osteoclasts from progenitor cells into mature multi-nucleated bone-resorbing cells (8,9).

Infection of murine osteoblasts with virulent *P. gingivalis* cells was found to induce the expression of RANKL, in contrast to infection with a gingipain-deficient *P. gingivalis* mutant (10). Similarly, sonicates from *P. gingivalis*, *T. denticola* and *T. socranskii* induced osteoclast formation in a coculture system of mouse calvaria-derived osteoblasts and bone marrow cells via a RANKL-dependent pathway (11).

In the present study, the effects of the gingipains on osteoclast differentiation were investigated in mouse bone marrow cultures. The cultures, harvested from C57BL6/J mice essentially as previously described (12), were incubated in the presence of human parathyroid hormone fragment (PTH fragment 1–34; Sigma, Sydney, NSW, Australia), a known osteoclastogenic factor, or active/inactivated forms of HRgpA, Kgp or RgpB, isolated as described previously (3). Osteoclast differentiation was quantified at the end of 10 days of culturing by counting the number of multinucleated cells positive for tartrate-resistant acid phosphatase (TRAP; Fig. 1). After 10 days of culture, PTH caused significant stimulation of osteoclast differentiation compared with medium alone. In contrast, HRgpA, RgpB and Kgp, either active or inactive, did not stimulate osteoclast differentiation. Low numbers of multinucleated TRAP-positive cells were observed in murine bone marrow cells treated with the gingipains and, when compared with media alone, no significant difference was found.

Okahashi *et al.* (10) showed that mouse primary osteoblasts produced RANKL, an osteoclastogenic cytokine, when infected with viable *P. gingivalis* cells but not with a gingipain-deficient strain, suggesting that the gingipains had a direct role in inducing RANKL responses in osteoblasts. Although we have shown that the gingipains do not directly induce osteoclast differentiation in mouse bone marrow cultures, it is possible that the gingipains in the periodontal environment contribute indirectly to osteoclast differentiation by activating other haemopoietically derived cells, such as T cells, B cells, neutrophils or macrophages, to release osteoclastogenic factors to mediate bone resorption. Recently, a link between T cell activation and bone resorption has been identified as being a subset of T-helper cells (Th17) that produce interleukin-17 (IL-17; 13), a T cell-derived pro-inflammatory cytokine that potently stimulates osteoclastogenesis (14). It was proposed that Th17 cells facilitate osteoclastogenesis through IL-17-mediated induction of RANKL on osteoblastic cells (13). The cellular source of RANKL in periodontal lesions has been identified as being activated T and B cells (15). Also, activation of Protease-Activated Receptors-2 (PAR-2) on oral epithelial cells induces the production of a pro-inflammatory cytokine, interleukin-6, which is a potent stimulator of osteoclast differentiation and bone resorption (16), and the activation of this receptor has been shown to be crucial to the bone resorption seen in this disease (17). Since the present study has demonstrated that the gingipains from *P. gingivalis* do not directly cause osteoclast differentiation, it is likely that the mechanisms used by the bacterium to elicit bone destruction are more complex than the previously envisaged direct effect of the gingipains on bone-derived cells (10) and might involve other molecules from *P. gingivalis*, such as lipopolysaccharide, inducing responses from key cells, such as macrophages, to cause the bone destruction associated with periodontal disease (18).

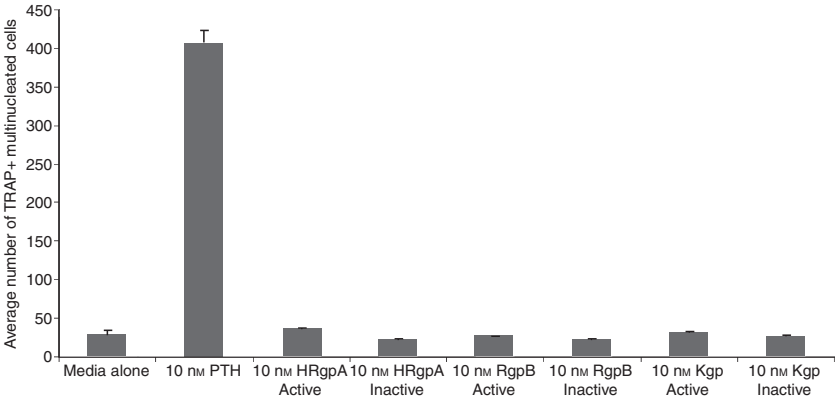

Fig. 1. Effects of the gingipains (RgpB, HRgpA or Kgp) on the number of TRAP-positive (TRAP+) multinucleated cells formed per well in mouse bone marrow cultures isolated from C57BL6/J mice. Mouse bone marrow cells were seeded in 24-well plates at 2×10^6 cells/mL (0.5 mL per well) and cultured in α-minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin for 10 days in the presence of 10 nM PTH, 10 nM HRgpA, 10 nM Phe-Pro-Arg-Chloromethylketone (PPACK)-treated HRgpA, 10 nM RgpB, 10 nM PPACK-treated RgpB, 10 nM Kgp and 10 nM PPACK-treated Kgp. Data are expressed as means ± SEM ($n \geq 5$).

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