Role of *Porphyromonas gingivalis*-Derived Fibroblast-Activating Factor in Bone Resorption

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A 24-kDa protein was isolated from the outer membrane vesicles of *Porphyromonas gingivalis* W50. This protein, referred to as fibroblast-activating factor (FAF), was examined for its bone-resorptive ability by the rat long-bone assay and the mouse bone marrow cell culture system. FAF resulted in a significant release of 45Ca from cultured bones, as well as the formation of tartrate-resistant acid phosphatase-positive monocytes.

These cultures were compared with control cells. FAF therefore might be considered a significant bacterially expressed protein which could affect and modulate the resorption or destruction of tissue and alveolar bone in the local periodontal environment.

It is well known that periodontal diseases are mediated by a selected group of bacteria resident in the subgingival microbiota (15–17). *Porphyromonas gingivalis* appears to be a major participant in the progression of the inflammatory events of periodontal disease. Since human gingival fibroblasts are major nonimmune cells of the periodontium, any adverse effect of the resident or pathogenic microbiota or their component parts (i.e., lipopolysaccharides [LPS], peptidoglycan, and outer membrane protein) on the human gingival fibroblasts could have a significant effect on gingival homeostasis.

In a previous investigation, Mihara and Holt (9) isolated and characterized a *P. gingivalis* outer membrane vesicle-associated protein which was biologically active for fibroblasts. This protein, with a relative molecular mass of 24 kDa, was referred to by Mihara and Holt (9) as fibroblast-activating factor (FAF). FAF was very active in human gingival fibroblast proliferation and in protein synthesis and displayed a similarity in function to several human-derived growth factors (10). Since progressing periodontitis is a result of the loss of the structural integrity of the connective tissue and the detachment of this tissue from the tooth matrix, one function of FAF might be to actively modulate connective tissue synthesis in inflamed and infected sites. In addition to the destruction of the connective tissue of the periodontium, the resorption or destruction of alveolar bone is a common outcome of periodontal disease progression. Therefore, in this study we examined the possibility that FAF plays a role in bone resorption in addition to having fibroblast-modulating effects.

FAF was isolated and purified from *P. gingivalis* W50 as described by Mihara and Holt (9). The purified FAF used in the bone resorption experiments described here enhanced [3H]thymidine uptake in human gingival fibroblasts by >400% compared with that by the control (data not shown).

Bone resorption and osteoclast formation were determined by the 45Ca-fetal rat long-bone assay (14) and the mouse bone marrow cell culture system (18), respectively. For bone resorption, pregnant rats were injected with 200 μCi of 45Ca (ICN Radiochemical, Irvine, Calif.) on the 18th day of gestation. Approximately 24 h later, the fetuses were removed and the mineralized shafts of the radii and ulnae were dissected free from the cartilaginous ends and placed in organ culture. After a 24-h preculture period to allow for the exchange of loosely bound 45Ca with stable calcium in the medium, FAF was added to the organ cultures, and incubation was continued. All affected cultures were compared with the control (minus FAF). At 120 h, there was a significant release of 45Ca (*P < 0.01*) compared with that by the control (Table 1). While the bone-resorptive ability of FAF was approximately one-half that of parathyroid hormone, it did result in the release of almost 50% 45Ca from the rat long bone (Table 1). While previous reports have indicated that the *P. gingivalis* LPS is a predominant bone-resorbing macromolecule (7, 11), silver staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of the FAF, chemical assay for specific LPS components, and heating (100°C, 15 min) were negative (data not shown). Since heating of FAF at 100°C for 15 min resulted in almost complete abrogation of FAF bone-resorbing activity (Table 1), the presence of contaminating LPS is not indicated.

Osteoclastic cells have been previously recognized as primary cells involved in bone-resorptive events (13). Since the osteoclast is believed to arise by monocytic fusion (19), any factor or event which has the potential to modify or alter

### Table 1. Effect of FAF on rat long-bone resorption

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Conc (μg/ml)</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no addition)</td>
<td>23 ± 1</td>
<td></td>
</tr>
<tr>
<td>FAF</td>
<td>0.75</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>FAF</td>
<td>1.5</td>
<td>47 ± 3b</td>
</tr>
<tr>
<td>Heated FAFa</td>
<td>1.5</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>PTH</td>
<td>0.05</td>
<td>94 ± 1</td>
</tr>
</tbody>
</table>

*All values were expressed as percent release of 45Ca from rat long bone into culture supernatant after 120 h of incubation. Results are means ± standard errors and are from three experiments run in duplicate.

a Significantly different from control at a *P* of <0.01.

b FAF was heated at 100°C for 15 min.

c PTH, parathyroid hormone.
TABLE 2. Effect of P. gingivalis W50 FAF on formation of TRAP(+)MNC$^a$

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Conc of FAF (µg/ml)</th>
<th>No. of TRAP(+)MNC$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no addition)</td>
<td>0 ± 0</td>
<td>326.7 ± 31.8</td>
</tr>
<tr>
<td>1,25D$_3$</td>
<td>0.15</td>
<td>268.0 ± 46.3$^c$</td>
</tr>
<tr>
<td>1,25D$_3$ + FAF</td>
<td>0.75</td>
<td>342.7 ± 58.6$^d$</td>
</tr>
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</table>

$^a$ Mouse bone marrow cell cultures were incubated for 6 days with 0.15 and 0.75 µg of FAF per ml in the presence of 1,25D$_3$.

$^b$ All values reveal the number of TRAP-positive cells in mouse bone marrow cell cultures (means ± standard errors). FAF alone (minus 1,25D$_3$) was negative for TRAP-positive cells. Results are from triplicate experiments run twice.

$^c$ Significant difference was determined at a P of <0.01.

$^d$ Significant difference was determined at a P of <0.05.

this differentiation should have a significant effect on bone resorption. For osteoclast studies, the modified murine marrow cell culture system developed by Takahashi et al. (18) was used. Femurs and tibiae of 4- to 6-week-old male ICR Swiss mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were removed aseptically and dissected free of adherent tissues. Marrow cells were collected from three to four mice by flushing the marrow cavity with a 27 G needle. The cells were washed twice with minimal Eagle’s medium and resuspended at a final concentration of 4 × 10$^6$ cells per ml in minimal Eagle’s medium supplemented with 10% fetal bovine serum (Hazleton, Lenexa, Kans.) without antibiotics. A 0.5-ml volume of this cell suspension was inoculated onto 24-well plates (Corning, New York, N.Y.) and stimulated with FAF at final concentrations of 0, 0.15, and 0.75 µg/ml in the presence of 10$^{-8}$ M 1,25-dihydroxyvitamin D$_3$ (1,25D$_3$) (18). Cultures were maintained at 37°C in humidified air. The cells were fed every 2 days with 0.3 ml of fresh minimal Eagle’s medium including FAF, 10% fetal bovine serum, and 1,25D$_3$. After 6 days, the cultures were washed in phosphate-buffered saline and fixed in 60% acetone in citrate buffer (pH 5.4) for 30 s. The plates were air dried and stained for tartrate-resistant acid phosphatase (TRAP) with a commercially available kit (Sigma Chemical Co., St. Louis, Mo.). Stained cultures were examined by light microscopy at ×40 magnification. TRAP-positive (red-staining) multinucleated cells (with five or more nuclei) [TRAP(+)MNC] were easily distinguished from other cells. All TRAP(+)MNC in each well were counted manually by scanning across the entire well in a systematic fashion (Table 2). 1,25D$_3$ significantly increased the number of TRAP(+)MNC in the marrow cell cultures at 6 days of incubation (Fig. 1). The addition of 1.5 µg of FAF per ml to the 1,25D$_3$ resulted in an almost 35% increase in TRAP(+)MNC, suggesting that the bone-resorptive ability of FAF might be caused in part by inducing differentiation from monocytes to osteoclasts.

Many of the surface macromolecules of both gram-positive and gram-negative bacteria have been found to induce in vitro bone resorption. Holt and Bramanti (6) have reviewed the reports on the role of the LPS from oral bacteria in bone resorption. Hausmann et al. (5) have provided an early observation on the role of LPS (endotoxin) in in vitro bone resorption. Hausmann et al. (3, 4) and Lenigraf et al. (8) have also demonstrated that lipoteichoic acids and peptidoglycans (3) are capable of resorbing bone in the rat calvarial assay. Muramyl dipeptides (1), outer membranes and isolated lipoproteins (11), and capsules from several of the gram-negative oral periodontopathogens have also been previously shown to resorb bone (19). However, while all of these
prokaryotic surface molecules are capable of in vitro bone resorption, the mechanisms by which they might accomplish this in vivo remain unknown. Since a direct interaction between these molecules and bone-regulatory cells might be a rare event, these bacterially derived components might function in the activities of a host-derived mediator which can then activate and/or replicate bone-resorptive activity. LPS, for example, has been previously shown to be capable of enhancing bone resorption by inducing osteoclast-activating factor (12), which was found to be identical with interleukin-1β, or prostaglandins (2). Therefore, the role of P. gingivalis FAF as a second messenger for bone-resorptive activity as well as a modulator of connective tissue structure by affecting cell proliferation, differentiation, and protein synthesis must be determined.

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