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Porphyromonas gingivalis Fimbria-Stimulated Bone Resorption Is Inhibited through Binding of the Fimbriae to Fibronectin

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Our most recent study demonstrated that fibronectin is one of the *Porphyromonas gingivalis* fimbria-binding proteins. In this present study, we demonstrate with mouse embryonic calvarial cells that *P. gingivalis* fimbria-stimulated bone resorption is inhibited by human fibronectin. The fibronectin inhibition was dose and culture time dependent and was completely neutralized by antifibronectin antibody. The inhibitory action of fibronectin depended on fimbrial interaction with the heparin-binding and cell-attachment domains in the fibronectin structure.

An important first stage in bacterial colonization is adherence of the bacterium to the host cells. In general, although it is well known that the bacterial cell surface components play an important role in adherence, many studies (8, 11, 13, 16, 17, 19–24) have shown that extracellular matrix proteins such as collagen, fibronectin, and laminin are able to bind to a variety of bacteria via various components on the bacterial surface.

Many investigators (9, 12, 14, 15, 18) have also demonstrated the ability of several extracellular matrix proteins to bind to periodontopathic organisms. An earlier study of ours (5) showed that *Porphyromonas gingivalis*, a predominant pathogen in periodontal disease, is able to bind to host cells via its fimbriae. Furthermore, we more recently demonstrated that fimbrillin, the major component of the fimbriae, is one of the fibronectin-binding proteins (14). These studies suggested to us that the matrix proteins function as a modulator in the pathogenesis of the organism via the fimbriae, because the binding of the bacterial cells to host cells is modulated by the interaction of fimbrillin with fibronectin.

On the other hand, recent interesting studies (3, 4) have demonstrated in vivo the pathogenic role of *P. gingivalis* fimbriae as a virulence factor in alveolar bone loss in periodontal disease. We (6, 10) also showed in vitro that the fimbriae function as a potent stimulator of bone resorption. Therefore, it is very important for understanding the pathogenic mechanism(s) of the organism to examine whether fibronectin actually functions as a modulator of the fimbria-stimulated bone resorption through its binding affinity. We demonstrate here that *P. gingivalis* fimbria-stimulated bone resorption is inhibited by fibronectin through its binding to heparin-binding and cell-attachment domains in the fibronectin structure.

Human fibronectin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Heparin-binding, gelatin-binding, and cell-attachment domains of human fibronectin and their respective monoclonal antibodies were obtained from Gibco BRL (Gaithersburg, Md.).

P. gingivalis ATCC 33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura et al. (25) as described previously (5). We showed earlier (7, 10) that the purified fimbria-induced biological activities could not be attributed to lipopolysaccharide contaminants in the preparation. Protein content of the fimbriae was measured by the method of Bradford (2).

The bone resorption assay used was described in detail in a previous paper (1). In brief, ICR mouse embryos at the age of 14 days (CLEA Japan, Tokyo, Japan) were dissected and their calvariae were harvested. The calvariae were rinsed and then digested at room temperature for 30 min in 10 ml of phosphate-buffered saline (pH 7.2) containing 0.1% bacterial collagenase (Sigma), 0.05% trypsin (Difco Laboratories, Detroit, Mich.), and 4 mM EDTA. The digested calvarial cells were washed three times with α-Eagle's minimum essential medium (α-MEM; Flow Laboratories, McLean, Va.) and then placed at a cell density of 5×10^5 cells/15 μl of a dentin slice (4 by 4 mm) in each well of a 24-well flat-type Falcon plastic plate. Then, after the calvarial cells had been incubated for 60 min in 10% fetal calf serum (FCS) (Flow Laboratories)-containing α-MEM, they were cultured for the desired times in 1 ml of α-MEM with or without the fimbriae or test samples. At selected times, the slices were scraped with a rubber policeman to remove cells to enable visualization of the dentinal surface and then washed with distilled water. The washed dentin slices were dehydrated with ethanol and sputter coated with gold. For evidence of osteoclastic bone resorption, the bone slices were examined with a T 200 electron scanning microscope (Japan Electronics Co., Tokyo, Japan). The number of excavations (pits) in the slices was also determined. Results were expressed as means ± standard errors (SE) of quadruplicate cultures. The significance of differences was examined by Student's *t* test.

Tartrate-resistant acid phosphatase (TRAP)-positive cells were detected as follows. The calvarial cells on dentin slices were fixed for 1 min with 10% formalin-ethanol after their cultivation for the desired times. The fixed cells were washed with distilled water and incubated for TRAP staining for 60 min at room temperature in Michaelis Veronal acetate buffer (pH 5.0) containing naphthol AS-BI phosphate (Sigma) as substrate, hexazonium pararosanilin as coupler, and 20 mM L-(+)-tartaric acid, as described previously (1). TRAP-positive cells were counted under a light microscope. Results were expressed as the means ± SE of quadruplicate cultures.

Firstly, we examined the effect of fibronectin on the fimbria-stimulated bone resorption. The fimbriae were pretreated for 2 h with fibronectin and then added to embryonic calvarial cells

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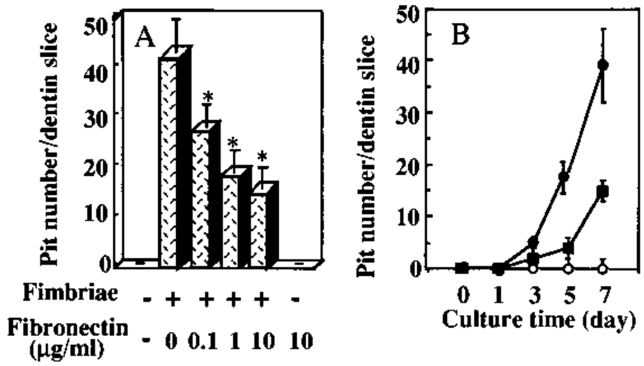

FIG. 1. Inhibitory effect of fibronectin on *P. gingivalis* fimbria-stimulated bone resorption. (A) Calvarial cells (5×10^5 cells per dentin slice) prepared from 14-day-old mouse embryos were inoculated onto dentin slices and incubated in 10% FCS-containing α-MEM in the absence or presence of *P. gingivalis* fimbriae (4 μg of protein/ml) pretreated or not with fibronectin at the indicated dose. After 7 days, pit number was measured. (B) Calvarial cells as described above were inoculated onto dentin slices and incubated in 10% FCS-containing α-MEM in the absence (○) or presence of *P. gingivalis* fimbriae (4 μg of protein/ml) pretreated (■) or not (●) with fibronectin (10 ng/ml). At the indicated times after the initiation of their treatment, the number of resorption pits was measured. The results are expressed as the means ± SE of quadruplicate cultures. *, $P < 0.01$ (versus the fimbria-treated group).

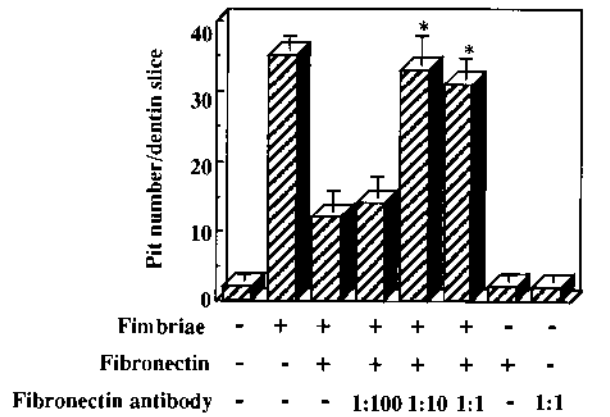

FIG. 2. Fibronectin inhibition of *P. gingivalis* fimbria-stimulated bone resorption is neutralized by fibronectin antibody. Calvarial cells (5×10^5 cells per dentin slice) prepared from 14-day-old mouse embryos were inoculated onto dentin slices and incubated in 10% FCS-containing α-MEM in the absence or presence of *P. gingivalis* fimbriae (4 μg of protein/ml) pretreated or not with fibronectin (10 ng/ml) that had been preincubated or not with antifibronectin antibody. After 7 days, the number of resorption pits was measured. The results are expressed as the means ± SE of quadruplicate cultures. *, $P < 0.01$ (versus the fibronectin-treated fimbria group).

on dentin slices. The bone-resorbing activity was measured at 7 days after the initiation of the culture. As shown in Fig. 1A, fibronectin inhibited the fimbria-stimulated bone resorption in a dose-dependent manner. Such inhibitory action was paralleled by a reduction in the number of cells positive for TRAP, a marker enzyme of osteoclasts (data not shown). Next we examined the kinetics of fibronectin inhibition of the fimbria-stimulated bone resorption by the calvarial cells. Fibronectin-pretreated fimbriae were added to the cell cultures on the dentin slices. After incubation for the indicated times, pit number and TRAP-positive cells were measured. As shown in Fig. 1B, fibronectin inhibited the fimbria-stimulated bone resorption in a culture time-dependent fashion. The same inhibition kinetics were also observed for TRAP-positive cells (data not shown).

To verify the specificity of the inhibitory action of fibronectin toward the fimbria-stimulated bone resorption, we examined whether the inhibition could be neutralized by anti-human fibronectin antibody. The fimbriae were treated with fibronectin that had been pretreated with fibronectin antibody and then were added to the calvarial cells on dentin slices. As shown in Fig. 2, the marked inhibitory action of fibronectin toward the fimbria-stimulated bone resorption was completely neutralized by the pretreatment with fibronectin antibody. These results strongly showed the specificity of fibronectin for the inhibition of the fimbria-stimulated bone resorption.

The fibronectin structure consists of heparin-binding, gelatin-binding, and cell-attachment domains. Therefore, we next examined which domain is involved in the inhibitory action of the matrix protein toward the fimbria-stimulated bone resorption. The fimbriae were separately pretreated with each fragment of fibronectin, and then the bone-resorbing activity was examined. The fimbria-stimulated bone resorption was significantly inhibited by pretreatment with heparin-binding or cell-attachment domains but was not affected when the gelatin-binding domain was used for pretreatment (Fig. 3). Therefore, finally, by using monoclonal antibodies to each domain, we verified the involvement of the above two domains in fibronectin inhibition of the fimbria-stimulated bone resorption. The fimbriae were treated with fibronectin that had been pretreated separately with monoclonal antibodies to each domain, and then the fimbria-stimulated bone resorption was assayed. Figure 4 shows that the inhibitory action of fibronectin toward the fimbria-stimulated bone resorption was significantly neutralized by pretreatment with antibodies to heparin-binding and cell-attachment domains. These data indicate that these two domains in the fibronectin structure play an important role in the inhibitory action of this matrix protein toward the fimbria-stimulated bone resorption.

Therefore, we showed here that fibronectin inhibited the fimbria-stimulated bone resorption that was measured as an increase in the number of the resorption pits. This inhibition may have resulted from inhibition of interleukin 1β and granulocyte-macrophage colony-stimulating factor expression in mouse embryonic calvarial cells by the fibronectin, because

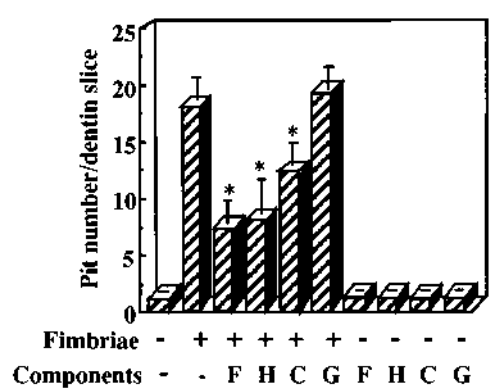

FIG. 3. Heparin-binding and cell-attachment domains are involved in fibronectin inhibition of the fimbria-stimulated bone resorption. Calvarial cells (5×10^5 cells per dentin slice) prepared from 14-day-old mouse embryos were inoculated onto dentin slices and incubated in 10% FCS-containing α-MEM in the absence or presence of *P. gingivalis* fimbriae (4 μg of protein/ml) pretreated or not with each domain (10 ng/ml). After 7 days, the number of resorption pits was measured. The results are expressed as the means ± SE of quadruplicate cultures. *, $P < 0.01$ (versus the fimbria-treated group). F, fibronectin; H, heparin-binding domain; C, cell-attachment domain; G, gelatin binding domain.

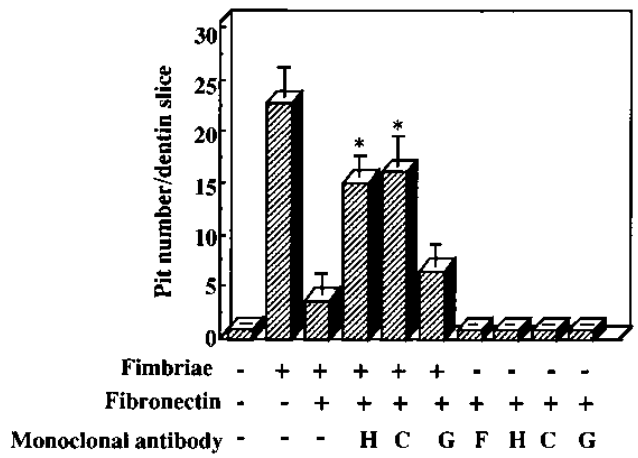

FIG. 4. Monoclonal antibodies against heparin-binding and cell-attachment domains neutralize fibronectin inhibition of the fimbria-stimulated bone resorption. Calvarial cells (5×10^5 cells per dentin slice) prepared from 14-day-old mouse embryos were inoculated onto dentin slices and incubated in 10% FCS-containing α-MEM in the absence or presence of *P. gingivalis* fimbriae (4 μg of protein/ml) pretreated or not with fibronectin (10 ng/ml) that had been incubated for 2 h with or without each monoclonal antibody. After 7 days, the number of resorption pits was measured. The results are expressed as the means $\pm$ SE of quadruplicate cultures. *, $P < 0.01$ (versus fibronectin-treated fimbria group). F, fibronectin; H, heparin-binding domain; C, cell-attachment domain; G, gelatin binding domain.

these cytokines are the predominant ones involved in bone resorption, as described previously (10). It is known that *P. gingivalis* is able to bind extracellular matrix proteins such as collagen, fibronectin, and laminin (9, 12, 14, 15, 18). However, as shown in our previous study (14), the fimbriae did not bind to collagen type I or V or to laminin. Therefore, the specificity of the fibronectin inhibition via binding to the fimbriae with high affinity may be a very significant factor in the regulation of *P. gingivalis* infection.

It is of interest to develop an antagonist that is able to neutralize *P. gingivalis* fimbria-mediated cell binding and pathogenesis. In this regard, it becomes important to understand the fimbria-binding domain in fibronectin structure, because, if the amino acid sequence of the essential binding domain is determined, it may then be possible to develop an antagonist to neutralize fimbria-mediated cell binding and pathogenesis. Our previous study (14) suggested that the inhibitory action of fibronectin toward the expression of a fimbria-induced cytokine gene depended on the interaction between the fimbriae and heparin-binding and cell-attachment domains in the fibronectin structure. This suggestion was confirmed by the present observations that the fimbria-stimulated bone resorption actually is inhibited by both domains. These findings suggest to us an important approach to develop an antagonist that can neutralize the fimbria-mediated pathogenesis in periodontal disease.

In summary, we have demonstrated that the extracellular matrix protein fibronectin is inhibitory toward *P. gingivalis* fimbria-stimulated bone resorption in vitro.

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