BACTERIAL PATHOGENESIS

Enhancement of hepatocyte growth factor (scatter factor) production by human gingival fibroblasts in culture stimulated with *Porphyromonas gingivalis* fimbriae

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Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a motogen, mitogen and morphogen produced by mesenchymal cells that mainly acts on epithelial cells and is involved in osteoclast stimulation. This study examined the possible enhanced production of HGF/SF by human gingival fibroblasts upon stimulation with killed cells of *Porphyromonas gingivalis* strain 381 and its representative bioactive cellular components, fimbriae and lipopolysaccharide (LPS). *P. gingivalis* whole cells enhanced the production of HGF/SF detected by ELISA in culture supernates of the fibroblasts. Fimbriae prepared from *P. gingivalis* exhibited powerful HGF/SF-inducing activity in a concentration-dependent manner, with peak activity observed at 100–200 µg/ml. The fimbriae-induced HGF/SF mRNA expression by the cells was also detected by reverse transcription-PCR. *P. gingivalis* LPS exhibited weak HGF/SF-inducing activity. The study also examined the HGF/SF-inducing activity of seven synthetic peptides corresponding to the segments of *P. gingivalis* fimbrial subunit protein. The peptides of residues 282–301 and 302–321, which exhibited antagonistic effects against *P. gingivalis* fimbriae-binding to human gingival fibroblasts in a previous study, showed weak activity, whereas other non-antagonistic peptides showed no significant activity. These findings indicated that *P. gingivalis* fimbriae enhanced production of HGF/SF by human gingival fibroblasts, whereas synthetic peptide segments of fimbrial subunit protein were not sufficient to exert the activity.

Introduction

Hepatocyte growth factor (HGF) was first purified from the plasma of patients with fulminant hepatic failure as a potent mitogen for hepatocytes [1] and is thought to be involved in regulation of liver function [2]. HGF was eventually identified as the same protein as the scatter factor (SF) [3] purified from conditioned medium of fibroblasts in culture, which enhances the movement and induces the dissociation of epithelial colonies [4, 5]. The receptor of HGF was demonstrated

to be the product of the *c-met* protooncogene [6, 7], and c-Met has been shown to be expressed on both epithelial and endothelial cells [5].

An earlier study demonstrated that human gingival fibroblasts released HGF/SF upon stimulation with interleukin (IL)-1 α / β and tumour necrosis factor (TNF)- α [8]. It was then found that bacterial cell-surface components such as lipoteichoic acid (LTA) from various gram-positive bacteria and a glycoprotein fraction (PGP) from the periodontitis-associated bacterium *Prevotella intermedia* enhanced the production of HGF/SF by human gingival fibroblasts, whereas lipopolysaccharides (LPS) examined to date – including that of *Pr. intermedia* – lacked the activity [9, 10]. It was also found that recombinant human HGF/SF exhibited mitogenic activity on human gingival epithelial cells (keratinocytes) [10]. HGF/SF has been implicated in bone resorption through the stimulation of osteoclasts

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[11, 12]. Therefore, HGF/SF may be involved in both destruction and reparative phases in periodontal diseases.

Porphyromonas gingivalis has been the most intensively studied bacterium in relation to periodontal diseases [13]. Among the various cell-surface components of the bacterium, fimbriae [14, 15] and LPS [16, 17] have been the most thoroughly studied as possible virulence factors for periodontal diseases. Both components are capable of stimulating gingival fibroblasts. *P. gingivalis* fimbriae activate human gingival fibroblasts to produce cytokines such as IL-1 and IL-8 [18, 19]. Specific binding of the fimbriae to human gingival fibroblasts was suggested [18] and the binding regions within the fimbrial subunit protein, fimbrillin, were demonstrated with synthetic peptides [20] (Table 1). It has been shown that LPS of *P. gingivalis* stimulates human gingival fibroblasts to produce various inflammatory cytokines such as IL-1, IL-6 and IL-8 under experimental conditions, whereas common LPS from Enterobacteriaceae such as *Salmonella* spp. and *Escherichia coli* did not exhibit such activity [21, 22]. A recent study observed that some human gingival fibroblasts carrying membrane CD14 release IL-8 upon stimulation with common LPS and synthetic lipid A [23]. *P. gingivalis* LPS and lipid A also activated CD14-bearing human gingival fibroblasts, probably *via* membrane CD14 molecules [19, 24]. Therefore, it is possible that fimbriae and LPS from *P. gingivalis* stimulate human gingival fibroblasts to enhance the production of HGF/SF.

In the present study, human gingival fibroblasts were stimulated with *P. gingivalis* fimbriae and LPS and the production of HGF/SF by the cells was examined. After obtaining positive results with fimbriae, the study investigated the active regions within fimbrillin of *P. gingivalis* by examining the activities of several synthetic peptide segments of *P. gingivalis* fimbrillin.

Materials and methods

Bacteria and their components

P. gingivalis strain 381 was grown anaerobically in GAM broth (Nissui Seiyaku, Tokyo, Japan) supple-

mented with haemin (Wako Pure Chemicals, Osaka, Japan) 5 mg/L and menadione (Wako) 10 µg/L at 37°C for 24 h. The bacterial cells were collected by centrifugation and washed twice with physiological saline and once with distilled water by centrifugation, then freeze-dried. In these procedures, the anaerobic bacteria were completely killed. A fimbrial protein with a mol. wt of 41 000 was isolated from freshly cultivated whole cells of *P. gingivalis* and purified chromatographically as described previously [25]. LPS was prepared by the hot phenol-water extraction method as described previously [26].

Synthetic peptides and IL-1α

The seven peptide segments listed in Table 1, which were based on the amino-acid sequence of native fimbrillin of *P. gingivalis* 381 deduced from the nucleotide sequence of fimbrillin, a fimbrial subunit protein proposed by Dickinson *et al.* [27], were synthesised as described previously [28]. These synthetic peptides were named FP381 (mm-nn): FP stands for fimbrial peptide, 381 stands for *P. gingivalis* strain 381 and mm-nn is the range of amino-acid residue numbers based on the report by Dickinson *et al.* [27]. The data on the antagonistic effects of these peptides against *P. gingivalis* fimbriae-binding to human gingival fibroblasts [20] are listed in Table 1. Recombinant human IL-1α (rHuIL-1α) supplied by Dainippon Pharmaceutical (Osaka, Japan) was used as a positive reference stimulant throughout the study.

Cells and culture conditions

Specimens of healthy human gingival fibroblasts were obtained from 9–12-year-old patients with their parents' informed consent, as described previously [21]. The cells used in this study were confirmed to highly express membrane CD14 by flow cytometry [23]. The cells were grown and maintained in alpha-modified minimal essential medium (α-MEM) (ICN, Amsterdam, The Netherlands) supplemented with fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA) 10% and kanamycin (Meiji Seiyaku, Tokyo) 200 µg/ml at 37°C in a humidified atmosphere of CO₂.

Table 1. The amino acid sequences of synthetic peptides corresponding to the segments of *P. gingivalis* 381 fimbrillin

Peptide	Position*	Amino-acid sequence*	Antagonistic activity [†]
FP381(61–80)	61–80	GKTLAEVKALTTELTAEENQE	NT
FP381(81–101)	81–101	AAGLIMTAEPKTIVLKAGKNY	–
FP381(142–161)	142–161	NIYTFVPEKIYGLIAKKQSN	–
FP381(182–201)	182–201	FNGAYTPANYANVPWLSRNY	–
FP381(216–241)	216–241	ENDYSANGGTTHTPTILCVYGLQKNG	NT
FP381(282–301)	282–301	NYTPKNKIERNHKYDIKLT	+
FP381(302–321)	302–321	TGPGTNNPENPITESAHLNV	+

NT, not tested.

*According to Dickinson *et al.* [27].

[†]Inhibitory activity on *P. gingivalis* fimbriae-binding to human gingival fibroblasts (quoted from Ogawa *et al.* [20]).

5% in air and were used at the fifth to tenth passage in the following assays.

Determination of HGF/SF in culture supernates of fibroblasts

Fibroblasts (2×10^4) were seeded into each well of 96-well culture plates in 100 μ l of α -MEM supplemented with FBS 10%. After overnight cultivation, the cells were washed and the medium was changed to α -MEM supplemented with FBS 1%. The cells were then cultured in triplicate with test materials for 24 h. Throughout this report, the concentrations of IL-1 α and the fimbriae are given in terms of protein, and those of whole bacterial cells, LPS and the synthetic peptides are given in terms of dry weight. The supernates were collected and stored at -20°C until use. The concentrations of immunoreactive HGF/SF in culture supernates were determined in triplicate with a commercial ELISA kit (Otsuka Pharmaceutical, Tokushima, Japan), as described by Tsubouchi *et al.* [29]. The assays were performed according to the manufacturer's instructions.

Reverse transcription (RT)-PCR

After experimental treatments with test materials, total cellular RNA was extracted from *c.* 2.5×10^6 cells with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesised from the RNA solution with M-MLV reverse transcriptase (Gibco-BRL) and oligo (dT)₁₂₋₁₈ primer (Gibco-BRL). To generate cDNA, 2.5 μ g of total RNA, 0.25 μ g of oligo (dT)₁₂₋₁₈ primer, First Strand Buffer (Gibco-BRL), 0.2 mM dNTPs, 500 units of M-MLV reverse transcriptase and 10 mM dithiothreitol (Gibco-BRL) were added to a total volume of 50 μ l. The reaction mixture was incubated for 1 h at 37°C followed by incubation for 5 min at 95°C . The resulting cDNA mixture was then amplified in a solution containing Taq polymerase and the following primers specific for human HGF/SF or GAPDH, which were prepared based on the cDNA sequences reported by Miyazawa *et al.* [30] and Tso *et al.* [31], respectively: HGF, 5'-CAAGCAATCCAGAGGTACGC-3' and 5'-GTCATGTCATGCTCGTGAGG-3'; GAPDH, 5'-TGAAGGTCGGAGCAACGGATTGTTGGT-3'; and 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The PCR mixture contained 5 μ l of cDNA mixture, 2 μ l of $10 \times$ PCR buffer, 0.2 mM dNTPs, 50 pmol of each primer and Ex Taq DNA polymerase (Takara, Ohtsu, Japan) 0.1 μ l in a total volume of 20 μ l.

Amplification was performed in a PCR thermal cycler (MP TP3000, Takara) as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min and then a final extension at 72°C for 3 min. The predicated size of the PCR products for HGF and GAPDH were 447 and 983 bp, respectively. GAPDH amplification was used as an internal control to confirm that the samples

contained similar amounts of cDNA. Amplified samples were visualised on agarose 2.0% gels stained with ethidium bromide and photographed under UV light. The bands of the photographs were scanned and then analysed with an ImageMaster 1D (Pharmacia Biotech, Uppsala, Sweden).

Statistical analyses

In most assays, the mean and SD were obtained, and the statistical significance of the difference between each test and its respective control was examined by Student's *t* test. Most experiments were performed more than twice, and representative results are presented.

Results

HGF/SF secretion from gingival fibroblasts stimulated with P. gingivalis whole cells

The study first examined the effects of *P. gingivalis* whole cells on the secretion of immunoreactive HGF/SF by human gingival fibroblasts. As shown in Fig. 1, *P. gingivalis* whole cells at 100 μ g (dry weight)/ml significantly stimulated the secretion of HGF/SF by the gingival fibroblasts. The HGF/SF level induced by the bacterial cells was similar to or stronger than that induced by the optimum concentration (10 ng of protein/ml) of the reference rHuIL-1 α , although a higher concentration was required to exhibit activity for the bacterial cells.

P. gingivalis fimbriae and LPS stimulate the secretion of HGF/SF by fibroblasts

P. gingivalis whole cells stimulated the production of HGF/SF by gingival fibroblasts. Therefore, the activities of representative cell-surface components of *P. gingivalis*, i.e., fimbriae and LPS – both of which are known to activate human gingival fibroblasts – were examined. As shown in Fig. 2, *P. gingivalis* fimbriae strongly stimulated the production of HGF/SF by fibroblasts with a typical bell-shaped dose-response curve. HGF/SF production was increased from 10 to 100 μ g (protein)/ml dose-dependently, then decreased to 1000 pg/ml. The level of HGF/SF induced by fimbriae 100 μ g/ml was more than two-fold higher than that induced by rHuIL-1 α 10 ng/ml. LPS exhibited significant but weak activity. Total RNA was then extracted from gingival fibroblasts stimulated with *P. gingivalis* fimbriae or rHuIL-1 α and RT-PCR was performed to define the levels of HGF/SF mRNA. A definite band was observed in the gel containing RNA extracted from gingival fibroblasts stimulated with *P. gingivalis* fimbriae at 1–100 μ g/ml (Fig. 3a). The results of ImageMaster 1D analysis indicated that the density of the bands increased in relation to the doses of fimbriae added and the band induced by 100 μ g of

Fig. 1. HGF/SF secretion from human gingival fibroblasts stimulated with *P. gingivalis* whole cells. Fibroblasts were cultured at a density of 2.0×10^4 cells/100 μl /well in 96-well plastic culture plates. Stimulants were added and incubated for 24 h in triplicate. Culture supernates were collected and the concentration of HGF/SF in the pooled specimens was determined by ELISA in triplicate assays. Values are means and SD. **Significantly different from the control (medium alone) as determined by Student's *t* test ($p < 0.01$). The results are representative of two different experiments.

Fig. 2. HGF/SF secretion from human gingival fibroblasts stimulated with *P. gingivalis* fimbriae and LPS. Experimental conditions were the same as described in the legend to Fig. 1. Values significantly different from control are indicated: * $p < 0.05$; ** $p < 0.01$. The results are representative of three different experiments.

Fig. 3. Expression of HGF/SF mRNA in human gingival fibroblasts. Fibroblasts were stimulated with various concentrations of *P. gingivalis* fimbriae or reference rHuIL-1 α (10 ng/ml) for 8 h. Total RNA was extracted and RT-PCR was carried out (a). Relative densities of the bands of HGF/SF mRNA shown in panel (a) were analysed with an ImageMaster 1D (b). Lane 1, medium alone; 2–4, *P. gingivalis* fimbriae (1, 10 and 100 μ g/ml, respectively); 5, rHuIL-1 α (10 ng/ml). The results are representative of two different experiments.

fimbriae/ml was about nine-fold denser than that induced by medium alone (Fig. 3b).

HGF/SF production by gingival fibroblasts stimulated with synthetic peptides of fimbrial protein from P. gingivalis

Next, to elucidate the regions responsible for the HGF/SF-inducing activity of fimbriae, the study examined seven synthetic peptide segments of *P. gingivalis* fimbrial protein. The peptide FP381(302–321) stimulated HGF/SF production by fibroblasts at concentrations of 10–1000 μ g (dry weight)/ml, although the activity was considerably weaker than that of reference rHuIL-1 α (Fig. 4). The peptide FP381(282–301) also exhibited weak activity at the highest concentration (1000 μ g/ml). The other peptides showed no significant activity.

Discussion

This study found that *P. gingivalis* whole cells, fimbriae and LPS enhanced the production of HGF/SF by human gingival fibroblasts in culture, and that the fimbriae exhibited the strongest HGF/SF-inducing activity reported to date. *P. gingivalis* fimbriae have been studied as a possible virulence factor of this bacterium in relation to periodontitis [14, 15]. The fimbriae are involved in the adherence of the bacterium to host cells, especially to human gingival fibroblasts

[18, 32, 33]. The fimbriae also stimulate human gingival fibroblasts to increase the production of inflammatory cytokines such as IL-1 and IL-8 [18, 19]. Therefore, IL-1 produced by fibroblasts in response to the fimbriae might in turn induce HGF/SF in the fibroblast cultures. However, the fimbriae should induce HGF/SF directly in the fibroblast cultures, because peak HGF/SF mRNA expression was noted after cultivation of the fibroblasts with the fimbriae for several hours, at which time IL-1 had not yet been released from the fibroblasts (data not shown). Ogawa *et al.* [20] demonstrated the specific binding region within fimbrillin of *P. gingivalis* on to human gingival fibroblasts in terms of the antagonistic effect of synthetic peptides corresponding to various fimbrillin segments. The data obtained in the present study were in accordance with the results of Ogawa *et al.* [20]; the antagonistic peptides FP381(282–301) and FP381(302–321) exhibited a definite HGF/SF-inducing activity in this study, whereas FP381(81–101), FP381(142–161) and FP381(182–201) showed no significant activity in either assay. Comparable results were not available in the antagonistic assay for FP381(61–80) and FP381(216–241), both of which showed no significant activity in this study. It must be noted that the fibroblast-activating effect of the synthetic peptides required higher concentrations than those required for their antagonistic effects. Thus, the higher order structures of fimbrillin or fimbriae might be required to exert the stimulating activity efficiently, and the segment peptides may not be sufficient to exert the activity.

Fig. 4. HGF/SF secretion from human gingival fibroblasts stimulated with various synthetic peptides corresponding to the segments of *P. gingivalis* 381 fimbriin. Experimental conditions were the same as described in the legend to Fig. 1. *, **Significantly different from control (* $p < 0.05$, ** $p < 0.01$).

A previous study reported that none of the LPS or lipid A specimens tested to date induced HGF/SF in human gingival fibroblasts in culture [10]. It must be emphasised that the inactivity of LPS (from *Salmonella abortusequi*) was confirmed both in high-CD14-expressing and low-CD14-expressing gingival fibroblasts (unpublished observations). Purified *Pr. intermedia* LPS was also inactive in this respect, although the crude LPS fraction extracted with hot phenol-water from the same bacterium exhibited definite HGF/SF-inducing activity [9]. The HGF/SF-inducing activity of the LPS fraction might be attributable to a bioactive glycopeptide, PGP, which exerted powerful HGF/SF-inducing activity [9]. There is a great deal of evidence suggesting similarities in the lipid A structures of *P. gingivalis* and *Pr. intermedia* [9, 16, 17]. Therefore, the activity of *P. gingivalis* LPS detected in the present study might also be attributable to the PGP-comparable minor component(s) in the LPS specimen. Another possibility is that the unique lipid A structure of the bacterium [34, 35], which is capable of activating human gingival fibroblasts to produce IL-8 [19],

stimulates the cells to produce HGF/SF in a manner different from that of common LPS and lipid A.

P. gingivalis is the most widely studied periodontopathic bacterium [13]. Therefore, the HGF/SF induced by the bacterial components might have a role in some periodontal diseases. In fact, it has been suggested that HGF/SF is responsible for osteoclast formation [12]. The HGF/SF receptor, c-Met, is expressed on both osteoclast-like cells and osteoblasts [11]. HGF/SF induces changes of shape and stimulates the chemotactic migration and proliferation of osteoclasts, and the cells then synthesise and secrete biologically active HGF/SF, suggesting the existence of autocrine and paracrine mechanisms of regulation of the osteoclasts and osteoblasts, respectively, by HGF/SF. The possible multiple functions of HGF/SF in gingival epithelial cells and endothelial cells may be involved in both destructive and reparative phases in periodontal diseases under different physiological and pathological conditions. HGF/SF has been shown to induce blood vessel formation *in vitro* [36] and *in vivo* [37], and may be

involved in tissue regeneration. At present, studies of HGF/SF in relation to periodontal diseases are still in the initial stages, and further findings are eagerly awaited.

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