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Histatin 5 Inhibits Apoptosis in Human Gingival Fibroblasts Induced by *Porphyromonas Gingivalis* Cell-surface Polysaccharide

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Abstract: The cell-surface components of *Porphyromonas gingivalis* have various biological activities. In the present study, we investigated the virulence of several cell-surface components prepared from *P. gingivalis* in human gingival fibroblasts (HGF). Furthermore the preventive effect of salivary protein histatin 5 was investigated. *P. gingivalis* polysaccharide (PS) significantly inhibited HGF proliferation, but lipopolysaccharide and outer-membrane protein did not. By using ELISA analysis, DNA fragmentation in HGFs was observed intracellularly when treated with the PS. These results suggest that the PS of *P. gingivalis* can induce apoptosis in HGF. Pretreatment of PS with histatin 5 restrained the inhibitory effect of PS on HGF proliferation. Histatin 5 also suppressed the apoptotic cell death in HGF induced by PS stimulation. The present study suggests that the PS of *P. gingivalis* can modulate the cell population in periodontal tissue, causing periodontitis by inducing HGF cell death through apoptosis. Also histatin 5 can inhibit the PS activity and may play an important part in the regulation of inflammatory periodontal diseases.

Key words: Porphyromonas gingivalis; Polysaccharide; Apoptosis; Human gingival fibroblast; Histatin 5

Introduction

Porphyromonas gingivalis is a gram-negative rod associated with the progression of human periodontal disease. *P. gingivalis* has a number of pathogenic components involved in colonization and invasion of the periodontal tissue (Beck et al., 1990; Okuda, 1993; Agarwal et al., 1995). We previously showed that 57-kDa exterior-membrane protein (57-kDa OMP) and cell-surface polysaccharide (PS) from *P. gingivalis* as well as lipopolysaccharide (LPS) from the organism were able to induce interleukin-6 (IL-6) and interleukin-8 (IL-8) from human gingival fibroblasts (HGFs) in vitro (Imatani et al., 2000; Imatani et al., 2001). Ochiai et al. (1997; 1999) have demonstrated that fatty acids of *P. gingivalis* exert inhibitory effects on T and B cells proliferation and apoptosis in murine thymocytes, splenic T cells, human Jurkat T cells, and human peripheral blood mononuclear cells. Another report has shown that preparation of *P. gingivalis* protease induced apoptotic cell death in HGFs (Wang et al., 1999). In our study we described the apoptosis inducing activity of a PS preparation from *P. gingivalis* on HGF.

Salivary proteins play an important part in the defense mechanism against various infections. The roles of salivary function have been studied in relation to colonization by oral bacteria (Gibbons, 1989; Gibson and Barrett, 1992). The salivary protein histatin 5 plays a protective role in elimination of some virulence factors (Imatani, 2000; Murakami et al., 1990; Nishikata et al., 1991). However, the functions of salivary proteins in controlling inflammation of periodontal diseases have not been adequately studied. In the present study, we investigated the protective role of salivary protein histatin 5 against the cytotoxicty of PS preparation from *P. gingivalis* on HGFs.

Materials and Methods

Preparation of Cell-surface

The bacterial strain selected for preparing cell surface components was obtained from *P. gingivalis* invasive strain ATCC 53977. LPS and PS were purified with phenol water extracts by means of gel filtration chromatography as described previously (Schifferle et al., 1989; Ochial et al., 1997). Briefly, the phenol-water extracts from the harvested cells were dialyzed against distilled water and lyophilized. The extracts were dissolved in Tris-HCl buffer. This solution was applied to a column of Sephacryl HR 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) and was analysed at 280 nm. PS formed a smear band at a high molecular weight position in the SDS-polyacrylamide gel electrophoresis (PAGE) with PAS staining. The fractions including LPS showed repeating ladder bands forming a typical LPS pattern. Appropriate fractions containing either LPS or PS were pooled, sodium chloride was added to a concentration of 0.15 M, and the PS was precipitated with 4 volumes of 95% ethanol and stored overnight. The precipitate was isolated by centrifugation, dissolved in water, dialyzed against water, and lyophilized.

The 57-kDa OMP was purified according to methods in previous papers (Blake and Gostchlich, 1982; Imatani et al, 2000). Briefly, the harvested cells of *P. gingivalis* were suspended in water, and hexadecyltrimethyl-amino bromide (CTB; Sigma Chemical

Co., St. Louis, MO, USA) was added. After centrifugation, the supernatant was discarded, and the precipitate was suspended in distilled water. The nucleic acid was removed, and the concentration of alcohol was then increased to 80%. The preparation was centrifuged. The precipitate was washed with alcohol and lyophilized. The preparation was resuspended in Tris-HCl with 0.05% *N*-tetradecyl-*N*, *N*-dimethl-3-ammonia-l-propanesufonate (Sigma Chemical Co.) and then were applied to a chromatograph column packed with DEAE-Toyopearl 650 S (Tosoh, Tokyo, Japan). The proteins were eluted with the NaCl gradient from 0 to 20 mM. The fractionated sample was applied to a column filled with TSK gel SW 3000 (Tosoh) for separation with the fast-protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). SDS-PAGE analysis showed a single protein band at approximately 57 kDa. The sample was lyophilized. We confirmed that LPS, PS, and 57-kDa OMP from *P. gingivalis* were not contaminated by each other by methods described previously (Imatani et al., 2000; Imatani et al., 2001). Additionally, no LPS contamination (Endotoxin Unit/ml <0.0001) was detected in the PS and 57-kDa OMP by the Endospecy technique (Seikagaku Corporation, Tokyo, Japan).

Fibroblast Culture

Our HGF cell line was derived by explant culture from clinically healthy gingiva collected with informed consent. HGFs were cultured in medium containing 10% fetal bovine serum (FBS; Boerhinger GmbH, Mannheim, Germany), L-glutamine (600μ g/ml), $NaHCO_3$, HEPES, penicillin (100 U/ml), and streptomycin (125μ g/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The 2-4 passage cells were seeded into 24 or 96-well chamber plates (Nunc, Naperville, IL, USA). These fibroblasts were used in this study.

Cell Proliferation Assay

HGF cellular proliferations following the addition of *P. gingivalis* cell-surface components were measured with an MTS (3-(4, 5-demethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-surlfophenyl)-2H-tetrazolium, inner salt) Assay Kit (Cell Titer 96™ AQ Assay Kit; Promega, Madison, WI, USA). When the HGF cells in 96-well chamber plates were preconfluent, the medium was replaced by one without FBS (100μl), and the cells were cultured for 24 h. The purified PS, LPS, and 57-kDa OMP preparations (5, or 10μ g/ml per well) were applied to the HGF cultures and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. The protein concentrations were determined as dry weights of lyophilized preparations per ml in phosphate buffered saline (pH 7.2, PBS). After incubation of the cells, 20μl of the combined MTS/PMS (phenazine methosulfate) solution was added to each well, and the plate was incubated for 30 min at 37°C in a humidified 5% CO_2 atmosphere. After the incubation of the cells, the solutions were mixed, and recorded from a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 490nm.

DNA Fragmentation Assay

DNA fragmentation was evaluated with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) using a MEBSTAIN Apoptosis Kit (Medical and Biological Laboratory Co., LTD., Nagoya, Japan). In the TUNEL method, 3'-OH DNA ends generated by DNA fragmentation is nick-end labeled with fluorescein-dUTP, mediated by TdT. HGF cells in 24-well chamber plates were stimulated with *P. gingivalis* components (30μ g/ml) and incubated for 24h at 37°C. After incubation, the cells were washed twice with PBS containing 0.2% bovine serum albumin (BSA). Then the fibroblasts were fixed with 4% paraformaldehyde at 4°C for 30 min and washed twice with PBS containing BSA. An aliquot of 200μl of ethanol was added to each cell chamber, which was then incubated for 30 min at -20°C to permeabilize it. After washing the fixed cells twice with PBS containing BSA, 30μl of TdT reaction regent (TdT buffer, FITC-dUTP and TdT in the ratio of 18:1:1) were added, and the system was incubated for 1 h at 37°C. Then the cells were washed twice with PBS containing BSA and suspended to the 500μl of PBS containing BSA. DNA fragmentations of the cells were evaluated by flow cytometry (FACS Caibur; Becton Dickinson, Sunnyvale, CA, USA).

Additionally, apoptosis was also determined with a Cellular DNA Fragmentation ELISA Kit (Boehringer GmbH) according to the manufacturer's instructions. This assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies. They were directed against DNA and histones, respectively, which allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates (Muir et al., 1990). Cellular DNA of HGF cells were labeled by 5-bromo-2'-deoxy-uridine (BrdU) by incubation at 37°C overnight. After labeling, these cells were plated in 96-well chamber (Nunc), stimulated with different concentrations of cell-surface components (1-30μ g/ml) and incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. The amount of BrdU-labeled DNA released into the cytoplasm of apoptotic cells was quantified by the ELISA system.

Inhibition of Histatin 5

In order to examine the inhibitory effects of histatin 5 taken from *P. gingivalis* PS on the HGF proliferation, PS was preincubated with synthetic peptide histatin 5 (20μ g/ml: Sigma Co.) at 37°C for 20 min. The purified PS preparations (10μ g/ml) preincubated with histatin 5 were applied to the HGF cultures and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. After incubation, cell proliferations were evaluated with the MTS Assay Kit.

In order to examine the inhibitory effects of histatin 5 on the induction of apoptotic cell death, the PS preparation (30μ g/ml) was preincubated with synthetic histatin 5 (1-20 μ g/ml) at 37°C for 20 min. HGFs were stimulated with the preincubated mixture and cultured at 37°C for 24 h. The intracellular DNA fragmentation of HGF was measured using the Cellu-

lar DNA Fragmentation ELISA system (Boehringer GmbH) described above. The DNA fragmentation rate after each histatin 5 treatment was determined as a percentage of the value of HGF cells incubated with *P. gingivalis* PS without histatin 5.

Statistics

The Mann-Whitney U test was used to identify statistically significant differences. A level of $p < 0.05$ was considered significantly different.

Results

Effect of *P. gingivalis* Cell-surface Preparations on HGF Cell Proliferation

The inhibition of cell proliferation was measured following stimulation with cell-surface components of LPS, PS or 57-kDa OMP from *P. gingivalis*. Results are summarized in Table 1. PS significantly inhibited proliferation of HGF ($p < 0.05$), but LPS and 57-kDa OMP showed no effect at the concentrations used in this study.

Apoptosis Induced by *P. gingivalis* Cell-surface Components

The apoptosis inducing activity of *P. gingivalis* preparations was evaluated by measuring the cellular DNA fragmentation by flow cytometric analysis. In the control fibroblasts, few apoptotic cells were found after 24 h incubation (Fig.1, A). Significant DNA fragmentation was found in the culture medium after treatment of the cells with PS of *P. gingivalis* (Fig.1, B). This apoptosis was confirmed with the Cellular DNA Fragmentation ELISA Kit. To determine whether the PS of *P. gingivalis* caused apoptosis, we examined the extent of DNA fragmentation induced by PS. Intercellular DNA fragmentation of HGF was detected when stimulating the cells with PS at a concentration of 30μg/ml (data not shown). These data suggest that PS of *P. gingivalis* induces apoptosis in HGF.

Inhibitory Activity of Histatin 5 on Cytotoxicity of *P. gingivalis* PS

The ability of histatin 5 to prevent the anti-proliferation activity of *P. gingivalis* PS against HGF was evaluated. Preincubation with histatin 5 significantly prevented the proliferation-inhibitory activity of *P. gingivalis* PS stimulation ($p < 0.05$, Table 2). However, simultaneous incubation (i.e. no preincubation) failed to inhibit the cytotoxic activity of *P. gingivalis* PS significantly.

Fig. 2 shows the inhibition effect of histatin 5 on the DNA fragmentation induced by *P. gingivalis* PS in HGF cells. Histatin 5 significantly inhibited the PS apoptosis inducing activity ($p < 0.05$) at a concentration of 20μg/ml.

Table 1. Inhibition of HGF proliferation stimulated with cell-surface components of *P. gingivalis*.

P. gingivalis components	Concentration (μg per well)	HGF proliferation (OD at 490nm)
None	-	0.77 ± 0.020
LPS	5	0.72 ± 0.038
	10	0.70 ± 0.041
PS	5	0.59 ± 0.064*
	10	0.57 ± 0.021*
57-kDa OMP	5	0.71 ± 0.036
	10	0.75 ± 0.042

HGFs were simulated with *P. gingivalis* components. *$p < 0.05$ compared with unstimulated HGF. The data shown are mean values of four duplicate experiments with standard deviations.

Table 2. Inhibitory activity of histatin 5 on HGF anti-proliferation effect of *P. gingivalis* PS.

Histatin 5 (10μg per well)	PS (10μg per well)	HGF proliferation (OD at 490nm)	Proliferation rate (%)
-	-	0.77 ± 0.020	(100)
-	+	0.57 ± 0.021*	74.2
+ (preincubation)	+	0.71 ± 0.059*	92.5
+ (simultaneous incubation)	+	0.62 ± 0.037	80.0

HGFs were treated with or without *P. gingivalis* PS and histatin 5. Preincubation with histatin 5 significantly restrained the activity of the *P. gingivalis* PS that inhibits HGF proliferation (*$p < 0.05$).

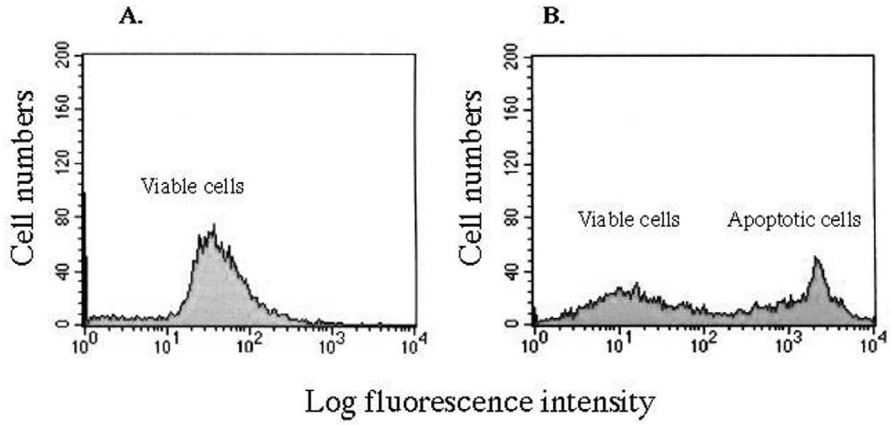

Fig. 1. Cytofluorimetric analysis of HGFs apoptosis. "A" is untreated HGF as negative control. "B" is treated with PS of *P. gingivalis*.

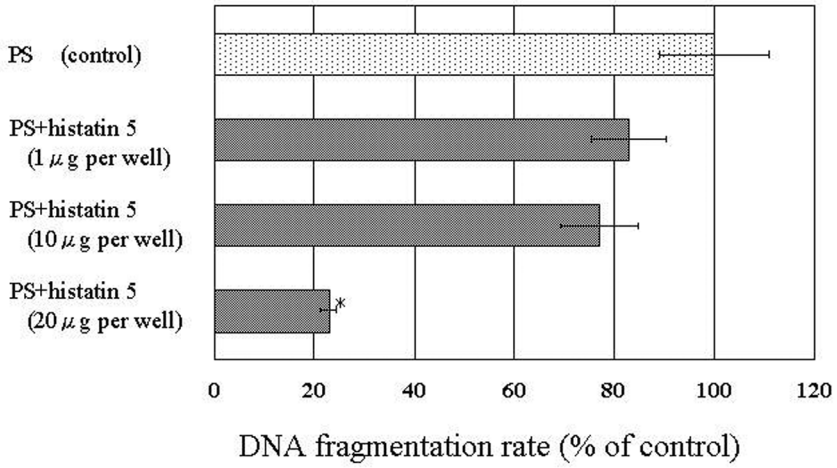

Fig. 2. Histatin 5 inhibition of DNA fragmentation of HGF cells stimulated with *P. gingivalis* PS (30μ g/ml). When the concentration of histatin 5 was 20μ g/ml, it inhibited the apoptotic cell death induced by PS of *P. gingivalis*. *Significantly different from control at $p<0.05$.

Discussion

HGF produces many kinds of cytokines in response to stimulation by some bacterial antigens, and plays an important role in the cytokine network in oral lesions (Imatani et al., 2000; Imatani et al., 2001; Sugawara et al., 1998; Wang et al., 2000). Our previous study (Imatani et al., 2001) demonstrated that *P. gingivalis* PS induced the production of the inflammatory cytokines from HGF. *P. gingivalis* LPS or 57-kDa OMP showed the same effect. In this study, *P. gingivalis* PS inhibited HGF cell proliferation, but *P. gingivalis* LPS and 57-kDa OMP were without influence. Furthermore, we precedently observed that *P. gingivalis* PS induced cell death by microscopic observations. In the present study, we confirmed that the PS preparation affected apoptosis. Apoptosis has been shown to play an important role in the control of the immune, hemapoietic, and developmental systems (Kerr et al., 1972). *P. gingivalis* LPS administration to mice was shown to induce apoptosis of lymphocytes in the spleen, lymph nodes, and thymus (Isogai et al., 1996). We detected intercellular DNA fragmentation of HGF after stimulation by PS at a concentration of 30μ g/ml. In contrast, a low dose of PS from *P. gingivalis* did not induce a significantly high rate of DNA fragmentation in HGF. In our previous paper, we demonstrated that the IL-1β and IL-8 production activity of *P. gingivalis* PS at low doses (1μ g and 10μ g) was higher than that of the control (Imatani et al., 2001). *P. gingivalis* PS may exhibit cytokine inducing activity at low doses and may induce apoptosis at high doses. Recently, Hirai et al. (2003) reported that *P. gingivalis* LPS did not induce significant apoptosis in all tested doses (1 to 100 μ g). We also clarified that the DNA fragmentation of HGFs was induced by PS stimulation, but not by the LPS of *P. gingivalis*. Our results suggest that the PS rather than LPS of *P. gingivalis* leads to the HGF apoptosis. The present study showed that apoptotic HGF cell death was induced mainly by PS.

Histatins are small, histidine-rich salivary polypeptides which exhibit antimicrobial activity. Histatins 1, 3 and 5 are capable of killing *Candida albicans* and *Streptococcus mutans* (Mackay et al., 1984). Our previous study (Imatani et al., 2000) demonstrated that histatin 5 plays a protective role in elimination of some of the virulence effects of *P. gingivalis* 57-kDa OMP on released inflammatory cytokines from HGF. In this study of the apoptosis-inducing activity of *P. gingivalis* PS, histatin 5 significantly inhibited its activity at 20μ g per well. A lower dose of histatin 5 (1 or 10μ g per well) showed only slight inhibition. In this study, the PS preparation was preincubated with histatin 5 for 20 min. A longer incubation time may be needed to induce an effective inhibitory activity with a lower dose of histatin 5. In this study, we also found that the protective effect of histatin 5 against cell proliferation inhibitory activity of *P. gingivalis* PS was effective at a

concentration of 20μ g/ml per well. When PS and histatin 5 mixtures stimulated HGF without preincubation, the inhibitory activities were less effective (Table 2). Previously, Murakami et al. (1991) have reported that histatin 5 binds to the cell surface of *P. gingivalis* rapidly and that the process is capable of reaching saturation. In this study, we demonstrated that preincubation of PS with histatin 5 was important. This fact suggests that histatin 5 interacts with the PS of *P. gingivalis*, but not with HGF. The present study indicates that histatin is an important salivary peptide for protecting against virulence factors of periodontopathic bacteria. However, as the entire role of salivary histatin 5 in relation to other salivary proteins in oral defense mechanisms has not yet been clarified, further study is necessary to understand the roles of salivary proteins.

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