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 (*$p < 0.05$).

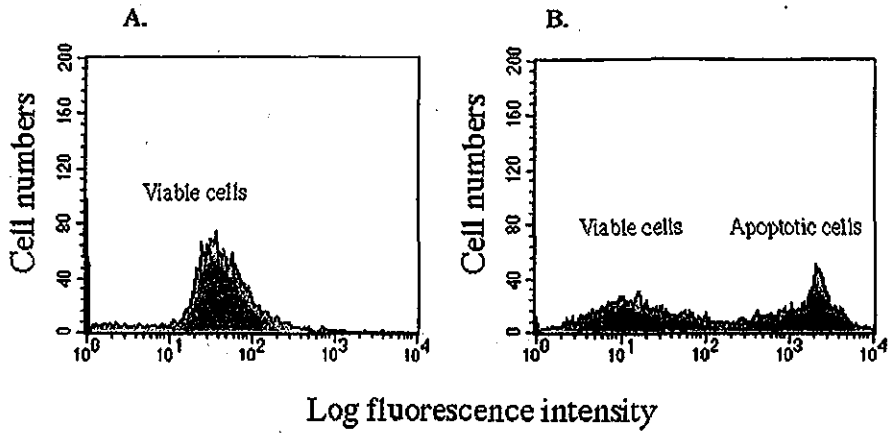

Fig. 1. Cytofluorimetric analysis of HGFs apoptosis. "A" is untreated HGF as negative control. "B" is treated with PS of *P. gingivalis.*

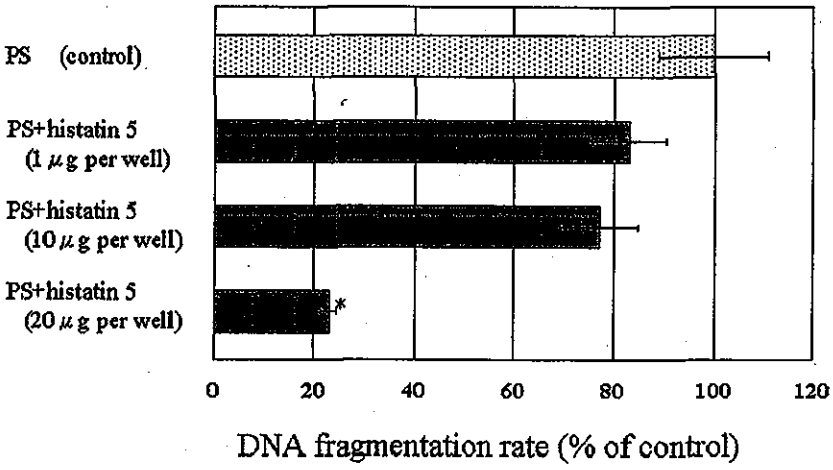

Fig. 2. Histatin 5 inhibition of DNA fragmentation of HGF cells stimulated with *P. gingivalis* PS (30μ g/ml). When the concentration of histatin 5 was 20μ g/ml, it inhibited the apoptotic cell death induced by PS of *P. gingivalis.* *Significantly different from control at $p<0.05$.

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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