bacteria, the organisms were anaerobically grown with [^{3}H]-thymidine at 37°C to the early stationary phase.

Preparation of cranberry juice

Cranberry juice powder (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA) was dissolved in water to a 25% concentration of cranberry juice following the manufacturer's instructions. Because a standard commercially available cranberry juice beverage contains around 25–27% juice, we selected 25% juice for this experiment. To avoid any effect of the low pH of cranberry juice on bacterial growth, the juice was dialyzed against distilled water (distilled water) in dialysis bags with a molecular mass cutoff point of 14,000 (Seamless Cellulose Tubing, 20/32, Viskase Sales Corp., Willowbrook, IL) at 4°C for 7 days. The non-dialyzable material was lyophilized. This non-dialyzable material was dissolved in trypticase soy broth and used in the biofilm formation assay as "high molecular weight constituents of cranberry juice".

Preparation of s-HA

Bacterial attachment to experimental pellicles was studied using s-HA as described by Gibbons et al. (4, 5). Human parotid saliva was collected with collecting devices from a healthy adult donor (16). The salivary flow was stimulated by an acid candy. The saliva was stored at –20°C and was used for the following experiments. A total of 5 mg of spheroidal hydroxyapatite beads (BDH Chemicals Ltd., Poole, England) were equilibrated in 200 μl buffered-KCl (0.05 M KCl, 1 mM potassium phosphate, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.0) at 4°C overnight. These hydroxyapatite beads were treated with 100 μl of saliva by use of a rotator (Model RT50 Taitec Co., Tokyo, Japan) at 5 r.p.m. for 60 min. They were then washed three times with buffered KCl (4, 5). Grown cells labeled with ^{3}[H]-thymidine were harvested by centrifugation. The harvested cells were washed three times in buffered KCl and then suspended in buffered KCl to produce a suspension containing 1.0×10^9 cells per ml.

Binding inhibition assay

A suspension of 100 μl containing a [^{3}H]-labeled bacterial suspension (1×10^9 cells/ml) was incubated with 500 μl of 25% cranberry juice in buffered KCl for 10 s, 10 min or 30 min. Buffered KCl without cranberry juice was used as a control. After washing once with buffered KCl, bacterial cells were suspended in 100 μl of same buffer. The suspension of 100 μl was incubated with s-HA in a rotator at 5 r.p.m. for 60 min. After washing three times with buffered KCl, the number of bacterial cells which had attached to s-HA was determined by direct scintillation counting. The experiments were done independently three times.

Hydrophobicity assay

Hydrophobicity was determined as described by Rosenberg et al. (14). Briefly, bacterial suspensions in PUM buffer, which contains $K_2PO_4 \cdot 3H_2O$ (22.2 g/l), KH_2PO_4 (7.26 g/l), urea (1.8 g/l), and $MgSO_4 \cdot 7H_2O$ (0.2 g/l), were adjusted to an optical density of approximately 0.5 at 400 nm using a 2000U Spectrophotometer (Hitachi, Tokyo, Japan). Duplicate samples of bacterial suspensions (1.2 ml in PUM buffer) were placed in tubes, and 600 μl of hexadecane was added. The tubes were vigorously mixed by vortex stirring for 60 s and left to stand for 15 min. The optical density at 400 nm (OD_{400}) of the aqueous phase was then measured. The percent hydrophobicity was calculated as follows: [(OD_{400} before mixing) – (OD_{400} after mixing)]/(OD_{400} before mixing) × 100. Each isolate was assayed twice, and the values obtained were averaged.

Biofilm formation assay

The inhibitory effect of cranberry juice on the biofilm formation of *S. sobrinus* 6715, *S. mutans* JC2, *S. criceti* E49, *S. sanguinis* ATCC 10556, *S. oralis* ATCC 10557, and *S. mitis* ATCC 9811 on the bottom of cell culture plates (SUMILON Multi Well Plate, Sumitomo Bakelite Co. Ltd, Tokyo, Japan) was examined. Biofilm assays were done using the protocol of Loo et al. (9). Briefly, strains of streptococci were cultured in trypticase soy broth supplemented with 100 μg/ml or 500 μg/ml of high molecular weight constituents of cranberry juice for 1 day under anaerobic conditions. Media and unattached bacterial cells were decanted from the wells, and the remaining planktonic or loosely bound cells were removed by rinsing with distilled water twice. The plates were then blotted on paper towels and air dried, and adherent bacteria were stained with 50 μl of 0.1% crystal violet for 15 min at room temperature. After rinsing twice with 200 μl of distilled water each time, bound dye was extracted from the stained cells by using 200 μl of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 595 nm (OD_{595}) with a microtiter plate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA).

Statistics

The Mann–Whitney *U*-test was used for all experiments in this study to identify statistically significant differences.

Results

Effects of cranberry juice on inhibiting adhesion to s-HA

The inhibitory effects of 25% cranberry juice on the adsorption of seven oral streptococcus strains are summarized in Table 1. The adherence rates of the tested oral streptococci to s-HA beads differed from strain to strain. The adsorption of *S. mutans* ATCC 10449 cells to the s-HA beads was strong, but the adsorption to *S. sobrinus* 6715 was markedly weaker. Momentary exposure to 25% cranberry juice significantly reduced the adherence of all tested oral streptococci except *S. sobrinus* 6715 to s-HA ($P < 0.01$).

Effect of cranberry juice on cell surface hydrophobicity

The effects of the addition of the cranberry juice on the cell surface hydrophobicity of tested streptococci are summarized in Table 2. The hydrophobicity also differed from strain to strain. The hydrophobicity reduction was found to be dependent on the concentration of cranberry juice. *S. mutans* Ingbritt, MT8148R and JC2 and *S. sobrinus* 6715 showed 40–60% hydrophobicity, *S. criceti* had the highest and *S. sobrinus* B13 a low hydrophobicity. The other oral streptococci exhibited more than 80% hydrophobicity. The addition of 12.5% cranberry juice significantly reduced the hydrophobicity of 10 of 11 strains of streptococcus. The addition of 25% cranberry juice significantly reduced the hydrophobicity of all the streptococcus strains tested ($P < 0.05$ to $P < 0.01$).

Effects of the high molecular weight dialyzable materials from cranberry juice on the formation of streptococcal biofilm

The data from the experiments examining the inhibitory effects of the high molecular weight dialyzable materials from cranberry juice on biofilm formation by oral

Table 1. Inhibitory effect of 25% cranberry on adsorption of oral streptococci to 5 mg S-HA beads

Strain	Time of exposure	Bacterial numbers adsorbed to s-HA ($\times 10^6$)	% inhibition of adsorption
S. sobrinus			
6715	Control[†]	1.79 ± 0.24	
	10 s	2.69 ± 0.91	−50.3
	10 min	0.91 ± 0.50	49.2
	30 min	0.66 ± 0.21*	63.1
B13	Control	3.71 ± 0.37	
	10 s	1.23 ± 0.33*	66.8
	10 min	1.12 ± 0.23*	69.8
	30 min	0.46 ± 0.24*	87.6
S. mutans			
MT8148R	Control	9.19 ± 1.89	
	10 s	2.45 ± 1.37*	73.3
	10 min	1.03 ± 0.22*	88.8
	30 min	1.29 ± 0.44*	86.0
JC2	Control	11.22 ± 2.16	
	10 s	2.46 ± 1.42*	78.1
	10 min	1.21 ± 0.44*	89.2
	30 min	1.03 ± 0.27*	90.8
Ingbritt	Control	4.04 ± 1.06	
	10 s	1.26 ± 0.29*	68.8
	10 min	0.58 ± 0.12*	85.6
	30 min	0.50 ± 0.04*	87.6
ATCC 10449	Control	20.30 ± 9.01	
	10 s	1.44 ± 0.64*	92.9
	10 min	1.43 ± 0.64*	93.0
	30 min	1.30 ± 0.39*	93.6
S. criceti			
E49	Control	12.52 ± 9.33	
	10 s	0.61 ± 0.90*	95.1
	10 min	0.51 ± 0.62*	95.9
	30 min	0.36 ± 0.33*	97.1
S. sanguinis			
ATCC 10556	Control	3.79 ± 0.44	
	10 s	0.65 ± 0.08*	82.8
	10 min	0.52 ± 0.10*	86.3
	30 min	0.58 ± 0.11*	84.7
S. oralis			
ATCC 10557	Control	5.93 ± 2.42	
	10 s	0.41 ± 0.18*	93.1
	10 min	0.53 ± 0.41*	91.1
	30 min	0.38 ± 0.23*	93.6
S. mitis			
ATCC 9811	Control	5.04 ± 3.19	
	10 s	0.92 ± 0.58*	81.7
	10 min	1.26 ± 0.95*	75.0
	30 min	0.90 ± 0.56*	82.1
S. gordonii			
Challis	Control	7.99 ± 5.44	
	10 s	3.05 ± 0.15*	61.8
	10 min	0.48 ± 0.16*	94.0
	30 min	0.37 ± 0.31*	95.4

Data of bacterial numbers adsorbed to s-HA are the means from three quintuple experiments with standard deviations.
*$P < 0.01$ as compared with control for respective bacteria.
[†]Buffered KCl was used for control (without cranberry).

Table 2. Effect of cranberry on cell surface hydrophobicity

Strain	Time of exposure	% of hydrophobicity
S. sobrinus		
6715	0	42.19 ± 11.90
	12.5	23.04 ± 3.76
	25	16.24 ± 10.04**
B13	0	24.07 ± 7.75
	12.5	−2.10 ± 4.50*
	25	−0.30 ± 5.71*
S. mutans		
MT8148R	0	58.52 ± 15.00
	12.5	28.90 ± 14.00**
	25	6.62 ± 16.45*
JC2	0	57.85 ± 13.01
	12.5	25.95 ± 1.14*
	25	12.43 ± 12.86*
Ingbritt	0	43.30 ± 7.37
	12.5	−1.28 ± 2.00*
	25	−4.44 ± 5.90*
ATCC 10449	0	88.79 ± 10.45
	12.5	17.50 ± 7.86*
	25	8.37 ± 6.10*
S. criceti		
E49	0	97.26 ± 1.32
	12.5	−3.93 ± 8.41*
	25	−6.10 ± 3.37*
S. sanguinis		
ATCC 10556	0	88.03 ± 5.44
	12.5	23.10 ± 3.30*
	25	8.02 ± 2.15*
S. oralis		
ATCC 10557	0	94.74 ± 2.52
	12.5	21.64 ± 6.50*
	25	2.83 ± 4.16*
S. mitis		
ATCC 9811	0	88.37 ± 4.61
	12.5	34.29 ± 10.05*
	25	−1.38 ± 2.14*
S. gordonii		
Challis	0	82.90 ± 8.86
	12.5	23.27 ± 32.41*
	25	−1.71 ± 6.10*

Data are the means from three duplicate experiments with standard deviation.
*$P < 0.01$ and **$P < 0.05$ as compared with 0 % of cranberry for respective bacteria respectively.

streptococcus strains are summarized in Table 3. The prepared high molecular weight constituents of cranberry juice clearly inhibited the biofilm formation of the streptococci. Biofilm formations by *S. mutans*, *S. criceti*, *S. oralis* and *S. mitis* were significantly inhibited by the cranberry constituents at both 100 μg/ml and 500 μg/ml concentration compared to control ($P < 0.01$). When the dose of the cranberry constituents was increased up to 500 μg/ml, the biofilm formations by *S. sobrinus* 6715 and *S. sanguinis* ATCC 10556 were significantly inhibited ($P < 0.05$).

Discussion

Cranberry juice has been demonstrated to inhibit the adherence of some bacteria (13, 32). Human daily consumption of cranberry juice can reduce urinary tract infections caused by *Escherichia coli* (1). High molecular mass constituents isolated from cranberry juice have been shown to inhibit the adhesion of *E. coli* and *H. pylori* (2, 13, 29). Howell et al. (7) found that high molecule mass proanthocyanidin (condensed tannin) from cranberry juice prevented the expression of the p-fimbriae of *E. coli* and inhibited its adherence activity. In addition, Weiss et al. (29) reported that a high molecular weight constituent of cranberry inhibited the adhesion and coaggregation activities of oral bacteria. These are known to be stable phenolic compounds that exhibit antiviral, antibacterial, antiadhesive, and/or antioxidant properties (18, 19). In an unpublished study, we found that our extracted high molecular weight

Table 3. Inhibitory effect of a high molecular weight constituent of cranberry on the biofilm formation of oral streptococci

Strain	Concentration of cranberry constituent (μg/ml)	Biofilm formation (OD_{595})
S. sobrinus		
6715	0 (Control)	0.311 ± 0.037
	100	0.290 ± 0.058
	500	0.278 ± 0.029**
S. mutans		
JC2	0 (Control)	0.337 ± 0.098
	100	0.221 ± 0.048*
	500	0.192 ± 0.026*
S. cricetí		
E49	0 (Control)	1.863 ± 0.252
	100	1.363 ± 0.309*
	500	1.229 ± 0.536*
S. sanguinis		
ATCC 10556	0 (Control)	0.209 ± 0.033
	100	0.192 ± 0.033
	500	0.180 ± 0.026**
S. oralis		
ATCC 10557	0 (Control)	0.920 ± 0.120
	100	0.531 ± 0.089*
	500	0.551 ± 0.188*
S. mitis		
ATCC 9811	0 (Control)	0.755 ± 0.204
	100	0.504 ± 0.101*
	500	0.522 ± 0.067*

Lyophilized high molecular weight constituent of cranberry was dissolved in trypticase soy broth. Data are means from three quintuple triplicate experiments with standard deviations.
*$P < 0.01$ and **$P < 0.05$ as compared with control for respective bacteria respectively.

constituents of cranberry juice did not kill *S. mutans* JC2 but that exposing oral streptococci to 25% cranberry juice for 10 s resulted in a significant reduction of their adsorption to s-HA beads. It is necessary to identify which major ingredients of this high molecular weight extract possess this inhibitory activity in a future study. Our results suggest that cranberry juice inhibits the colonization of streptococci in the initial phase of biofilm formation.

Cell surface hydrophobicity is one of the important factors involved in oral bacterial adherence to the tooth surface (27). Westergren et al. (30) showed that surface hydrophobicity-lacking mutant strains of *S. mutans* and *S. sanguinis* could not adhere to s-HA beads. The hydrophobicity of *S. mutans* is believed to be mainly associated with its cell surface proteins (12). Matsumoto et al. (10) have reported that oolong tea extract polyphenols may inhibit bacterial adherence to the tooth surface by reducing the cell surface hydrophobicity of mutans streptococci. The present study revealed that the cell surface hydrophobicity of some oral streptococci was reduced by the addition of the cranberry juice and that the reduction was dependent on the concentration of the juice. It is probable that the cranberry juice components bond to and/or mask the hydrophobic protein (s) on the cell surface of oral streptococci.

Dental plaque is a biofilm composed of polyspecies of bacteria, and the adhesive ability of these microorganisms seems to be an important pathogenic factor. Therefore, we investigated the inhibitory effect of cranberry juice on the biofilm formation of oral streptococci. The high molecular weight constituents of cranberry juice inhibited the biofilm formation of oral streptococci, including cariogenic strains, suggesting that the daily use of cranberry juice could inhibit dental plaque development.

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A 43-kDa protein of *Treponema denticola* is essential for dentilisin activity

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Abstract

A protease of *Treponema denticola*, dentilisin, is thought to be part of a complex with 43- and 38-kDa proteins. A sequence encoding a 43-kDa protein was located in the 3′ region of the *prcA* gene upstream of the dentilisin gene (*prtP*). The 43-kDa protein was apparently generated from digestion of PrcA. To clarify the function of the protein, we constructed a mutant of the 43-kDa protein following homologous recombination. The mutant lacked detectable dentilisin activity. Immunoblot analysis demonstrated that the dentilisin protein was degraded in the mutant. The results of real-time polymerase chain reaction suggested that *prtP* mRNA expression in the mutant was somewhat decreased compared with the wild-type strain. These data suggest that the 43-kDa protein is involved in the stabilization of the dentilisin protein.

Keywords: *Treponema denticola*; Protease; Operon; Stabilization

1. Introduction

Treponema denticola is predominantly detected in lesions of adult periodontitis and is associated with the development of this disease [1–3]. Recently, we reported that the DNA locus of *T. denticola* ribosomal RNA was amplified by polymerase chain reaction (PCR) in samples isolated from atherosclerotic lesions [4]. These microorganisms express several potential pathogenic factors such as an immunosuppressive factor, a major outer sheath protein and a serine protease [5–7]. The surface protease, which has been described as a chymotrypsin-like protease, is a major pathogenic factor of these microorganisms [8–12]. Previously, we characterized a serine protease gene (*prtP*) which encodes the protease domain of a chymotrypsin-like protease complex from *T. denticola* ATCC 35405 and designated the protease dentilisin [13]. Dentilisin is a 72-kDa protein in its mature form and hydrolyzes fibronectin, laminin, fibrinogen, type IV collagen, immunoglobulins, and bioactive peptides [14]. In addition, this protease was proposed to be associated with the adherence activity of *T. denticola* to fibronectin [15] and disruption of epithelial junctions [8]. The chymotrypsin-like protease is also suggested to express cytotoxic effects on porcine periodontal epithelial cells and to play a role in the adherence activities of the microorganism [9]. Based upon these properties, dentilisin apparently plays an important role in *T. denticola* colonization of, and migration into, host tissues.

Proteins of 43 kDa and 38 kDa were co-purified with dentilisin in the process of purification. These proteins associate together with dentilisin and the complex did not separate on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-denaturing conditions [13,16]. The gene encoding the 43-kDa protein is located immediately upstream of the *prtP* gene [13]. Therefore, the two proteins might be coordinately expressed leading to rapid association. Recently, Lee et al. [17] reported that the 43-kDa and 38-kDa proteins are expressed initially as a single protein which is processed into two proteins after translation. In the present study, we constructed a 43-kDa protein-deficient mutant to evaluate the role of this protein in dentilisin activity.

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Table 1
Bacterial strains and plasmids used in this study

	Relevant characteristics	Source or reference
Bacterial strains		
Treponema denticola ATCC 35405	Em^s	[25]
Treponema denticola K1	*prtP*::Em^r	[11]
Treponema denticola KO5	*prcA*::Em^r	This study
Escherichia coli HB101		
Plasmids		
pMCL191	Cm^r, medium-copy-number plasmid containing MCS and *lacZ* of pUC19	[11]
pV2198	Em^r	[20]
p431	Cm^r, pMCL191 containing the 5′ half of 43-kDa protein coding region of *prcA*	This study
p432	Cm^r, p431 with the 3′ half of 43-kDa protein coding region of *prcA*	This study
p43KO5	Em^r, Cm^r	This study
pKaz16	Km^r	[13]

2. Materials and methods

2.1. Microorganisms and plasmids

The microorganisms and plasmids used in this study are listed in Table 1. *T. denticola* ATCC 35405 was propagated in TYGVS medium [18] and was incubated at 37°C under anaerobic conditions as described previously [19].

2.2. Northern blot analysis and determination of the 5′ end of the gene

Total RNA from *T. denticola* was isolated by Trizol (Gibco BRL, Grand Island, NY, USA) extraction of late exponential phase cultures of *T. denticola*. The RNA was next treated with RNase-free DNase (Qiagen, Valencia, CA, USA) and isolated by RNeasy (Qiagen). The resulting RNA was electrophoresed through 1.0% agarose gels containing 2.2 M formaldehyde, 0.04 M 3-morpholino propanesulfonic acid (pH 7.0), 10 mM sodium acetate, 1.0 mM EDTA, and transferred to Hybond-N^+ paper (Amersham Pharmacia, Piscataway, NY, USA) by capillary transfer. RNA probes (2746-bp fragment of *prtP* corresponding to nucleotides 1641–4117 [13], Fig. 1A) were labeled with digoxigenin-dUTP using a DIG RNA labeling kit (SP6/T7) (Boehringer, Indianapolis, IN, USA). Hybridization and detection were performed as described previously [11]. To identify the transcription initiation site for the mRNA of the 43-kDa protein, 5′ rapid amplification of cDNA ends (RACE) reverse transcription (RT) PCR (Promega, Madison, WI, USA) was performed using primers 5UNIV, race 1 and race 2 (Table 2) according to the manufacturer's instructions. The fragment obtained was sequenced using oligonucleotide primers with the ABI 310A DNA sequencer (PE Biosystems, Foster City, CA, USA).

2.3. Construction of the 43-kDa protein mutant

The process for construction of the 43-kDa protein-deficient mutant is illustrated in Fig. 1A. The sequences of

Table 2
Oligonucleotide primers used in this study

Name of primer	Sequence	Locus
43KEC	5′-GGGGGGAATTCAATAGTAGGCTACGACGTTGCA-3′	*prcA*
43KPN	5′-GGGGGGGTACCGTTTTCGGGGGCTTTAAGGGA-3′	*prcA*
43BAM	5′-CCCCCGGATCCCGGCGGCCGAAGCTGCAAATA-3′	*prcA*
43PST	5′-CCCCCCTGCAGCTATATCCTTATCGTCAGGCTT-3′	*prcA*
TDRT1	5′-GTGTATTGGCGGTTGGAGCTA-3′	*prtP*
TDRT2	5′-CGACATAGGCCAAAGGAGCAT-3′	*prtP*
MspRT1	5′-GATGATTTTGGTGCTCGTGGGC-3′	*msp*
MspRT2	5′-CTGTTGTTCCTTTAGCTTTCCATG-3′	*msp*
TDTaq1	5′-TATGGGTAACGAAGGCCGTTTTCTCAGCCGAACCCGG-3′	*prtP*
TDTaq2	5′-AGCTCCAACCGCCAATACACTGCGGCATCACTTCGTGT-3′	*prtP*
TDTAQMAN	5′-CAGCCTATCCTGCAGCCTTCCCGAGGGACTGGTGCAGTTAGGCAGCG-3′	*prtP*
TD161	5′-ATGCGAATAAGCCCCGG-3′	16S rRNA
TD162	5′-CGCTCGCCCCTTACGTG-3′	16S rRNA
TD16TAQMAN	5′-ATTACGTGCCAGCAGCCGCGG-3′	16S rRNA
Race 1	5′-CAACGCTGAAGACGAAGGCTGC-3′	*prcA*
Race 2	5′-TCCCGATCCTCCCGTATAACCG-3′	*prcA*
5UNIV	5′-CTAATACGACTCACTATAGGGC-3′	

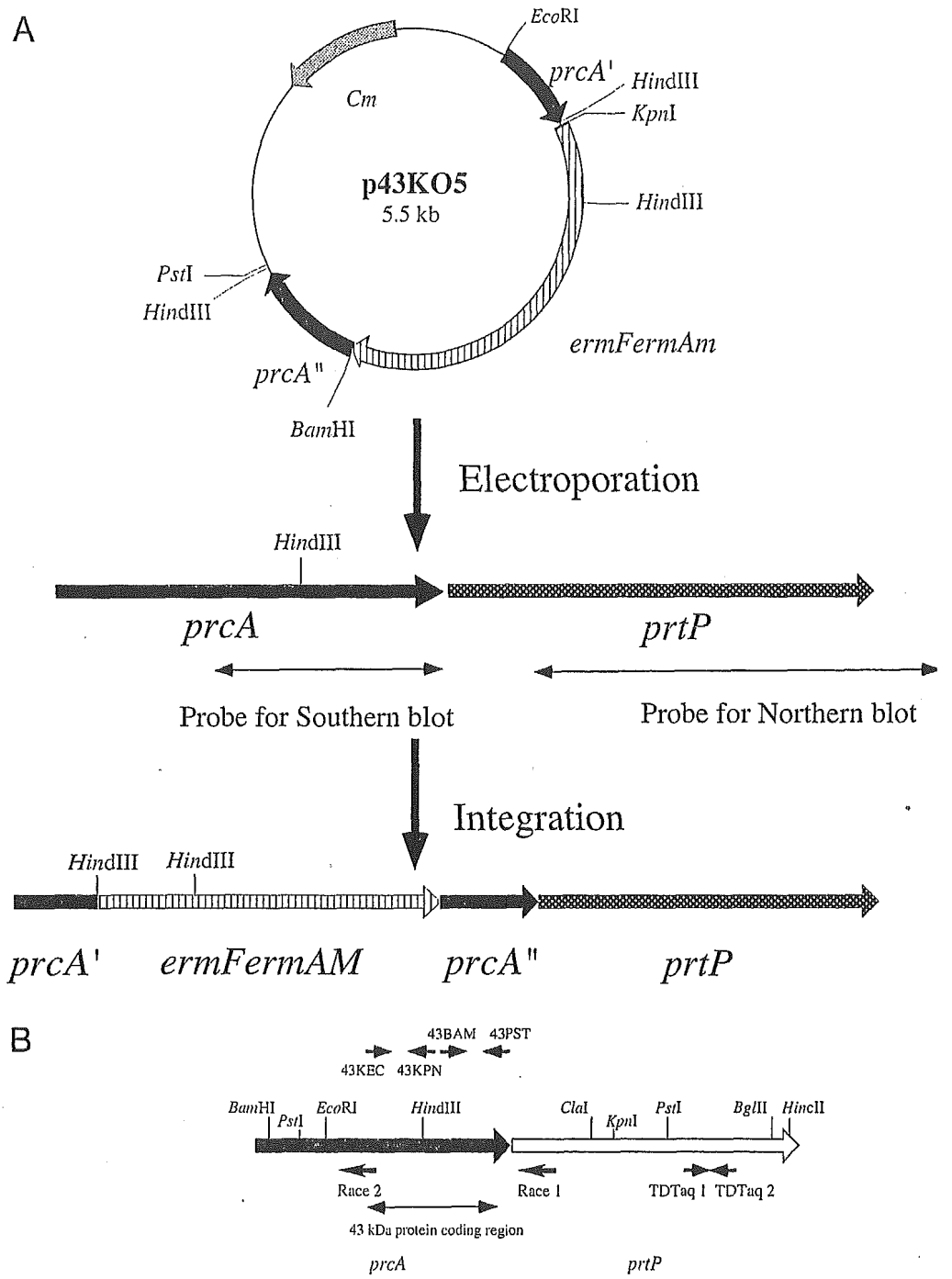

Fig. 1. A: Construction of the the 43-kDa protein mutant. Plasmid p43KO5 was linearized with *Eco*RI and electroporated into *T. denticola* 35405. B: Position of primers for *prcA* and *prtP*.

the 43-kDa protein were amplified by PCR using the synthetic oligonucleotide primers listed in Table 2. Amplification was performed as described previously [11]. Primers 43KEC, 43KPN, 43BAM and 43PST have *Eco*RI, *Kpn*I, *Bam*HI and *Pst*I restriction sites, respectively, at their 5′ ends (Fig. 1B). The amplified *Eco*RI-*Kpn*I fragment which contained the 5′ half of the 43-kDa protein coding region was inserted into pMCL191, and the plasmid obtained was designated p431. Next, the *Bam*HI-*Pst*I fragment which contained the 3′ region of the 43-kDa protein coding region was inserted into p431 producing plasmid p432. An *ermF-ermAM* cassette [20] was then isolated from plasmid pVA2198 following *Kpn*I-*Pst*I digestion and inserted into p432. The resulting plasmid, p43KO5, was linearized following *Eco*RI digestion and utilized in electroporation. Electroporation was carried out as described previously [21]. One mutant, designated KO5, was selected for further evaluation. Dentilisin activity was measured by *N*-succin-

yl-L-alanyl-L-alanyl-L-phenylalanine-*p*-nitroanilide hydrolysis as described previously [13]. To confirm the mutation, Southern hybridizations were performed as described previously [11]. The intact 43-kDa protein coding region was amplified with 43KEC and 43PST primers (Fig. 1B) and labeled with digoxigenin-dUTP using a PCR DIG probe synthesis kit (Boehringer Mannheim, Ridgefield, CT, USA) following the manufacturer's protocol.

2.4. Immunoblot analyses

Rabbit antiserum against dentilisin for immunoblot analysis was prepared as described previously [13]. The 72-kDa dentilisin protein band was observed by immunoblotting using *T. denticola* 35405 and the antiserum but no comparable band was observed in the dentilisin-deficient mutant [11]. We also confirmed the specificity of the antibody using purified dentilisin (data not shown). SDS–PAGE and immunoblotting were performed as reported earlier [11].

2.5. Evaluation of the expression of mRNA

To evaluate possible polar effects of the insertion of the *ermF-ermAM* cassette into the 43-kDa protein coding region, the relative expression of *prtP* mRNA was evaluated by RT-PCR and real-time PCR. Total RNA samples from *T. denticola* 35405 and KO5 were isolated as described above. RT-PCR was performed with the one-step RNA PCR kit (TaKaRa Biomedicals, Otsu, Japan) using primer pairs for *prtP* (TDRT1 and TDRT2) and *msp* (MspRT1 and MspRT2). Reverse transcription was carried out at 50°C for 30 min followed by inactivation at 95°C for 2 min. The PCR conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1.5 min. Taqman probes and PCR primers for 16S rRNA and *prtP* were designed by Primer Express (PE Biosystems). Quantification of mRNA was performed with real-time PCR using the Taqman probe and a TaqMan EZ RT-PCR Kit (real-time PCR, PE Biosystems). Briefly, the cDNA of *prtP* and 16S rRNA were synthesized from total RNA with Tth DNA polymerase at 50°C 30 min with primers TDTaq1 and TDTaq2 (Fig. 3B), and TD161 and TD162, respectively (Table 2). The PCR reaction was then performed with Tth DNA polymerase and Taqman probes, TDTAQMAN and TD16TAQMAN for *prtP* and 16S rRNA, respectively. The PCR conditions were initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s and 60°C annealing and elongation for 1 min. The quantities of the PCR products were measured by fluorescent activity using the ABI Prism 7700 sequence detector (PE Biosystems) in each PCR step. The 16S rRNA sequence was amplified as the endogenous RNA control and mRNA expression of each sample was normalized on the basis of its real-time PCR products compared with 16S rRNA. The final results were expressed as *prtP* gene expression relative to 16S rRNA as follows: relative expression = [(*prtP* in the mutant)/(*prtP* in the wild-type)]/[(16S rRNA in the mutant)/(16S rRNA in the wild-type)].

The nucleotide sequence of the *prcA* (*dagA*) gene has been assigned DDBJ accession number AB049977.

3. Results

3.1. Analysis of the dentilisin operon

To determine if the genes for the 43-kDa protein, which is located immediately upstream of the *prtP* gene [13], and dentilisin were cotranscribed, the size of the mRNA transcribed from this locus in strain 35405 was determined using the *prtP* gene as a probe. As shown in Fig. 2, the size of the mRNA of the *prtP* gene was approximately 5 kb. This size is sufficient to encode both the 43-kDa protein gene and *prtP* transcripts. In addition, several other smaller transcripts were also detected. Furthermore, the amplified products of the 5′ RACE reaction were approximately 2.2 and 1.0 kb (Fig. 3). This difference in size agrees with the distance between the race 1 and race 2 primers, which are located 133–154 bp from the start codon of *prcA* and 114–135 bp from the start codon of the *prtP* gene, respectively. The results from the sequencing of this region showed that one open reading frame existed upstream of *prtP*. This open reading frame is identical to *prcA* [17] which was identified during the course of the present investigation. The amplified fragment from the mRNA of the dentilisin operon was cloned and several clones were sequenced. The sequencing results of the clones indicated that the mRNA is initially transcribed

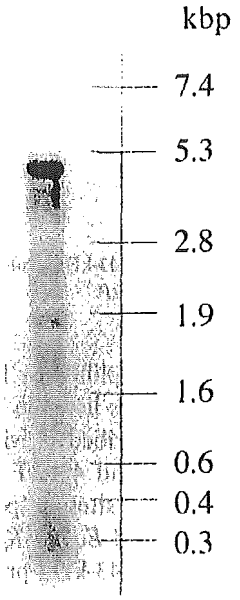

Fig. 2. Northern blot analysis of *prtP* expression in *T. denticola* 35405. The RNA probe was labeled with digoxigenin and T7 polymerase.

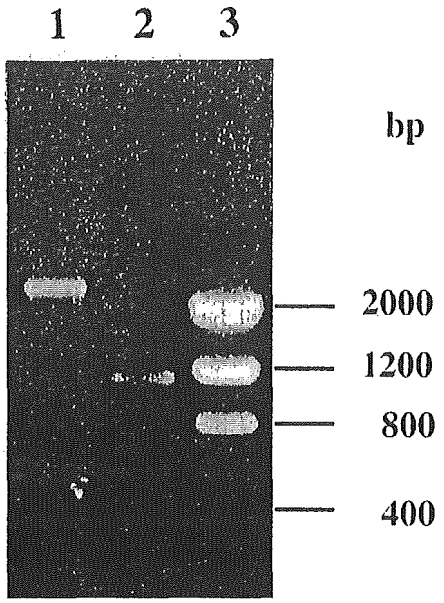

Fig. 3. Amplification of the 5′ terminus of mRNA encoding *prcA* and *prtP* in *T. denticola* 35405. Lane 1, amplified by primers 5UNIV and race 1; lane 2, amplified by primers 5UNIV and race 2; lane 3, molecular size marker.

at an A residue approximately 52 bases upstream of the ATG codon, although this has yet to be confirmed. This result suggested that transcription of the *prtP* operon was initiated approximately 2.2 kb upstream of the open reading frame of *prtP* and confirmed that the *prtP* and 43-kDa protein genes are organized in an operon.

3.2. Construction of the 43-kDa protein-deficient mutant

To determine the role of the 43-kDa protein in the physiology of *T. denticola*, an isogenic mutant defective in the 43-kDa protein coding region in *prcA* was constructed by allelic exchange mutagenesis (Fig. 1A). The 2.1-kb *ermF-ermAM* cassette was inserted into the *Kpn*I-*Bam*HI site of plasmid p432. The recombinant plasmid, p43KO5, was then linearized with *Eco*RI and electroporated into strain 35405. Since the plasmid was linearized, erythromycin-resistant transformants should arise as a result of a double-crossover event between the regions flanking the *erm* cassette and the wild-type gene on the chromosome. This would result in the insertion of the *ermF-ermAM* cassette into the middle of the 43-kDa protein region.

We obtained 11 Em^r colonies following 7 days of incubation. The efficiency of the recombination events was approximately 1.1 colonies per μg DNA. We isolated seven of the putative mutants and designated them KO1–KO7. To confirm the predicted recombination event, Southern blot analysis was carried out using KO5 (Fig. 4). Bands of 2.3 and 3.0 kb were observed for the wild-type 35405 strain (lane 1). On the other hand, 2.3- and 4.5-kb bands were observed for mutant KO5. The increase of 1.5 kb in the size of the larger band agreed with the size of the 3′ *Hin*dIII fragment from the inserted cassette and the orientation of the insertion of the cassette was confirmed by sequencing (data not shown). These results confirmed that the predicted recombination event had occurred resulting in the interruption of the wild-type 43-kDa protein coding region with the antibiotic resistance gene cassette. The insertion was also confirmed using PCR primers 43KEC and 43PST (data not shown). Identical results were also observed for the other six mutants. Therefore, one mutant, KO5, was chosen for further analysis.

3.3. Immunoblot analysis of the 43-kDa protein-deficient mutant

The dentilisin activities of sonicates of the 43-kDa protein-deficient mutant and the wild-type strain were 0.009 ± 0.002 (OD_{410} mg $protein^{-1}$ h^{-1}) and 3.36 ± 0.11 (OD_{410} mg $protein^{-1}$ h^{-1}), respectively. In addition, the effects on dentilisin of the mutation in the 43-kDa protein coding region were also examined by immunoblot analysis using anti-dentilisin rabbit serum which reacted strongly with dentilisin and weakly with approximately 50-kDa and 30-kDa proteins reported previously [11]. The protein profile of mutant KO5 is similar to that of *T. denticola* K1, the *prtP*-deficient mutant. As shown in Fig. 5, dentilisin was detected as a 72-kDa protein in the strain 35405 extract. Interestingly, the 72-kDa band was not detected in the 43-kDa protein-deficient *T. denticola* mutant KO5 while 60-kDa and 47.5-kDa bands were prominent. The cross-reactive band of approximately 30 kDa in the parental strain also was not detected in the mutant strain.

3.4. Expression of prtP mRNA

To determine if inactivation of the 43-kDa protein cod-

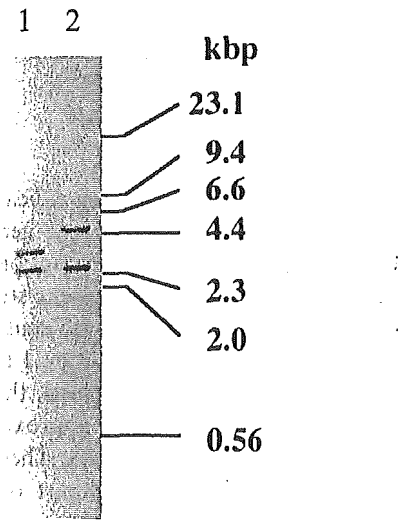

Fig. 4. Southern blot analysis of the 43-kDa protein-deficient mutant. Lane 1, *Hin*dIII-digested *T. denticola* 35405; lane 2, *Hin*dIII-digested KO5 genomic DNA. The probe was labeled with digoxigenin using PCR.

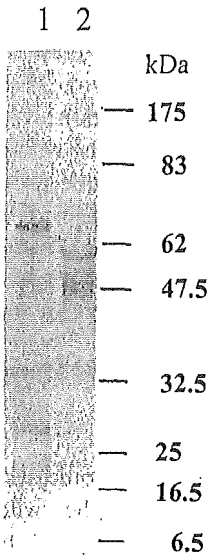

Fig. 5. Immunoblot analysis of the 43-kDa protein-deficient mutant. Lane: 1, *T. denticola* 35405; lane 2, mutant KO5. Antibody: anti-dentilisin rabbit serum.

ing region affected transcription of the *prtP* gene in KO5, RT-PCR was performed using total RNA from strains 35405 and KO5. As shown in Fig. 6, the *prtP* gene was detected by RT-PCR in both the parental strain and the mutant. Expression of the *prtP* gene is relatively weak compared with the *msp* gene which encodes the major outer sheath protein of this microorganism. No band for *prtP* mRNA was detected in the lanes without reverse transcriptase. The expression of mRNA from the *ermF-ermAM* cassette to *prtP* was confirmed by RT-PCR (data not shown). From the results from real-time PCR, the ratio of *prtP* mRNA/16S rRNA in the wild-type strain was approximately $1.0–4.0\times10^{-3}$ from early exponential phase to stationary phase. However, the expression level in the mutant was approximately one-half of that in the wild-type (mean ± S.D. of the expression of the *prtP* in the mutant is 42.4 ± 7.17% of the wild-type strain).

4. Discussion

Recent results in our laboratory indicated that the DNA sequence between the *prcA* and *prtP* genes did not reveal the presence of an obvious transcription terminator [13]. Northern blot analysis in the present study demonstrated that the mRNA expressed from the dentilisin gene is large enough to contain both *prtP* and *prcA* transcripts. The 5′ RACE RT-PCR results confirmed the cotranscription start site of these two genes as approximately 52 bp upstream of the ATG codon of *prcA*. These results together suggest that the two genes are part of a single operon structure. Purification of dentilisin resulted in the identification of two proteins, 43-kDa and 38-kDa proteins, which are associated with the 72-kDa dentilisin [13,16]. Lee et al. [17] showed that the PrcA protein was cleaved after translation and the resulting fragments were 39 kDa and 43 kDa in size in *E. coli*. They also showed that the intact PrcA protein (70 kDa) was detected in a dentilisin-deficient mutant. The molecular mass of the purified dentilisin complex was approximately 100 kDa and it consisted of 72-kDa, 42-kDa and 38-kDa proteins. The complex under non-denaturing conditions exists as duplex bands demonstrated previously [13]. These results suggested some kind of interaction between dentilisin and PrcA. Further analysis will be required to determine the mechanism of complex formation and role of PrcA in dentilisin activity.

Only insignificant levels of dentilisin activity were detected in the 43-kDa protein coding region knockout mutant. In addition, Western blot analysis revealed that the 72-kDa protein was not present. In the present results, RT-PCR analysis with the Taqman probe showed that the expression of the *prtP* gene in the 43-kDa protein-deficient mutant was 42.4% of the wild-type level. Therefore, these results suggested that the absence of detectable levels of the 72-kDa dentilisin protein was not the result of the attenuation of transcription of the *prtP* gene. However, we could not formally rule out effects on translation of the gene in the 43-kDa protein-deficient mutant. Nevertheless, since the 43-kDa protein has been normally associated with dentilisin, the most likely explanation for these results is that the 43-kDa protein stabilizes dentilisin from proteolytic degradation. It is also possible that in the absence of the 43-kDa protein, dentilisin may be subject to autodegradation. A protein band at approximately 28 kDa reactive with anti-dentilisin was observed in the wild-type strain; however, it was absent in the 43-kDa protein-defi-

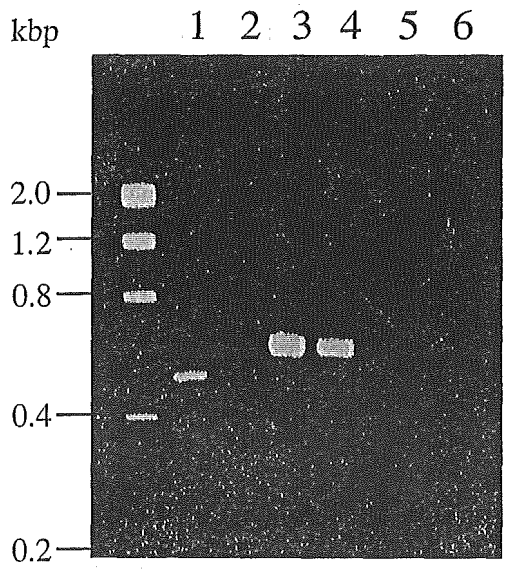

Fig. 6. RT-PCR analysis of *msp* and *prtP*. Lane 1, RT-PCR of the *prtP* gene of *T. denticola* 35405; lane 2, RT-PCR of the *prtP* gene of mutant KO5; lane 3, RT-PCR of the *msp* gene of strain 35405; lane 4, RT-PCR of the *msp* gene of mutant KO5; lane 5, RT-PCR of the *prtP* gene of *T. denticola* 35405 without reverse transcriptase; lane 6, RT-PCR of the *prtP* gene of mutant KO5 without reverse transcriptase.

cient mutant. This band may represent a degraded product of dentilisin and its absence in the mutant is compatible with autodigestion of dentilisin to produce a 28-kDa truncated protein. The increased intensities of the 60-kDa and 47.5-kDa bands in the mutant may correspond to degradation products of dentilisin, although the possibility of other cross-reactive bands has not yet been ruled out. These results suggest that the 43-kDa protein could play a chaperone function [22] in the secretion of dentilisin to the surface of *T. denticola*. However, most chaperone proteins have not been found in the final processed forms of secreted proteins so this potential role for the 43-kDa protein also appears unlikely.

Many *T. denticola* proteins were observed in high molecular mass complexes following non-denaturing SDS–PAGE analysis [23]. Haapasalo et al. [24] reported that the oligomeric form of Msp was resistant to hydrolysis by proteinase K but the monomers were protease-sensitive in buffer containing SDS. It is likely that dentilisin complex formation was also attenuated in the 43-kDa protein knockout mutant. Further analysis is required for the quantitation of dentilisin although a faint reactive band was observed by immunoblotting using anti-dentilisin antibody in the 43-kDa protein-deficient mutant. In this regard, decreased high molecular mass protein complex formation was observed in the dentilisin-deficient mutant [11]. Therefore, based on the present results, it would be predicted that the 43-kDa protein mutant should also be altered in Msp complex formation. However, further analysis is required to investigate the role of the 43-kDa protein in Msp complex formation.

The present results, taken together, suggest that the co-transcription of PrcA with dentilisin allows for rapid formation of protein–dentilisin complexes. This may stabilize dentilisin and protect the enzyme from degradation. The role of the associated 38-kDa protein in this processes is currently under investigation in our laboratory. In addition, the relationship of the dentilisin to Msp complex formation in *T. denticola* still remains to be determined.

Acknowledgements

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Short Communication

Cystatin SA, a cysteine proteinase inhibitor, induces interferon-γ expression in CD4-positive T cells

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Abstract

Recently, it has been demonstrated that family 2 cystatins upregulate interleukin-6 production by human gingival fibroblasts. In the present study, we investigated the effects of cystatin SA on cytokine production by helper T cells. Human CD4-positive T cells were cultured with phytohemagglutinin in the presence or absence of 0.1 μM recombinant cystatin SA1 or SA2. When the amounts of interleukin-4 (IL-4) and interferon-γ (IFNγ) were analyzed in an ELISA system after stimulation with either cystatin, no significantly increased levels of IL-4 were detected. However, the amounts of IFNγ were significantly increased after stimulation with the cystatins. Our results suggest that salivary family 2 cystatins are involved in immune responses through the cytokine network.

Keywords: cystatin; IFNγ; immune response; saliva; T cell.

Cystatins are in general tight-binding inhibitors of cysteine proteinases such as papain, ficin, and cathepsins (Barrett et al., 1986; Abrahamson, 1994; Saitoh et al., 1998). It has been demonstrated earlier that some cystatins possess antimicrobial activity (Korant et al., 1986; Björck et al., 1990; Takahashi et al., 1994; Naito et al., 1995; Blankenvorde et al., 1996, 1998; Collins et al., 1998). Several investigators have also suggested that some cystatins are effector molecules of immunomodulation (Leung-Tack et al., 1990; Verdot et al., 1996, 1999; Hartmann et al., 1997; Kato et al., 2000). Gingival tissues are always bathed in saliva, and cystatins S, SN, SA and D are abundant in saliva (Dickinson, 2002). Salivary cystatins demonstrate very different properties *in vitro*. Baron et al. (1999) have shown that cystatin SA is able to inhibit cathepsins C and L (K_i values of 1.1 μM and 7.3 nM, respectively), whereas cystatin S has little or no inhibitory activity toward these same cysteine proteases *in vitro*. It has been noted that there are genetic polymorphisms at the *CST2* locus coding for human cystatin SA and two alleles, *CST2*1* (f=0.965) and *CST2*2* (f=0.035), of this gene locus have been characterized (Saitoh et al., 1998). Recently, the family 2 cystatins have been shown to upregulate interleukin-6 (IL-6) production by human gingival fibroblasts (Kato et al., 2000). One of our previous studies (Kato et al., 2002) demonstrated that cystatins SA1 and SA2 adhere to human fibroblasts, and that this event results in tyrosine phosphorylation and upregulation of the release of IL-6-mediated enhancement of NF-κB activity. Verdot et al. (1999) have demonstrated that chicken cystatin induces the synthesis of TNFα and IL-10 and that these cytokines stimulate the production of nitric oxide by interferon-γ-activated mouse peritoneal macrophages. In the present study, we investigated the effect of salivary family 2 cystatins on cytokine production by helper T cells.

We had already established a system for preparing large amounts of recombinant cystatins SA1 and SA2 (Saitoh et al., 1998), and therefore all the experiments in the present study were carried out using these recombinant cystatins as salivary cystatins. Cystatin SA1 is identical to cystatin SA (Isemura et al., 1986), and cystatin SA2 is a variant that carries two amino acid substitutions: ^{59}Gly→Asp and ^{120}Glu→Asp (Shintani et al., 1994; Haga and Minaguchi, 1999). Human peripheral blood mononuclear cells (PBMC) from healthy volunteers were separated using Ficoll-Hypaque gradients. $CD4^+$ T cells were obtained from PBMC by positive selection using magnetic beads coated with anti-CD4 antibodies (Dynabeads CD4; Dynal Inc., Lake Success, USA). Briefly, PBMC were incubated with antibody-coated beads for 1 h at room temperature, and $CD4^+$ T cells were selected using a magnet. The magnetic beads were finally removed from the $CD4^+$ T cells using DETACHaBEAD (Dynal). The purity of the $CD4^+$ T cells isolated by this method is usually >99% with <1% monocytes. $CD4^+$ T cells were cultured in complete RPMI 1640 medium containing 10% fetal calf serum, penicillin, streptomycin, and glutamine in a humidified atmosphere with 5% CO_2 at 37°C. T cell preparations were used within 4 h of isolation. $CD4^+$ T cells (10^5/well) were cultured with phytohemagglutinin (PHA; 2 μg/ml) in the presence or absence of recombinant cystatin SA1 (0.1 μM) or SA2 (0.1 μM) and recombinant human interleukin-12 (rhIL-12; 10 pg/ml; Genzyme/Techne, Minneapolis, USA). Cell culture flat-bottom 96-well plates were used in this study. The concentration of cystatins SA1 and SA2 used here,

Table 1 IL-4 and IFNγ levels in supernatants of PHA-treated $CD4^+$ T cells stimulated by cystatin SA1 or SA2.

	Cytokine concentration (pg/ml)			
	IL-4		IFNγ	
	17 h	48 h	17 h	48 h
Control	6.2±5.1	5.4±3.2	12.3±2.5	15.2±5.1
Cystatin SA1 (0.1 μM)	5.8±3.5	6.4±5.3	18.9±3.1	32.2±5.4[a]
Cystatin SA2 (0.1 μM)	5.2±1.7	5.5±2.5	16.2±2.2	28.2±3.1[a]

[a] $p<0.05$ vs. control (Mann-Whitney test).
Data are mean values±standard deviations.
PHA: phytohemagglutinin.

0.1 μM, corresponds to the physiological concentration in saliva (Henskens et al., 2000). The amounts of IL-6 produced by human gingival fibroblast cell lines were significantly increased after stimulation with cystatins SA1 and SA2 at this concentration (Kato et al., 2000). Culture supernatants were isolated at 17 h and 48 h and analyzed for interleukin-4 (IL-4) and interferon-γ (IFNγ) by an ELISA system (Endogen Inc., Woburn, USA). Statistical analysis was performed by Mann-Whitney *U* test, and *p* values less than 0.05 were considered significant. When the amounts of IL-4 and IFNγ were analyzed after stimulation with cystatin SA1 or SA2, no significantly increased levels of IL-4 were detected (Table 1; data are presented as mean±standard deviations). However, the amounts of IFNγ at 48 h were significantly increased after stimulation with cystatin SA1 or SA2 at 0.1 μM ($p<0.05$). PHA-activated $CD4^+$ T cells produced IFNγ after stimulation with cystatin SA1 or SA2. Generally, immune responses are categorized as T helper type 1 (Th1)- and T helper type 2 (Th2)-type activities. IFNγ is considered to account for an important characteristic of the Th1 response (Boem and Howard, 1997). Because IL-12 is a potent inducer of IFNγ production and Th1 differentiation (Lamont and Adorini, 1996; Magram et al., 1996), the addition of rhIL-12 to the cell cultures enhanced the production of IFNγ by $CD4^+$ T cells (Table 2). The addition of cystatin SA1 or SA2 to the cultures enhanced the production of IFNγ by $CD4^+$ T cells. Costimulatory molecules are essential for inducing maximal T cell cytokine secretion, proliferation, and induction of effector function (Croft and Debey, 1997). In the present study, the human gingival fibroblast cell line IKG-1 (Kato et al., 2002) was used as the source of accessory cells. IKG-1 cells were seeded at a density of 10^5 cells/well in the cell culture medium for 1 day before coculture. Coculturing of PHA-activated $CD4^+$ T cells with IKG-1 fibroblasts greatly increased the levels of IFNγ (Table 2).

In order to elucidate the binding of salivary cystatins to $CD4^+$ T cells, anti-cystatin SA1 mouse serum (Kato et al., 2002) was used. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 to 20% gradient micro-slab gels (Daiichi Pure Chemical Co., Tokyo, Japan) with the Laemmli buffer system (Laemmli, 1970). $CD4^+$ T cells were dissolved in the loading buffer and applied onto the gels. For immunoblotting analysis, the separated proteins were transferred to PVDF membranes (Millipore Co., Bedford, USA) using Trans-Blot SD (Bio-Rad Laboratories). Each membrane was washed with phosphate-buffered saline (pH 7.2)/0.05% Tween 20 (PBS-T) three times for 15 min and then placed in a blocking solution of PBS-3% skim milk and incubated for 1 h at room temperature. The membrane was incubated with the recombinant cystatin SA1 overnight at 4°C. The control membrane was incubated in PBS without the recombinant cystatin. After washing, the membranes were incubated with anti-cystatin SA1 mouse serum (diluted 1:1000 with PBS) for 2 h at 37°C and washed three times with PBS-T. Non-immunized mouse serum was used as a control serum. After being probed with the antiserum and washed with PBS-T, the membranes

Table 2 IFNγ levels in supernatants of PHA-treated $CD4^+$ T cells.

	rhIL-12	HGF	IFNγ concentration (pg/ml)	
			17 h	48 h
Control	−	−	12.3±2.5[a]	15.2±5.1[c]
	+	+	18.7±12.3	73.5±22.5[d]
Cystatin SA1 (0.1 μM)	−	−	18.9±3.1	32.2±5.4[e]
	+	−	24.6±10.3	50.5±15.9[e]
	+	+	75.4±16.1[b]	139.3±20.2[f]
Cystatin SA2 (0.1 μM)	−	−	16.2±2.2	28.2±3.1[e]
	+	−	21.8±8.4	32.8±16.3[e]
	+	+	70.9±12.2[b]	121.1±15.2[f]

([a]) vs. ([b]), ([c]) vs. ([d]): $p<0.01$ (Mann-Whitney test).
([c]) vs. ([e]): $p<0.05$ (Mann-Whitney test).
([c]) vs. ([f]): $p<0.001$ (Mann-Whitney test).
Data are mean values±standard deviations.
PHA: phytohemagglutinin.
rhIL-12: recombinant human interleukin-12.
HGF: human gingival fibroblast cell line IKG-1.

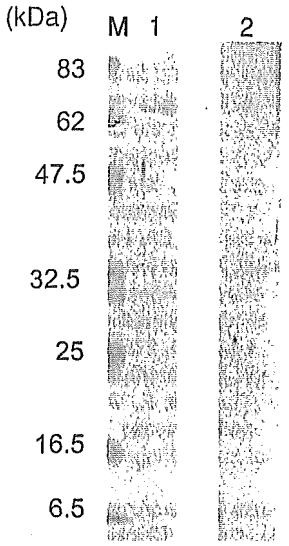

Figure 1 Immunoblotting analysis for binding of cystatin SA1 to CD4$^+$ T cells.
CD4$^+$ T cells were dissolved in the loading buffer and applied onto the gel. The separated proteins were transferred to PVDF membranes, which were incubated with the recombinant cystatin SA1 overnight at 4°C. The control membrane was incubated without cystatin. After washing, the membranes were incubated with diluted anti-cystatin SA1 mouse serum. After incubation, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulins. Finally, the membranes were developed. Lanes: M, molecular mass markers; 1, incubation with cystatin SA1; 2, incubation without cystatin SA1.

were incubated with horseradish peroxidase-conjugated anti mouse immunoglobulins (IgG, IgM and IgA; Cappel Laboratories, Cochranville, USA). Finally, the color reaction on the membranes was developed. Immunoblotting analysis using anti-cystatin SA1 serum revealed that cystatin SA1 bound to CD4$^+$ T cells had a molecular mass of approximately 70 kDa (Figure 1, lane 1), suggesting that cystatin SA adhered to CD4$^+$ T cells and that this binding resulted in upregulation of the release of IFNγ. The anti-cystatin SA1 mouse serum did not react with CD4$^+$ T cell lysate (Figure 1, lane 2). When the control serum was used in the immunoblotting assay, no reactive band was found (data not shown). Our previous study (Kato et al., 2002) has demonstrated that cystatins SA1 and SA2 adhere to human fibroblasts through cell surface molecules, mainly CD58. The target protein of cystatin SA in CD4$^+$ T cells may be immunoglobulin superfamily adhesion molecule(s).

Halfon et al. (1998) have proposed that cystatin F (leukocystatin) plays an immunomodulatory role. It is highly probable that family 2 cystatins can play a role in the immune defense system. The present study indicates that cystatins SA1 and SA2 upregulate IFNγ production in CD4$^+$ T cells. Lower levels of IFNγ and interleukin-2 (IL-2) have been shown to be present in periodontal disease lesions (Gemmel and Seymour, 1994, 1998). The induction of IFNγ by salivary cystatins may be important in the protection of the oral cavity against dysregulation of cytokine networks. The results presented here suggest that salivary family 2 cystatins are involved in immune responses through the cytokine network.

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Need for Procedural Details in Detection of Periodontopathic Bacterial DNA in the Atheromatous Plaque by PCR

We read with interest the recent paper published by Ishihara and colleagues (1) in which the authors detected the presence of DNA from periodontopathic bacteria in stenotic artery plaques and in samples obtained from periodontal pockets. This group has already published a report describing the detection of *Treponema denticola* in atherosclerotic lesions by using both PCR and polyclonal serum-based immunofluorescence assay techniques (2).

We agree with the conclusion of Ishihara et al. that these results, taken together with the large amount of already-published epidemiological, microbiological, and clinical data, give support to the hypothesis that periodontopathic bacteria could, in some way, play a role in the pathogenesis of the atheromatous plaque. Anyway, we feel that some additional details could add value to the data presented in this paper.

At first, the title of the paper refers to "carotid coronary artery plaque," whereas in the text the authors wrote that ". . . we sought to detect. . .DNA from stenotic coronary artery plaques. . .," and no mention is made anywhere to vascular samples obtained from the carotids. This is in our opinion a point that need to be clarified, since the presence of bacteria could be different in the two vascular segments, and if no specimens from the carotids were studied, there is no reason to include this word in the title of the paper. In addition, in case the authors have been using only vascular fragments obtained from the coronary artery, it could be very useful to describe which segment of these arteries was studied and what have been both the clinical diagnosis and surgical procedures for the patients studied, since it is quite uncommon for the stenotic segment of the coronary artery to be removed during a bypass procedure with a vascular graft. Moreover, the addition of some details about the well-known risk factors for atherosclerosis, like cholesterol plasmatic level and smoking, for the patients that gave the vascular fragments used could increase the significance of these results. Another point that in our opinion needs to be clarified is the real significance of the negative controls used in this study. In fact, 2 clinically unrelated negative vascular samples are quite a disproportionate number when correlated to the 51 specimens obtained from the atherosclerotic patients. In addition, one of these two negative control vascular samples gave a positive result for the presence of *Actinobacillus actinomycetemcomitans* and for *Campylobacter rectus*, with the results of the dental examination of these two patients being totally negative and with, as far as we know, the etiology of the Kawasaki syndrome not being strictly related to any bacterial infection. These findings, which demonstrated the presence in the coronary artery of DNA from periodontopathic bacteria in a nonatherosclerotic and periodontally healthy patient, could suggest that these microorganisms are unrelated to the presence of atheromatous plaque. A last point that should benefit from additional details is the statistical analysis performed to assess the correlation between the rates of detection for DNA in periodontal and vascular specimens. In fact, it is quite difficult to understand why the correlation was statistically significant only in the case of detection of *Porphyromonas gingivalis* and *C. rectus* DNA and not for other bacteria, such as, for instance, *T. denticola*, which was detected in a very high percentage of samples (67.7 and 29.4% in subgingival and artery specimens, respectively, in patients showing four or more periodontal lesions). Further clarification of the above points would allow the reader to consider more deeply the results and conclusions of this interesting study, which could improve knowledge of the various factors that may have a role in atherogenesis.

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Authors' Reply

In the comment, Sambri and colleagues pointed out that no mention is made anywhere to vascular samples obtained from the carotids. In our experiment, the specimens were taken from the coronary artery wall at the time of bypass grafting (1). We isolated the segment upstream from the coronary artery lesion during the bypass procedure with a vascular graft. We had not always prepared "atheromatous plaque" for each sample. Most of the samples were obviously degenerated, but they sometimes looked quite normal. However, these samples usually contained diffuse atherosclerosis lesions from stenotic artery plaque even when appearing normal. Furthermore, the sampled coronary vascular surface is too small to evaluate both the existence of microorganisms and the histological conditions. In this experiment, we used all of the specimens for

detection of microorganisms because we intended to clarify the relationship between the existence of oral pathogens in periodontal pockets and stenotic plaque. In this context, the phrase "carotid coronary stenotic artery plaque" is correct.

We examined total cholesterol levels in plasma from 35 out of 51 patients and found that 6 patients' levels were higher than the basal range for healthy adults. We also examined the smoking experience of 30 of 51 patients in the study. Present smokers (22.9%) belonged to the patient group possessing four or more periodontal pockets. The ratio of smoker and past smoker in patients with fewer than four periodontal pockets and for those harboring four or more periodontal pockets was 57.7 and 44.4%, respectively. The plasma cholesterol level was 196.2 ± 36.18 mg/dl. This value is in the range for normal patients. The levels for patients with fewer than four periodontal pockets and for those harboring four or more periodontal pockets were 194.6 ± 39.35 and 200.5 ± 26.79 mg/dl, respectively. No statistically significant difference was observed between the two groups. This indicated that the increase in the detection rate for periodontal pathogens in stenotic lesions is associated with the extent of periodontal lesions.

One of the comments questioned the significance of the control samples. It is true that we have to use a relatively large number of control samples, but samples from healthy sites cannot be obtained because of ethical considerations. In our experiment, we intended to compare the detection levels for periodontopathic bacteria from the coronary artery in patients with different periodontal conditions. We included the Kawasaki syndrome patients to show the low detection rate for periodontopathic bacteria as additional preliminary information because Kawasaki syndrome is not directly related to any bacterial species, and the patients usually do not suffer from periodontitis. We did not treat the data as an additional negative control. *A. actinomycetemcomitans* and *C. rectus* were detected from one patient, but neither was detected from the other patient. The number of bacterial species detected for Kawasaki syndrome patients was apparently low compared with that for the 51 stenosis patients. To verify the detection rates, we intended to obtain samples from patients with Kawasaki disease, but we could not utilize more patients in our experiment. Further analysis, increasing the sample size from patients with Kawasaki disease, is obviously required.

The results indicated that only *P. gingivalis* and *C. rectus* were related to detection in the oral cavity and coronary artery samples. Other microorganisms, such as *T. denticola*, were not correlated with detection in both the oral cavity and coronary artery. *T. denticola* is also detected in high numbers in stenotic lesions. The microflora of periodontal lesions is not the same in each lesion. In this experiment, we sampled two sites/patient but not from all periodontal lesions. It is possible that an individual sampled periodontal pocket does not harbor a specific microorganism. Increasing the number of sampling sites will be required to clarify these issues.

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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-