

# Parotid secretory protein is expressed and inducible in human gingival keratinocytes

Hideki Shiba<sup>1,2</sup>, S. G. Venkatesh<sup>1</sup>,  
Sven-Ulrik Gorr<sup>1</sup>, Germán Barbieri<sup>1</sup>,  
Hidemi Kurihara<sup>2</sup>, Denis F. Kinane<sup>1</sup>

<sup>1</sup>Oral Health and Systemic Disease, University of Louisville School of Dentistry, Louisville, Kentucky, USA and <sup>2</sup>Department of Periodontal Medicine, Division of Frontier Medical Science, Hiroshima University Graduate School of Biomedical Sciences, Minami-ku, Hiroshima, Japan

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**Background:** Parotid secretory protein (PSP) is a major salivary protein that is thought to possess both antibacterial and anti-inflammatory activity. A major question is whether PSP expression can be regulated by humoral factors and bacteria. Periodontitis is an inflammatory lesion initiated by interaction between gingival keratinocytes and periodontopathogenic microorganisms such as the Gram-negative anaerobe *Porphyromonas gingivalis*. Cytokines and sex hormones have been implicated in the progression of various forms of periodontal diseases.

**Materials and methods:** We investigated the expression of PSP and its regulation in primary cultures of human gingival keratinocytes (HGK). HGK at the third or fourth passage were exposed to heat-killed *P. gingivalis*, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and 17 $\beta$ -estradiol. The PSP mRNA levels were examined by real-time polymerase chain reaction (PCR). The protein expression of PSP was confirmed by immunofluorescence.

**Results:** Heat-killed *P. gingivalis*, TNF- $\alpha$  and 17 $\beta$ -estradiol all resulted in increased HGK levels of mRNA for PSP as determined by real-time PCR analysis. Immunofluorescence demonstrated increased PSP localized within the cytoplasm of HGK following exposure to killed *P. gingivalis*.

**Conclusion:** The present study has demonstrated for the first time that PSP is expressed in keratinocytes and that it can be up-regulated by bacteria and humoral factors. Thus PSP may have a role in the innate defense system at the gingival epithelial surface.

Hideki Shiba, Oral Health and Systemic Disease, University of Louisville School of Dentistry, 501 S Preston, Louisville, Kentucky 40292, USA  
Tel: +1 502 852 3175  
Fax: +1 502 852 1317  
E-mail: h0shib02@louisville.edu and bashihi@hiroshima-u.ac.jp

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Parotid secretory protein (PSP) is a major salivary protein (1–3) that is also termed short palate, lung and nasal epithelium clone 2 (SPLUNC2) (4). PSP belongs to family of mammalian lipid-binding proteins that includes bactericidal/permeability-increasing protein and lipopolysaccharide-binding protein (4–6). PSP-derived peptides inhibit the binding of endotoxin to lipopolysaccharide-binding protein

and inhibit the endotoxin-stimulated secretion of tumor necrosis factor (TNF)- $\alpha$  from macrophages (3). Thus, PSP may function as an oral antibacterial and anti-inflammatory protein, but the question remains as to whether PSP can be regulated by environmental or endogenous factors.

Periodontitis is an inflammatory lesion initiated by periodontopathogenic, predominantly Gram-negative

bacteria that colonize the gingival sulcus region. Gingival keratinocytes are the primary defense against the bacterial infection. Gingival keratinocytes respond to the bacteria by producing inflammatory factors such as TNF- $\alpha$ , interleukin-8 and antimicrobial peptides such as  $\beta$ -defensin-1 and -2 (7–9). The antimicrobial and inflammatory responses to the bacteria are considered important in regulating the

inflammation that characterizes gingivitis and periodontitis. An important question is whether gingival epithelium or indeed any keratinocytes can express PSP constitutively or inducibly.

*Porphyromonas gingivalis* is considered an important periodontopathogenic bacterium (10). Estrogen and TNF- $\alpha$  are known to be involved in the progression of periodontal inflammation (11–14). In the present study, we investigated the PSP expression and its regulation by *P. gingivalis*, TNF- $\alpha$  and 17 $\beta$ -estradiol in cultures of human gingival keratinocytes (HGK) to address whether PSP was present in gingival keratinocytes and whether it could be induced by bacteria and soluble agents.

## Material and methods

### Preparation of cells

Two portions of healthy gingival tissues, obtained surgically following wisdom tooth extraction, were collected from two subjects in a protocol approved by the IRB at the University of Louisville. The gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C and HGK were isolated as previously described (7). The cell suspension was centrifuged at 120 g for 5 min, and the pellet was suspended in MCDB153 medium (pH 7.4) (Sigma, St. Louis, MO, USA) containing 10  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 10  $\mu$ M 2-mercaptoethanol, 10  $\mu$ M 2-aminoethanol, 10 nM sodium selenite, 50  $\mu$ g/ml bovine pituitary extract, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 50 ng/ml amphotericin B (medium A) (7). The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO<sub>2</sub>/95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured.

### Bacterial strains and conditions

*P. gingivalis* 33277 was kindly provided by Dr Don Demuth (University of Louisville). The organisms were grown at 37°C in trypticase soy broth supplemented with 1 g of yeast extract,

5 mg of hemin and 1 mg of menadione per liter under anaerobic conditions of 85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5%CO<sub>2</sub> for 2 days. After cultivation, the bacteria were harvested by centrifugation, washed three times in phosphate-buffered saline, heat-inactivated for 1 h at 60°C and suspended in MCDB153 medium containing 10  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 10  $\mu$ M 2-mercaptoethanol, 10  $\mu$ M 2-aminoethanol and 10 nM sodium selenite (medium B).

### RNA preparation

HGK in cultures at the third or fourth passage were harvested, seeded at a density of 5  $\times$  10<sup>4</sup> cells/6-well culture plate coated with type I collagen, and maintained in 2 ml of medium A. After 6 days, the confluent cultures were washed twice with phenol red-free Hank's solution (pH 7.4) and heat-killed *P. gingivalis* (5  $\times$  10<sup>7</sup> cells/ml) suspended in 2 ml of medium B were added to the HGK cultures. Incubation was carried out for 0, 3 and 24 h. HGK were also exposed to 17 $\beta$ -estradiol (Sigma) at 1 or 10 nM and TNF- $\alpha$  (R & D Systems, Minneapolis, MI, USA) at 1 or 10 ng/ml for the same time periods. These concentrations are typical of those used for other published keratinocyte studies (15, 16). RNA from each culture was extracted using TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) and quantified by spectrometry at 260 and 280 nm.

### Reverse transcriptase–polymerase chain reaction (RT-PCR) and sequencing PCR products

Total RNA (4  $\mu$ g) was reverse-transcribed using oligo (dT) 15 (Roche Diagnostics Corp., Indianapolis, IN, USA). Reverse transcription was performed for 60 min at 42°C and stopped by heat inactivation for 5 min at 99°C. The primers for PSP were designed according to the human PSP sequence (GenBank, Accession no. AF432917). Using the upstream (5'-ATGCTTCAG CTTTGGAACTTGTTCTCCTG-3') and downstream (5'-GATGAGGGTT TGCAGCTGGGTTTTGTGCTG-3') primers, human PSP cDNA was amplified under the following

conditions: 94°C for 30 s, 63°C for 1 min, 72°C for 2 min for a total of 35 cycles. The PSP cDNA was sequenced using CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA, USA).

### RT-PCR

RT-PCR with cDNA was performed with an ABI 7900 system (Applied Biosystems, Foster City, CA, USA). TaqMan probes, sense primers and antisense primers for gene expression of human PSP were purchased from Applied Biosystems along with probes and primers for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Using a Universal Master Mix (Applied Biosystems), the reactions were carried out according to the manufacturer's protocol.

### Cellular localization of PSP by confocal laser scanning microscopy

HGK in cultures at the third or fourth passage were harvested, seeded at a density of 1  $\times$  10<sup>4</sup> cells per well on a chamber slide (Nalge Nunc International, Naperville, IL, USA) coated with type I collagen and maintained in 0.5 ml of medium A. After 6 days, the confluent cells were washed thrice with phenol red-free Hank's solution (pH 7.4) and exposed to heat-killed *P. gingivalis* 33277 (5  $\times$  10<sup>7</sup> cells/ml) suspended in 0.5 ml of medium B. After incubation for 24 h, the medium and killed bacteria were removed and the cells were prepared for labeling with antibody to the C-terminal peptide of human PSP. Custom polyclonal antibody to the C-terminal peptide of human PSP, raised in rabbit, was obtained from Sigma Genosys (The Woodlands, TX, USA) (3). The antiserum obtained from the fourth bleed was directly employed for immunostaining of the cells. All incubations and washes were carried out in phosphate-buffered saline at room temperature. The cells were washed three times after each incubation. The cells were fixed for 20 min in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 15 min. Blocking was performed with 1% bovine

serum albumin for 60 min. The cells were incubated for 60 min with rabbit antiserum to human PSP C-terminal peptide at a dilution of 1 : 50 (3). Preimmune rabbit serum was also utilized as a negative control. The cells were then incubated with the fluorescein-labeled secondary antibody (sheep anti-rabbit IgG FITC conjugate, 1 : 1000 dilution) (Silenus Labs Pty. Ltd, Melbourne, Australia) for 60 min. Immunofluorescent images were obtained with a Confocal Laser Scanning Microscope (FV500) (Olympus, Melville, NY, USA).

### Statistical analysis

Statistical analyses of the data were performed using the Student's *t*-test.  $p < 0.01$  was accepted as the threshold for statistical significance.

### Results

The primary cultures of HGK were > 99% keratinocytes under the present conditions as evidenced morphologically by microscopic examination (data not shown) (7). Furthermore, the keratinocyte phenotype was further confirmed by immunoblotting with anti-cytokeratin type I and II as described in a previous study (7).

PCR amplification of HGK cDNA with PSP specific primers showed a single band of 747 bp (data not shown). Sequence analysis of the PCR product showed complete identity with the reported human PSP sequence (data not shown). Figure 1 shows PSP expression in HGK following exposure to heat-killed *P. gingivalis* 33277. A low level of basal expression of PSP mRNA was observed in HGK. However, upon exposure to the bacteria, there was an induction in the mRNA levels of PSP. Maximum expression was observed at 3 h, followed by a drop to near-basal levels at 24 h (Fig. 1A). This result was confirmed by confocal immunofluorescence microscopy. Immunofluorescence analysis for PSP expression showed a punctuate cytoplasmic localization of the protein, suggesting that the protein is localized in cytoplasmic organelles and vesicles (Fig. 1B, a). Following exposure to *P. gingivalis*

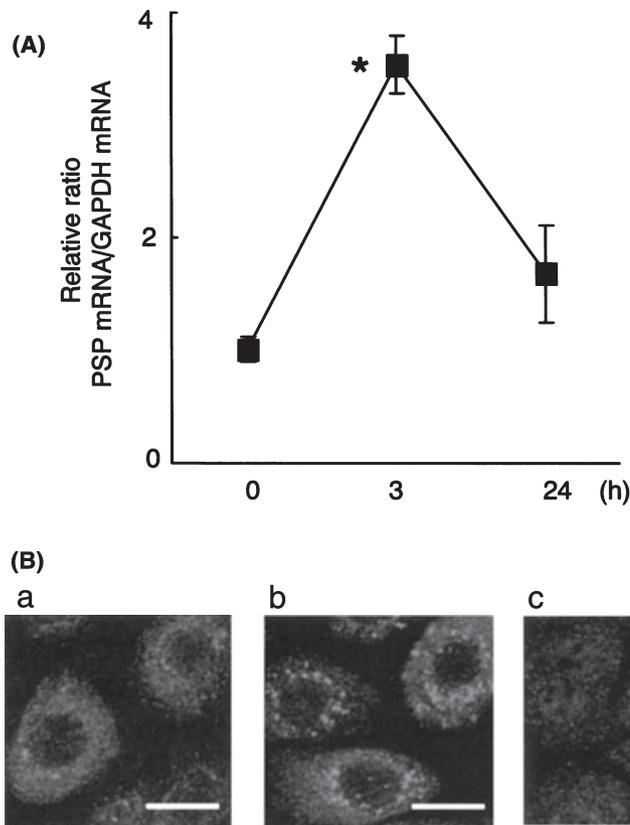


Fig. 1. Parotid secretory protein (PSP) expression in human gingival keratinocytes (HGK) cultures stimulated with killed *Porphyromonas gingivalis*. (A) PSP mRNA expression. HGK were exposed to killed *P. gingivalis* 33277 ( $5 \times 10^7$  cells/ml) for the indicated times before the end of incubation on day 7. PSP mRNA expression was determined by real-time polymerase chain reaction (PCR). The graph shows the ratio of PSP mRNA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA from the results of real-time PCR. Values are the means  $\pm$  SD of three cultures. Differs significantly ( $*p < 0.01$ ) from no exposure to killed *P. gingivalis* 33277. (B) Cellular localization of PSP. HGK were exposed to killed *P. gingivalis* 33277 ( $5 \times 10^7$  cells/ml) for 24 h before the end of incubation on day 7. Immunofluorescent images were obtained by confocal laser scanning microscopy. (a) control HGK incubated with rabbit anti-PSP serum; (b) killed *P. gingivalis* 33277-exposed HGK with rabbit anti-PSP serum; (c) killed *P. gingivalis* 33277-exposed HGK with preimmune rabbit serum. Bars: 100  $\mu$ m.

33277, an increase in the level of protein expression was observed at 24 h (Fig. 1B, b). This immunoreactivity was not observed when preimmune rabbit serum was substituted for the PSP serum on *P. gingivalis* 33277-exposed HGK (Figs 1B, c).

A dose-dependent effect on PSP mRNA expression was observed upon exposure of the cells to TNF- $\alpha$ . At a concentration of 10 ng/ml of TNF- $\alpha$ , PSP mRNA levels increased in a time-dependent manner (Fig. 2A). However, at a concentration of 1 ng/ml of TNF- $\alpha$ , little effect was noted on PSP expression (Fig. 2A). A dose depend-

ent effect was also observed when the cells were exposed to 17 $\beta$ -estradiol (Fig. 2B). Maximum expression of PSP mRNA was seen at 3 h followed by a drop to near basal level at 24 h (Fig. 2B).

### Discussion

The interaction between epithelial cells and microorganisms is the crucial initial stage of bacterial infection and subsequent inflammation. The present study shows for the first time that PSP is expressed in gingival keratinocytes. Furthermore, PSP expression was

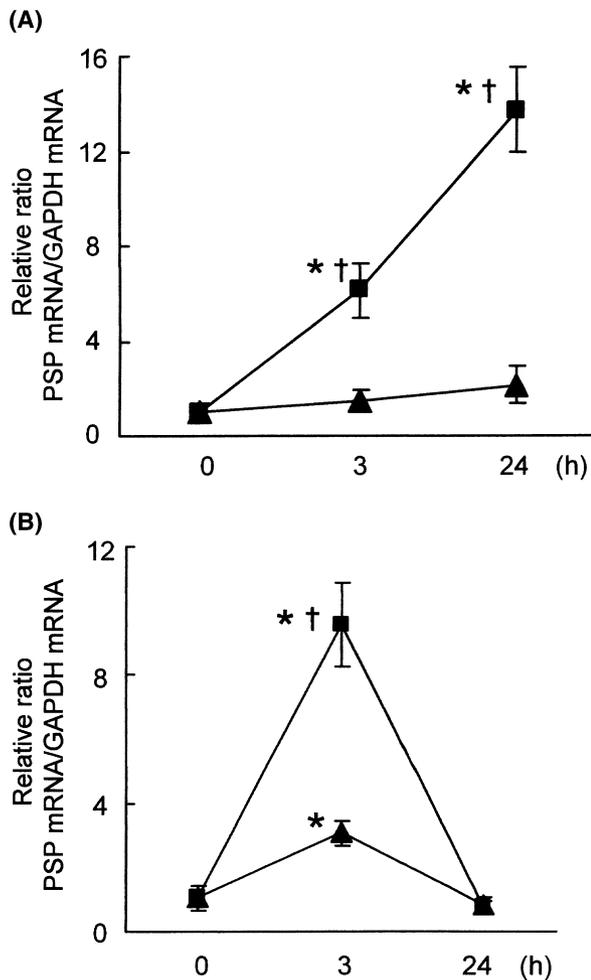


Fig. 2. Effects of tumor necrosis factor (TNF)- $\alpha$  (A) and 17 $\beta$ -estradiol (B) on parotid secretory protein (PSP) mRNA in cultures of human gingival keratinocytes (HGK). HGK were exposed to 1 ng/ml ( $\blacktriangle$ ) or 10 ng/ml ( $\blacksquare$ ) TNF- $\alpha$  and 1 nM ( $\blacktriangle$ ) or 10 nM ( $\blacksquare$ ) 17 $\beta$ -estradiol for the indicated times before the end of incubation on day 7. PSP mRNA expression was determined by real-time polymerase chain reaction. The graphs show the ratio of PSP mRNA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Values are the means  $\pm$  SD of three cultures. Differs significantly ( $*p < 0.01$ ) from no exposure to TNF- $\alpha$  or 17 $\beta$ -estradiol. Differs significantly ( $\dagger p < 0.01$ ) from TNF- $\alpha$  at 1 ng/ml or 17 $\beta$ -estradiol at 1 nM.

increased following challenge with bacteria, cytokines and sex hormones. As PSP possesses antibacterial and anti-inflammatory activities (3), it may have a role in the innate defense system at the gingival surface and play an important role during the initial stage of inflammation.

Previous studies have shown that cell wall extracts of *Fusobacterium nucleatum* induce  $\beta$ -defensin-2 mRNA in human gingival epithelial cells, although *P. gingivalis* extract did not (15). In the present study, the cocultures of HGK and killed *P. gingivalis*

resulted in increased levels of PSP mRNA in the HGK. It is feasible that PSP expression in HGK may be differentially regulated by various components of the cell surface of periodontopathogenic bacteria.

$\beta$ -Defensin-2 and -3 are expressed in epithelial cells and have antimicrobial activity (17, 18). The expression of these peptides, and now PSP, can be induced by TNF- $\alpha$  (15, 19), suggesting that anti-inflammatory peptides are up-regulated during the early stages of inflammation. Thus, although defensins and PSP may respond to different initial exposures,

the further response to pro-inflammatory cytokines may be similar.

Previous studies have shown that 17 $\beta$ -estradiol inhibits the production of interferon-gamma-inducible protein 10, monocyte chemoattractant protein-1 and RANTES in the human foreskin keratinocytes (16). Periodontitis subjects with sufficient serum level of estradiol show less gingival inflammation than those with deficient levels in osteopenic/osteoporotic women in early menopause, although bone mineral density of the lumbar spine is not different between the two groups (14). Thus, estradiol is suggested to suppress the development of inflammatory skin disease and periodontal inflammation. In the present study, 17 $\beta$ -estradiol increased PSP mRNA levels in HGK. PSP may be involved in the suppression of inflammation by estradiol.

The present study has demonstrated for the first time PSP expression and its regulation in human gingival keratinocytes. PSP could be an important anti-inflammatory and antimicrobial peptide involved in the innate defense at the gingival epithelial surface.

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