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Short communication

Porphyromonas gingivalis gingipains mediate the shedding of syndecan-1 from the surface of gingival epithelial cells

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Andrian E, Grenier D, Rouabhia M. Porphyromonas gingivalis *gingipains mediate the shedding of syndecan-1 from the surface of gingival epithelial cells.*
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Porphyromonas gingivalis gingipains are thought to be critical virulence factors in periodontitis. Increased serum levels of the soluble ectodomains of surface effectors have been reported to occur during bacterial infections. In the present study, we show that the cell surface proteoglycan syndecan-1 was highly expressed on human gingival epithelial cells. Treatments with *P. gingivalis* culture supernatants consistently mediated the shedding of syndecan-1 from the surface of epithelial cells. Concomitantly, the amount of soluble syndecan-1 detected in the culture medium increased significantly in a time-dependent manner. However, neither a heat-inactivated supernatant nor a supernatant from a gingipain-deficient mutant had a significant effect on syndecan-1 shedding. Such a shedding process may play an important role in the bacterial invasion of periodontal tissue and the modulation of host defences.

Key words: epithelial cells; periodontitis; *Porphyromonas gingivalis*; proteinase; proteoglycans; syndecan-1

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Porphyromonas gingivalis is a gram-negative anaerobic bacterium that is considered a key etiologic agent of chronic periodontitis (13). Among the virulence factors expressed by this oral pathogen, cysteine proteinases referred to as Arg- and Lys-gingipains have long been considered potential contributors to the pathogenic process of periodontitis (15, 18). Two genes code for Arg-gingipains (*rgpA* and *rgpB*). The Lys-gingipain is encoded by a single gene (*kgp*) (25). Over the last few years, increasing numbers of reports have shown that *P. gingivalis* gingipains play a critical role in the growth of *P. gingivalis* (12), the perturbation of host defences (15, 18), and the destruction and invasion of host tissues (1, 30).

In vitro studies have shown that *P. gingivalis* can invade and replicate within transformed and primary cultures of human gingival epithelial cells (8, 22) as well as in multilayered pocket epithelium (32). Recently, we demonstrated that *P. gingivalis*, through a contribution of gingipain activities, can migrate across the basement membrane and reach the underlying connective tissue (1). However, the exact mechanisms by which gingipains of *P. gingivalis* contribute to bacterial dissemination have not been clearly determined. Recent studies have reported that gingipains have a cytotoxic effect on gingival epithelial cells (17) and have the capacity to degrade epithelial cell–cell junction complexes, including intercellular adhesion molecule ICAM-1, vascular cell adhesion molecule VCAM-1, antigen molecule VLA-4, as well as adherence junction molecules such as catenins (41, 44). Gingipains also degrade transmembrane adhesion molecules involved in signal transduction pathways, such as E-cadherin and N-cadherin (19). Alteration of these adhesion molecules by gingipains could affect epithelial barrier functions and facilitate tissue and cellular invasion by *P. gingivalis*. These observations support the concept that *P. gingivalis* can gain access to the deeper structures of connective tissues via a paracellular pathway by degrading epithelial intercellular adhesion molecules or by modulating the host cell cytoskeleton when taken up by epithelial cells. Oleksy et al. (27) reported that, in addition to deregulating inflammatory cytokine networks by degrading key cytokines such as interleukin-6 (IL-6), *P. gingivalis* gingipains are capable of down-regulating IL-6 signaling transduction by the shedding, degradation, and inactivation of IL-6 receptors. Although numerous studies have reported the

involvement of *P. gingivalis* gingipains in the degradation of mammalian cell surface proteins, their inactivation via a shedding mechanism has been poorly investigated. Shedding is a mechanism by which cells down-regulate (co)-receptors and convert membrane-anchored proteins into soluble effectors, which can compete for ligands with their membrane-anchored counterparts (3). The extracellular domains of numerous mammalian cell surface proteins can be shed in a biologically active soluble form through a proteolytic cleavage process (3, 6). Recent studies have demonstrated that microbial pathogens can activate the shedding of mammalian cell surface components by exploiting the host cell shedding machinery (28, 29). Interestingly, ectodomain levels have been found to increase in fluids following tissue injury and inflammation (11, 40). Syndecan-1 is known to function as a coreceptor for various growth factors and matrix molecules such as basic fibroblast growth factor (bFGF) (20), collagen (4), and fibronectin (33). In the present study, the ability of *P. gingivalis* gingipains to promote the shedding of syndecan-1, a transmembrane heparan sulphate proteoglycan (HSPG) expressed by most epithelial cells, including gingival epithelial cells (16, 39), was investigated.

Gingival epithelial cells were isolated from a human palatal biopsy of a healthy patient using the procedure described by Rouabhia & Deslauriers (31). The epithelial cells were cultured in 75-cm^2 tissue culture flasks in Dulbecco's Modified Eagle–Ham's F-12 medium (3 : 1, v/v) (DMEM–Ham's) (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 9 mM triiodothyronine, 10 ng/ml epidermal growth factor, 100 IU/ml penicillin, 25 μg/ml streptomycin, 10 mM cholera toxin, and 5% (v/v) foetal calf serum (FCS). Cell cultures were incubated in an 8% CO_2 atmosphere at 37°C until the epithelial monolayer reached approximately 90% confluence. The cells were detached from the flask by trypsinization (0.05% trypsin-0.01 M EDTA) prior to use.

Adherent monolayers of epithelial cells were exposed to *P. gingivalis* culture supernatants, which contain both soluble and vesicle-associated gingipain activities. *P. gingivalis* ATCC 33277 and the derivative gingipain-deficient mutants KDP128 (*rgpA*–, *rgpB*–, *kgp*–), KDP112 (*rgpA*–, *rgpB*–, *kgp*+) and KDP129 (*rgpA*+, *rgpB*+, *kgp*–) were grown anaerobically at 37°C for 24 h in Todd-Hewitt Broth (BBL, Microbiology Systems, Cockeysville, MD) supplemented with vitamin K (1 μg/ml) and hemin (10 μg/ml). Mutants KDP128, KDP112, and KDP129, kindly provided by K. Nakayama (Nagasaki University, Japan), were constructed using suicide plasmids as described previously (25, 34). To prevent the appearance of revertants and to ensure the correct genotype, tetracycline (0.7 μg/ml) and erythromycin (10 μg/ml) were added to culture plates for growing mutant KDP128. The phenotype of the mutants was confirmed prior to each experiment by testing their ability to cleave the chromogenic substrates for Arg-gingipain (benzoyl-Arg-*p*-nitroanilide) and Lys-gingipain (N-*p*-tosyl-glycine-proline-lysine-*p*-nitroanilide) as previously described (5). Whereas the mutants KDP112 (*rgpA*–, *rgpB*–, *kgp*+) and KDP129 (*rgpA*+, *rgpB*+, *kgp*–) lacked Arg-gingipain and Lys-gingipain activities, respectively, the mutant KDP128 (*rgpA*–, *rgpB*–, *kgp*–) failed to exhibit any gingipain activities. Bacteria-free culture supernatants were obtained following three consecutive centrifugations (10,000 × *g* for 10 min) of the bacterial cultures. The culture supernatants were diluted 1 : 2 in DMEM–Ham's serum-free media containing 0.2 mM dithiothreitol and were used to stimulate gingival epithelial cells at 37°C. The epithelial cells were also treated with 0.5 mM 4α-phorbol-12-myristate 13-acetate (PMA; Sigma-Aldrich, Oakville, ON, Canada) in DMEM–Ham's serum-free medium, as positive control for syndecan-1 shedding. PMA is an agonist of syndecan-1 shedding (3, 10). Moreover, we previously demonstrated that PMA is a potent inducer of syndecan-1 release from human gingival epithelial cell surface (2). Preliminary experiments showed that serum starvation had no effect on epithelial cell viability.

Immunofluorescence was used to locate the syndecan-1 on the gingival epithelial cell surface (Fig. 1). A suspension containing 10^5 epithelial cells (100 μl) was added to the wells of a 24-well plate containing sterilized 13-mm-diameter glass coverslips. The plate was incubated overnight at 37°C to establish adherent monolayers prior to the stimulation assay using *P. gingivalis* ATCC 33277 culture supernatant and KDP128 culture supernatant. After a 15 h stimulation period, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) containing 2.8 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS, pH 7.4). The cells were incubated for 45 min with a mouse monoclonal antihuman syndecan-1 antibody (clone B-B4 from the U266 cell line; BioSources International Inc., Camarillo, CA), washed three times with PBS

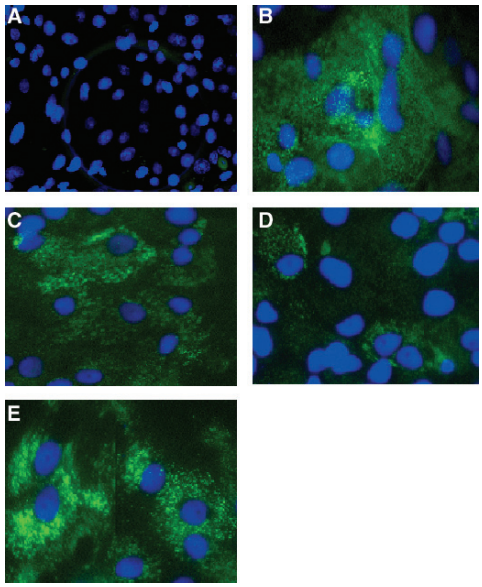

Fig. 1. Indirect immunofluorescent localization of syndecan-1 on the surface of gingival epithelial cells. Cells were stimulated with *P. gingivalis* ATCC 33277 culture supernatant (panel C), KDP128 culture supernatant (panel E), or PMA (panel D). After a 15 h stimulation period, the cells were fixed, permeabilized, and stained with mouse monoclonal antihuman syndecan-1 antibody and secondary fluorescein-isothiocyanate-conjugated goat antimouse IgG antibody. Immunofluorescent staining was also performed on unstimulated cells (panel B). Nuclei were stained with Hoescht 33258. Cells labelled with FITC-conjugated IgG only were included as controls for nonspecific staining (panel A). Data are representative of three individual experiments.

and subsequently incubated for 45 min with the secondary fluorescein-isothiocyanate-conjugated goat antimouse IgG antibody (Chemicon International Inc., Temecula, CA) diluted 1 : 100 in PBS–BSA (bovine serum albumin). Nuclei were stained with 0.5 μg/ml Hoechst 33258 (Sigma-Aldrich) for 10 min. After additional rinses in PBS, the coverslips were mounted cell-side down on glass microscope slides using 30% glycerol-2% gelatin containing 2.5% 1,4-diazabicyclo [2.2.2] octane to reduce the fading of the fluorescence. Cells incubated with FITC-conjugated IgG only were included as controls for nonspecific staining (Fig. 1A). Immunofluorescent images were obtained with a Nikon Eclipse 6600 microscope (excitation filters: FITC 450–490 nm, Hoescht 330–380 nm) equipped with a Spot RT slide camera (Diagnostic Instruments Inc., Sterling Heights, MI). The photomicrographs were captured using SPOT Advance version 3.1 and processed using IMAGE PROPLUS version 4.1.0.0. As shown in FIG. 1B, syndecan-1 is highly expressed on gingival epithelial cells. This agrees with previous studies, which have shown that syndecans are expressed in periodontal tissue (45). Epithelial cells treated with *P. gingivalis* ATCC 33277 (Fig. 1C) showed a significant loss of cell surface syndecan-1 comparable to that obtained with the PMA treatment (Fig. 1D). In contrast, the gingipain-null mutant KDP128 did not cause a marked decrease in syndecan-1 immunostaining of the cells (Fig. 1E). Epithelial cell viability was not affected by the bacterial culture supernatant treatments, suggesting that the release of syndecan-1 into the medium was not related to cell death.

The amounts of soluble syndecan-1 shed into the culture medium, following the treatments with culture supernatants from *P. gingivalis* ATCC 33277 and the derivative gingipain-deficient mutants KDP128 (*rgpA*–, *rgpB*–, *rgpB*–), KDP112 (*rgpA*–, *rgpB*–, *kgp*+), and KDP129 (*rgpA*+, *rgpB*+, *rgpB*–), were evaluated by ELISA. Epithelial cells were seeded into 96-well plates at an initial density of 2×10^4 cells per well and settled overnight to form adherent monolayers. Subconfluent monolayers were rinsed twice with serum-free DMEM–Ham's medium. The cells were exposed to *P. gingivalis* culture supernatants, as described above, for 5, 15, and 24 h at 37°C. Following each time period of stimulation, culture media were harvested and assayed for syndecan-1 shedding. The levels of soluble syndecan-1 in the culture media were determined using a sCD138 (syndecan-1) ELISA kit (Cell Sciences, Diaclone Research, Norwood, MA) according to the manufacturer's instructions. Medium from untreated cells served as a negative control to determine the constitutive level of syndecan-1 shedding.

The amount of syndecan-1 constitutively shed from the gingival epithelial cells was estimated at 129.8 ± 7.8 ng/ml after a 24 h incubation period. As shown in Fig. 2, treatment with *P. gingivalis* ATCC 33277 culture supernatant significantly increased the level of soluble syndecan-1 into the culture medium in a time-dependent manner, with a 14.5-fold increase over a 24 h stimulation period compared to the control. However, the shedding of syndecan-1 was completely abolished by heating (60°C for 10 min) the *P. gingivalis* ATCC 33277 culture supernatant prior to the stimulation. This suggests that a heat-sensitive bacterial component was involved in the shedding of syndecan-1 from the gingival epithelial cell surface.

The contribution of Arg- and Lys-gingipain activities to syndecan-1 shedding was then assessed. No significant increase in the levels of soluble syndecan-1 shed in the medium was observed following the treatment of the epithelial cells with the single, double, and null-gingipain mutants KDP129, KDP112, and KPD128, respectively, compared to the wild-type strain (Fig. 3). This suggests that Arg- and Lys-gingipains act in synergy for mediating syndecan-1 shedding. The increase in soluble syndecan-1 in the conditioned media correlated with the observed loss of syndecan-1 from the gingival epithelial cell surface following treatments with *P. gingivalis* ATCC 33277. The above observations support studies reporting loss of syndecan-1 expression by periodontal pocket epithelial cells and detection of the syndecan-1 ectodomain in dermal wound fluids in inflamed periodontal tissues (10, 39).

An increased release of glycosaminoglycans from chronically inflamed periodontal tissue has been observed in gingival crevicular fluid (35). To investigate the effect of *P. gingivalis* gingipains on the release of proteoglycans other than syndecan-1, cell surface proteoglycans expressed by gingival epithelial cells were radiolabelled by adding free [^{35}S]sulphate (Amersham Biosciences Inc., Baie d'Urfé, QC, Canada) at a final concentration of 50 μCi/ml in sulphate-free medium supplemented with 5% FCS. After a 24 h incubation, free-[^{35}S]sulphate was removed by washing the cells three times with serum-free medium. The cells were then treated with either *P. gingivalis* ATCC 33277 or KDP128 culture supernatant diluted 1 : 2 in serum-free DMEM–Ham's supplemented with 0.2 mM dithiothreitol at 37°C, or with PMA (0.5 mM). After a 24 h stimulation period, the conditioned media containing released [^{35}S]-labelled proteoglycans were collected and centrifuged ($1000 \times g$ for 5 min), and then analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (21) followed by autoradiography. As shown in Fig. 4, a major band with a molecular mass of

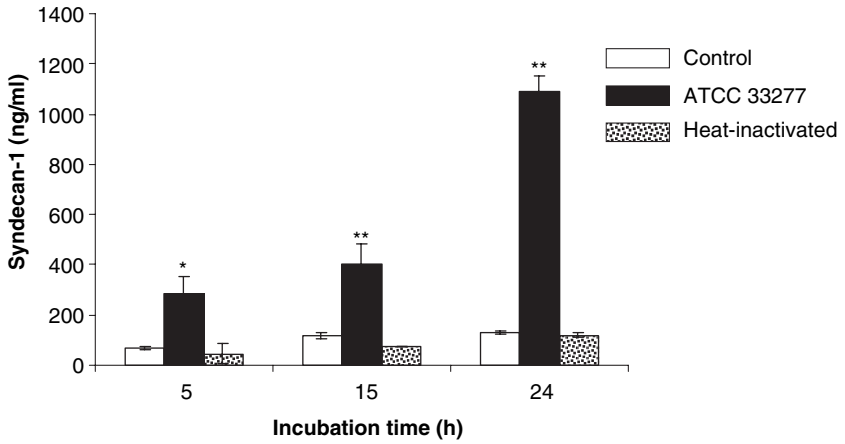

Fig. 2. Time-dependent effect of *P. gingivalis* ATCC 33277 culture supernatant on syndecan-1 shedding from gingival epithelial cells. Gingival epithelial cells were stimulated with either *P. gingivalis* ATCC 33277 culture supernatant or heat-treated bacterial culture supernatant (60°C for 10 min). After 5, 15, and 24 h of stimulation, culture media were collected and the amounts of soluble syndecan-1 were measured by ELISA. The results are reported as the means ± SDs of triplicate assays. Single and double asterisks indicate significant differences between stimulated and unstimulated cells ($P < 0.05$ and < 0.01, respectively).

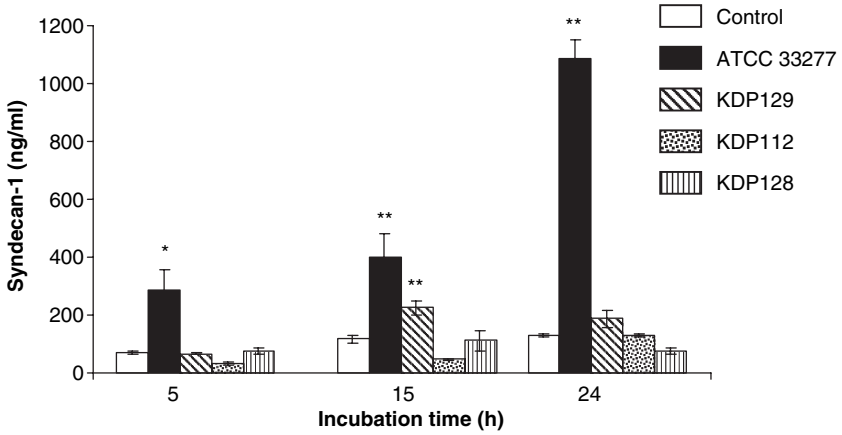

Fig. 3. Comparative analysis of the effect of the culture supernatants of *P. gingivalis* ATCC 33277 and its derivative gingipain-deficient mutants KDP128, KDP112 and KDP129 on the shedding of syndecan-1 from the gingival epithelial cell surface. After 5, 15, and 24 h of stimulation, culture supernatants were collected and the amounts of soluble syndecan-1 were measured by ELISA. The results are reported as the means ± SDs of triplicate assays. Single and double asterisks indicate significant differences between stimulated and unstimulated cells ($P < 0.05$ and < 0.01, respectively).

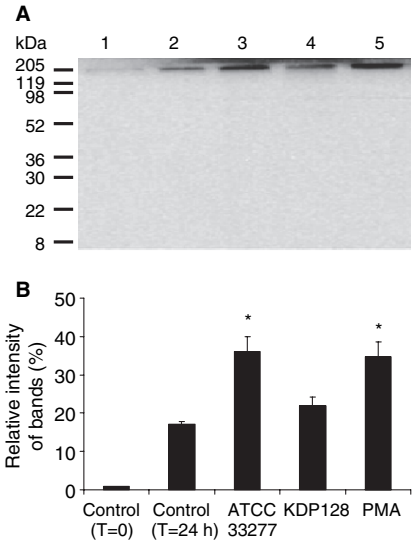

Fig. 4. SDS-PAGE (12% resolving gel) and autoradiography analysis of epithelial cell surface [^{35}S]-labelled proteoglycans released following a treatment with *P. gingivalis* culture supernatant. Gingival epithelial cells were labelled with [^{35}S]sulphate for 24 h and then stimulated with PMA, or the culture supernatants of *P. gingivalis* ATCC 33277 and *P. gingivalis* KDP128. After 24 h of stimulation, the culture media were collected and analyzed by SDS-PAGE and autoradiography. A) Lanes: 1, untreated cells at T = 0; 2, untreated cells after 24 h of incubation; 3, cells treated with *P. gingivalis* ATCC 33277 culture supernatant for 24 h; 4, cells treated with *P. gingivalis* gingipain-null mutant KDP128 culture supernatant for 24 h; 5, cells treated with PMA for 24 h. The molecular mass markers (in kDa) are indicated on the left. B) The relative intensity of the bands were determined using SCION IMAGING SOFTWARE (Scion Corporation). The results are reported as means and SDs. A value of 1% was assigned to the control at T = 0. A single asterisk indicates significant differences between stimulated and unstimulated cells after a 24-h period of stimulation ($P < 0.05$).

approximately 200 kDa was detected. Based on the quantification of relative intensity of the band, PMA and *P. gingivalis* ATCC 33277 culture supernatant were significantly more effective than the culture supernatant of the gingipain null mutant KDP128 in inducing the release of the high molecular mass proteoglycan. This 200 kDa band was also observed in the conditioned medium of untreated cells, suggesting a constitutive shedding. The total amount of soluble [^{35}S]-labelled proteoglycan released following stimulation of gingival epithelial cells was also quantified by counting the radioactivity released into the culture medium using a multipurpose scintillation counter (Beckman Coulter, Fullerton, CA). Stimulation of the gingival epithelial cells by PMA, *P. gingivalis* ATCC 33277 supernatant, and KDP128 supernatant resulted in the release of 34.9%, 31.4%, and 28.0%, respectively, of the total isotope incorporated by cells compared with 12.8% for the control (data not shown). The radioactivity quantified in the culture medium may include not only the 200 kDa band but also higher molecular mass [^{35}S]-labelled proteoglycans that did not enter the resolving gel. This hypothesis is supported by Larjava (23), who observed high molecular mass proteoglycans (> 200 kDa) in the conditioned medium of periodontal fibroblasts. Moreover, low molecular mass [^{35}S]sulphate-oligosaccharides, poorly [^{35}S]sulphate-glycosylated proteoglycans, or proteoglycans deglycosylated by *P. gingivalis* glycosidases (36) may be not detectable by the radioactive procedure used. This may explain the absence of the band that may correspond to syndecan-1. Further studies will investigate the major [^{35}S]-labelled proteoglycan components released by *P. gingivalis*.

Our results revealed that *P. gingivalis* can induce the shedding of syndecan-1 and possibly other proteoglycans from the gingival epithelial cell surface. The shedding of syndecan-1 likely involves gingipain-mediated proteolytic cleavage. This is supported by the fact that no shedding was observed with a gingipain-null mutant. Syndecan-1 ectodomain shedding has been reported to be highly regulated and accelerated by physiological agents that mediate cellular responses to stress. These agents involve distinct intracellular signaling pathways leading to proteolytic cleavage mediated by a cell surface proteinase sensitive to tissue inhibitor of matrix metalloproteinases (TIMP)-3 or by a non-matrix metalloproteinase (10, 14). Gingipains may stimulate gingival epithelial cell signal transduction, leading to the secretion of matrix metalloproteinases (MMPs); in concert, these two enzyme activities may cause syndecan-1 shedding. This hypothesis is supported by previous studies, which demonstrated that a purified thiol-proteinase of *P. gingivalis* related to Arg-gingipain A stimulated MMP secretion by mucosal epithelial cells (7), human gingival fibroblasts (38, 42) and neutrophils (38). Several studies have reported the direct contribution of MMPs to the shedding of syndecan-1 and other cell surface proteins (9, 10, 24). Other hydrolytic enzymes secreted by *P. gingivalis*, such as heparinase and chondroitin sulphatase (36), may also participate in the shedding of syndecan-1 from the epithelial cell surface. In addition, we recently demonstrated that *P. gingivalis* lipopolysaccharide induces the shedding of syndecan-1 from human gingival epithelial cells (2). Lipopolysaccharide appears to promote syndecan-1 shedding by stimulating IL-1β production, which may in part activate intracellular signaling pathways leading to the secretion of MMPs.

Since high levels of proteoglycan metabolites in gingival crevicular fluid from advanced periodontitis patients have been reported to reflect active tissue destruction periods (37, 43), investigations of the pivotal role of *P. gingivalis* in the shedding of cell surface proteoglycans may provide information on the mecha-

nisms involved in the pathology of periodontal diseases. Syndecans are the major form of cell surface heparan sulphate proteoglycans that mediate cell–cell and cell–extracellular matrix adhesion and are implicated in the binding and modulation of soluble and insoluble ligands (4). Syndecan shedding is a regulated host response to tissue injury and shed syndecan has been reported to function as a regulator of inflammation (11). Consequences of the loss of syndecans from the epithelial cell surface upon up-regulation of syndecan-1 ectodomain shedding could affect the epithelial cell integrity as well as the ability of tissues to respond to injury. Our results suggest that *P. gingivalis* gingipains contribute to the shedding of syndecan-1 from the epithelium cell surface. This phenomenon may be important in the invasion of periodontal tissue and the modulation of host defences as syndecan-1 shedding was recently reported to enhance the microbial virulence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (28, 29).

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