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A novel molecule, SLURP-1, enhances the survival of periodontal ligament fibroblasts

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Background and Objective: The mechanism behind the survival of periodontal ligament fibroblasts is critical for the maintenance of periodontal ligament tissue. However, the number of known proteins that are involved in this action is limited. The aim of this study was to examine the role of a novel molecule, secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein 1 (SLURP-1), in periodontal ligament fibroblast survival.

Material and Methods: Human periodontal ligament fibroblasts were isolated from eight healthy human donors using established protocols. Gene expression for SLURP-1 was analysed using the reverse transcriptase-polymerase chain reaction, while protein expression was examined by immunoblotting with a SLURP-1 antibody. In addition, the apoptotic effect was examined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay.

Results: Messenger RNA for SLURP-1 was expressed in the periodontal ligament, gingival fibroblasts, oral keratinocytes and bone. Moreover, the protein was secreted by both periodontal ligament and gingival fibroblasts. Functional analysis revealed that SLURP-1 substantially enhanced cell survival in periodontal ligament fibroblasts by the anti-apoptotic signal phosphatidylinositol 3-kinase.

Conclusion: These findings suggest that SLURP-1 may play an important role in the control and maintenance of the periodontal ligament by protecting the periodontal ligament fibroblasts from apoptosis.

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The periodontal ligament is a highly specialized connective tissue that plays a critical role in tooth function. When this tissue is damaged, often through periodontitis, it leads to a complex biological process regulated by cytokines and growth factors. More specifically, these factors target the periodontal ligament fibroblastic cells that reside in the tissue (1,2). Several proteins have been identified, including platelet-derived growth factor (3), transforming growth factor β (4), epidermal growth factor (5) and nerve growth factor (6). The ultimate goal in periodontal research is not only to understand the function of these factors but also to discover other proteins that can aid in regeneration and control of the periodontal ligament.

Apoptosis or programmed cell death is a physiological process characterized by specific cellular and molecular changes within the cell (7). This event plays a key role in the development of the periodontium, including the periodontal ligament, as well as the progression of oral disease and inflammation (8). Previous studies have emphasized the clinical significance of apoptosis that is linked to the loss of attachment, an early feature of periodontitis (9).

Secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein 1 (SLURP-1) is categorised into the Lymphocyte 6 (Ly-6) antigen superfamily, which has been implicated in a wide variety of

functions, including T-cell activation (10). Until recently, the Ly-6 family was envisaged to be one large superfamily consisting of membrane-bound proteins, specifically the glycosylphosphatidylinositol (GPI) membrane-linked proteins. However, there is now evidence to suggest that the Ly-6 superfamily is divided into two subfamilies: one is the original membrane-linked anchor (11–14) and the other is the secreted form, consisting of SLURP-1, -2 and -3 (15–17). The biological function of SLURP-1 is still unknown, since study has been limited to its role during skin development and disease, including the developing epidermal keratinocyte (15). There are also preliminary studies investigating SLURP-1 function in oral epithelium and keratinocytes (18). In these cells, the protein abolished the tumorigenic effects of nitrosamine in malignant gingival keratinocytes. SLURP-2 has been shown to be involved in prevention of apoptosis by specifically down-regulating pro-apoptotic markers in oral keratinocytes (18). Like SLURP-1, most of the studies have concentrated on skin development. In contrast, SLURP-3 is more of an enigma, with this protein originally identified as 'caltrin', a calcium transport inhibitor rather than a member of the Ly-6 family (17). However, caltrin is now known to be part of the Ly-6 family, with studies demonstrating the expression and purification of this protein *in vitro* (16,17).

It is possible that SLURP-1 may have a larger role in the maintenance of the oral cavity, and this prospect warrants further investigation. In this study, we examined the gene expression of SLURP-1 in various human cells and tissues and then ascertained the function of the protein in periodontal ligament fibroblasts in relation to cellular survival.

Material and methods

Cell culture

All cells were maintained in Dulbecco's modified Eagle's medium (Thermo-Trace, Melbourne, Australia) supplemented with L-glutamine (200 mM Gibco BRL, Melbourne, Australia), 10% fetal bovine serum (5000 units) (Gibco BRL), penicillin and streptomycin (5 mg/mL; ICN Biomedical, Aurora, OH, USA).

All cells cultured from human sources were obtained with appropriate informed consent and were approved by the Human Ethics Committee at the University of Western Australia. Periodontal ligament fibroblasts were isolated from eight healthy human donors, both male and female, aged between 12 and 14 years, showing no clinical signs of periodontitis. In addition, cells were scraped from the middle of the premolar tooth (extracted for orthodontic reasons) root to exclude contamination from the gingivae and dental pulp. Human gingival fibroblasts were prepared from the explants of normal gingival tissues obtained from eight 6-year-old patients. Human oral keratinocytes were purchased from ScienCell (Carlsbad, CA, USA). Human osteoblastic-like cells (hFOB 1.19) and human oesophagus Het1-A cells were purchased from ATCC (Manassas, VA, USA). Human periodontal ligament and gingival fibroblasts were obtained, cultured and validated according to established methods described elsewhere (19–22). All the experiments were carried out at passages 4–7.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA fraction for cultured human periodontal ligament and gingival fibroblasts, oral keratinocytes and hFOB 1.19 was isolated with RNeasy isolation columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Total RNA (2 μg each) was reverse transcribed and the subsequent cDNA amplified. In addition, human cDNA samples consisting of brain, spleen, kidney, seminal vesicle, thymus, long and calvarial bones were purchased from Origene (Rockville, MA, USA) and Zyagen (San Diego, CA, USA). Polymerase chain reaction was performed for 30 cycles within the linear range of amplification. The primer pair for PCR included: SLURP-1 (forward, 5′-CTCTCATCACTTCTGAGCAC-3′; reverse, 5′-GCGTGGGGTATGGAAGG-3′) and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; reverse, 5′-CATGTGGGCCATGAGGTCCACCAC-3′). Polymerase chain reaction and RT-PCR reagents were obtained from Promega (Madison, WI, USA).

Immunoblotting analysis

To determine whether periodontal ligament,gingival fibroblasts, intact Het-1A (negative control) and oral keratinocytes (positive control) secrete SLURP-1, concentrated supernatants of all cell types were collected over a 10 d period. Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. Blots were incubated in 5% dry skim milk in Tris-buffered saline–Tween (TBS–Tween) for 1 h and subsequently washed twice with TBS–Tween for 5 min. The blots were then probed with H00057152-B01 SLURP-1 primary antibody (Abnova, Taipei, Taiwan) diluted 1:100. Blots were washed three times for 5 min with TBS–Tween, and incubated with PAB0096 IgG secondary antibody (Abnova) at a final dilution of 1:2500 in TBS–Tween containing 1% skim milk. Finally, the blots were washed twice with TBS–Tween for 5 min and twice with TBS for 5 min. The antibody reactivity was detected by the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Production and purification of recombinant SLURP-1

Cloning of human SLURP-1 and production of the recombinant protein were performed as previously described (15). Briefly, normal human skin cDNA (Biochain, Hayward, CA, USA) was used to obtain the PCR product of SLURP-1. The cDNA was inserted into the pET SUMO vector (Invitrogen, Carlsbad, CA, USA) followed by transformation into competent BL21-star *Escherichia coli* cells (Invitrogen) and subsequently purified

using the His tag TALON Co^{2+} resin purification kit (ClonTech, Palo Alto, CA, USA) as previously described (16). The N-terminal 6-His tag was then removed using the SUMO protease enzyme (Invitrogen).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay

Periodontal ligament fibroblasts were grown in 75 mm culture flasks and subsequently seeded onto five six-well plates. The cell density used for all experiments was approximately 1×10^4–1.2×10^4 cells. Cells were pre-incubated with 5 mM of the pro-apoptotic agent camptothecin (23,24) with or without recombinant SLURP-1 (rSLURP-1). In addition, 100 nM of phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (Cell Signaling Technology, Taipei, Taiwan) was also added in specific experiments. To determine the optimal concentration of rSLURP-1 to produce an effect on periodontal ligament fibroblasts, a range of doses between 10 and 200 ng/mL was added. Control or untreated groups consisted of periodontal ligament fibroblasts not exposed to any agent. Labelling of fragmented DNA in cultured periodontal ligament fibroblasts was performed with the TUNEL assay using the ApopTag apoptosis detection kit (Intergen Co., Purchase, NY, USA) according to the manufacturer's protocol for monolayer cultures. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. Cells were washed and incubated with blocking solution (0.3% H_2O_2 in phosphate-buffered saline) at 4°C for 15 min and then treated with equilibrium buffer at room temperature for 10–15 s, followed by TUNEL reaction mixture for 60 min at 37°C. Cells were incubated with anti-digoxigenin peroxidase for 60 min at 37°C and the staining colour of the immunolabelling was developed with diaminobenzidine substrate solution. These apoptotic cells appeared as dark brown cells. The numbers of apoptotic and non-apoptotic cells were counted from five fields per well at ×400 magnification using a phase contrast microscope attached to a Nikon imaging system (Nikon, Mclville, NY, USA). Apoptosis was analysed by two investigators in a blinded fashion without knowledge of the experimental group.

Statistical analysis

All experiments were performed in triplicate. Unpaired Student's *t*-test was used to analyse differences between two specific groups. Differences were considered significant if the *p*-value was < 0.05.

Results

Tissue distribution of SLURP-1

Secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein 1 mRNA expression in various human tissues and cells was examined by RT-PCR. The SLURP-1 was expressed in oral keratinocytes, periodontal ligament and gingival fibroblasts (Fig. 1). Expression was also seen in brain and kidney tissues. The SLURP-1 mRNA was expressed in long bone, calvarial bone, hFOB1.19, spleen, seminal vesicle and thymus. Overall, the expression of SLURP-1 in human tissues was similar to that reported in previous studies (25,26).

Protein expression of SLURP-1

A SLURP-1 antibody was used to detect the presence of the protein in 50-fold concentrated supernatants of periodontal ligament and gingival fibroblasts (Fig. 2). The SLURP-1 protein was detected in both periodontal ligament and gingival fibroblasts.

Anti-apoptotic effect of SLURP-1 on periodontal ligament fibroblasts

To examine whether SLURP-1 has any affect on periodontal ligament fibroblast survival, the cells were treated with camptothecin with and without rSLURP-1 and subsequently stained using the TUNEL assay as described in the Material and methods section. The optimal concentration of rSLURP-1 on periodontal ligament fibroblasts was ascertained by a dose-dependent experiment (Fig. 2). The percentages indicate the proportion of live cells compared with untreated control cultures. At 10, 20, 40 and 80 ng/mL there was no statistically significant effect on periodontal ligament fibroblasts. However a positive effect was seen at 100–200 ng/mL. Therefore, 100 ng/mL was chosen for the rest of the experiments. Interestingly, the number of live

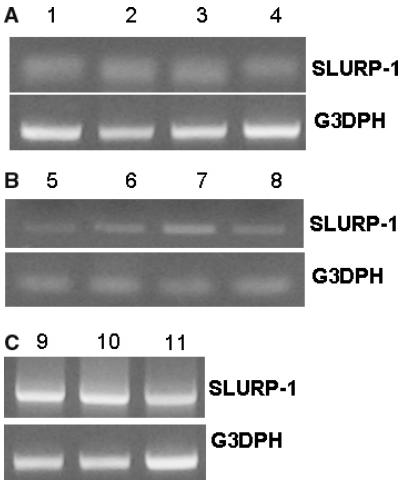

Fig. 1. Expression of SLURP-1 in human bone-associated cells and tissues (A), general tissues (B) and oral-specific cells (C). Total RNA was isolated from specific human cells and tissues, transcribed to cDNA and amplified using SLURP-1 specific primers. The SLURP-1 is expressed in periodontal ligament and gingival fibroblasts and oral keratinocytes. Lanes: 1, long bone; 2, calvarial bone; 3, hFOB1.19; 4, spleen; 5, seminal vesicle; 6, thymus; 7, kidney; 8, brain; 9, oral keratinocyte; 10, periodontal ligament fibroblasts; and 11, gingival fibroblasts. The data are representative of three independent experiments.

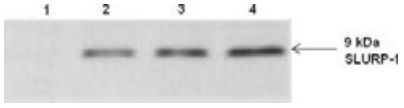

Fig. 2. Visualization of the 9 kDa SLURP-1 protein in concentrated supernatants of Het1-A cells (lane 1, negative control), periodontal ligament (lane 2), gingival fibroblasts (lane 3) and oral keratinocytes (lane 4, positive control). Supernatants were collected and concentrated. A SLURP-1 antibody was used to visualize the presence of the SLURP-1 protein in the supernatants.

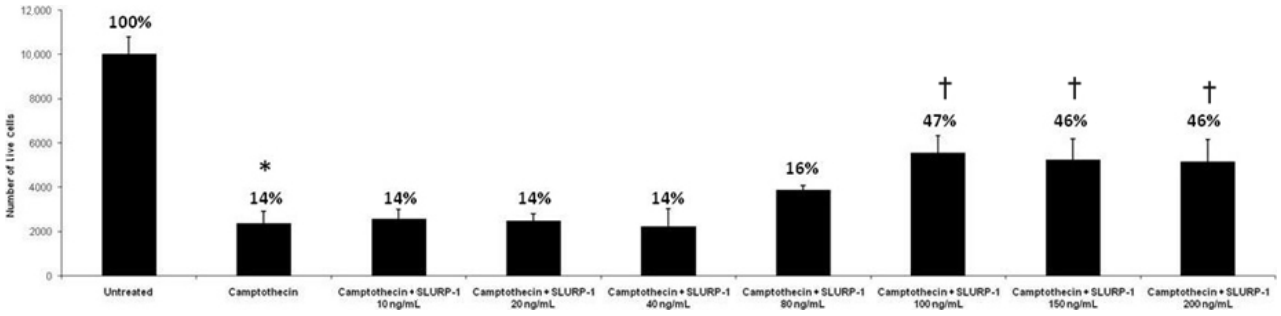

Fig. 3. Detection of apoptosis using TUNEL. Camptothecin treatment reduced the number of live cells to 14%, compared with untreated control cells,. The addition of 100 ng/mL SLURP-1 to camptothecin-treated cells significantly increased the number of live cells. Concentrations below 100 ng/mL produced no effect. The percentages indicate the proportion of live cells compared with untreated control cells. *$p < 0.01$ vs. untreated control; †$p < 0.01$ vs. camptothecin alone. Data shown are the means ± SEM (live cell number) for three independent cultures.

cells decreased from 100 (untreated) to 14% when periodontal ligament fibroblasts were pre-incubated with camptothecin (Fig. 3). However, when rSLURP-1 was added after camptothecin pretreatment, the number of live cells increased to 47%. This indicates a strong protective effect by SLURP-1 on periodontal ligament fibroblasts. Whether inhibition of PI3K negates the function of SLURP-1 was examined using wortmannin, a PI3K inhibitor (Fig. 4). Similar results were observed, with the addition of camptothecin decreasing the number of live cells to 14% and the addition of SLURP-1 increasing the number of live cells to 47%. However when the cells were pre-incubated with wortmannin this protective effect was completely lost, with the number of live cells being the same as cells treated with camptothecin only (14%).

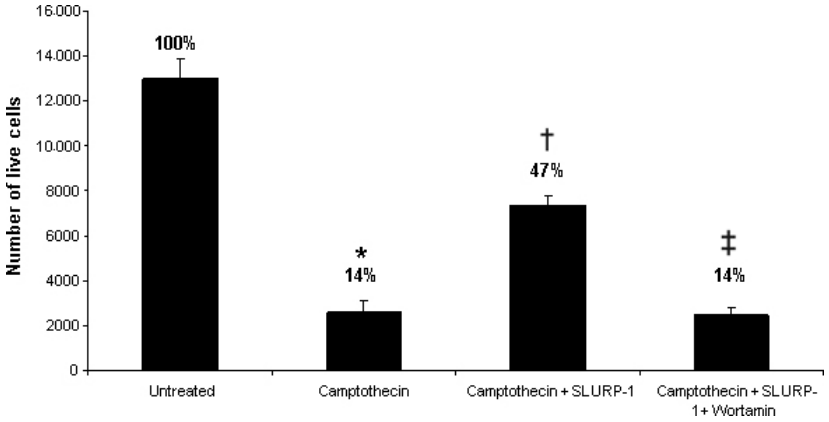

Fig. 4. Detection of apoptosis in the presence of PI3K inhibitor. When wortmannin was added to cells treated with camptothecin and 100 ng/mL of SLURP-1, the protective effect of SLURP-1 was completely abolished. The percentages indicate the proportion of live cells compared with untreated control cultures. *$p < 0.01$ vs. untreated controls; †$p < 0.01$ vs. camptothecin alone; ‡$p < 0.01$ vs. camptothecin with SLURP-1. Data shown are the means ± SEM (live cell number) for three independent cultures.

Discussion

It is known that periodontal ligament fibroblasts have specialized functions for repair, remodelling and regeneration of the adjacent tissues (20,21,27) as well as during inflammatory periodontal diseases (28). Although the exact mechanisms behind these functions have not been fully elucidated, a host of studies have suggested that specific factors are involved (1,2). These proteins may have a paracrine mode of action, such as platelet-derived growth factor, which is produced primarily by platelets but can function on periodontal ligament fibroblasts, or an autocrine mode of action, such as nerve growth factor, which is expressed by periodontal ligament fibroblasts and acts on the same cells (1,29). Owing to the myriad factors that have been identified, it is fundamental that their function and mechanism of action is fully understood. In addition, identifying novel factors that may have a significant role in periodontal ligament regeneration is equally important. One such novel factor is SLURP-1, which was originally identified in epidermal keratinocytes and epithelial cells (15,25). For the first time, this study has investigated the role of SLURP-1 in periodontal ligament fibroblast survival. Our results suggest that SLURP-1 is expressed in periodontal ligament fibroblasts and, via the PI3K signal, exerts an anti-apoptotic effect on these cells.

Reverse transcriptase-PCR analysis revealed expression of the *SLURP-1* gene in all of the human tissues and cells analysed, including periodontal ligament, gingival fibroblasts and oral keratinocytes. This apparently ubiquitous expression pattern was also seen in a previous study examining SLURP-1 expression in murine tissues (26). Interestingly, SLURP-1 has been examined in human tissues previously but was limited to skin, exocervix and lung (25). Our study is the first to examine a wide range of human tissues and cells. The *SLUPR-1* gene is

expressed in periodontal ligament and gingival fibroblasts, and the resulting secreted protein product can be found in the supernatants of the same cells. The absence and presence of the SLURP-1 protein in Het1-A cells and oral keratinocytes, respectively, is in agreement with previously published studies (18). Moreover, with previous studies having shown expression and protein production of SLURP-1 in gingival keratinocytes (18,30), SLURP-1 may indeed play an important role in the oral cavity and possibly in disease.

Functional examination using TUNEL indicated that, upon SLURP-1 stimulation, there is a large reduction in apoptotic cells. Interestingly, when wortmannin (a PI3K inhibitor) is added to cells, the action of SLURP-1 is completely abolished, with the number of live cells returning to the same levels as for camptothecin treatment alone. This indicates that SLURP-1 exerts a strong protective effect on periodontal ligament fibroblasts and that this particular anti-apoptotic function operates through the PI3K signal. Although this protective effect is seen in other cells, this is the first study to examine it in cells within the periodontium. Our functional results contradict previously published reports that claim a pro-apoptotic effect for SLURP-1 (15). It has been suggested that SLURP-1 upregulates specific pro-apoptotic markers, such as caspase 3 (15). The difference in results may be due to the use of two different cell types: epidermal keratinocytes for Arredondo and colleagues (15) and periodontal ligament fibroblasts for our study. Interestingly, when SLURP-1 was tested in oral keratinocytes, rather than those from an epidermal origin, the data revealed that the protein negates the tumourigenic effect of the nitrosamine 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone, suggesting a protective effect (30). It is noteworthy that the function of SLURP-1 in periodontal ligament fibroblasts is similar to the function of SLURP-2 in oral keratinocytes (18,30). SLURP-2 prevents apoptosis by the down-regulation of pro-apoptotic markers such as Bax, Bad and caspase 3. Since the differential function of SLURP-1 and SLURP-2 in keratinocytes is explained by the interaction of different nicotine acetylcholine receptor subtypes, it would be interesting to ascertain whether nicotine acetylcholine receptors are also expressed in periodontal ligament fibroblasts, since they are also expressed in epidermal and oral keratinocytes. The effect of SLURP-1 on the periodontal ligament fibroblasts may translate into distinct outcomes of periodontal treatments if its function and mechanism are further investigated. If its function can be established, then it may support the concept that clinical application of SLURP-1 may enhance periodontal wound healing and regeneration by specifically modifying periodontal ligament cell turnover.

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