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Local expression of lipopolysaccharide-binding protein in human gingival tissues

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Background: Lipopolysaccharide-binding protein (LBP) functions as a crucial molecule in innate host defense responses to bacterial challenge through neutralization of bacterial lipopolysaccharide (LPS) and activation of cellular responses.

Objectives: This study was to investigate the expression profile and levels of LBP in gingival tissues and their associations with periodontal health and disease.

Methods: Gingival biopsies were collected from 44 chronic periodontitis patients, including periodontal pocket tissues (PoTs) and the adjacent healthy gingival tissues (HT-Ps), as well as from 15 periodontally healthy subjects as controls (HT-Cs). The peptide and mRNA of LBP were detected by semi-quantitative immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR), respectively.

Results: LBP peptide was detected in 90.9% of PoTs (20/22), 84.6% of HT-Ps (11/13) and all HT-Cs (7/7). The expression of LBP was mainly confined to the cytoplasm of granular and keratinized layers of gingival epithelium, spreading from the oral sulcular epithelium to oral epithelium with the expression density decreasing gradually from coronal to apical portion. LBP peptide was also found on endothelial surfaces and/or inside the lumens of blood vessels in connective tissues. The mean LBP expression levels in HT-Cs were significantly higher than those in HT-Ps and PoTs. LBP mRNA was detected in 55% of PoTs (11/20), 55% of HT-Ps (11/20) and 75% of HT-Cs (6/8).

Conclusions: We for the first time found the expression of LBP peptide and mRNA in human gingival tissues. Local expression of LBP in gingival tissues might contribute to periodontal homeostasis.

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Lipopolysaccharide (LPS) has been shown to play an important role in the pathogenesis of various infections caused by gram-negative bacteria (1). LPS is an endotoxin component of outer membrane in gram-negative bacteria (2). It is a complex molecule consisting of three covalently linked regions – lipid A, core polysaccharide and O-specific chains. It acts as a potent stimulus to a variety of host cells, resulting in the expression of cytokines, adhesive proteins and proinflammatory molecules (3). Previous studies have shown that LPS-initiated inflammatory responses are strongly enhanced by LPS-binding protein (LBP) (4).

LBP is an acute-phase reactant, predominantly derived from the liver. It is a 60–65 kDa protein present in blood at a concentration of approximately 2–20 μg/ml in healthy individuals (5). The plasma level of LBP can rise dramatically after inflammatory challenge, such as sepsis induced by gram-negative bacteria (6). Moreover, LBP can also be detected in the lung, which may account for many of its biological effects (7). It is known that LBP binds LPS through the

recognition of lipid A (8) and the binding domain of LBP comprises amino acids 89–97 (9). As a lipid transfer protein, LBP functions in keeping with its sequence homology to the lipid transferases phospholipid transfer protein and cholesterol ester transfer protein. It can also co-purify with high-density lipoprotein particles, resulting in the neutralization of LPS toxicity (10).

LPS does not injure host tissues directly but rather through the actions of induced endogenous mediators of inflammation, such as IL-1 and TNF-α. Membrane CD14 (mCD14) is a 55 kDa glycoprotein that serves as a receptor for the complex of LPS-LBP (11). LBP and mCD14 are therefore regarded to be two major participants in innate host immune responses to LPS (12). Activation of macrophages and granulocytes mediated by LPS is more potent in the presence of LBP and resulted in more rapid cytokine production than LPS alone (13). It is found that LBP potentiates the stimulation of CD14-positive cells in response to LPS by 100–1000 fold (14). These observations corroborate that LBP may play a crucial role in clearance and killing of gram-negative bacteria within injured tissues (15). Thus LBP may serve to both neutralize LPS and enhance its biological activities. The present study for the first time described the LBP expression of human gingival tissues in periodontal health and disease.

Material and methods

Subjects

Forty-four Chinese adults with a mean age of 48.5 ± 8.9 years (22–65 years) were recruited for the study. Inclusion criteria were: (i) presentation of untreated advanced chronic periodontitis, with probing depth ≥ 5.0 mm, clinical attachment loss ≥ 3.0 mm and radiographic evidence of alveolar bone loss on at least two teeth per quadrant, excluding the third molars; (ii) healthy systemic condition; (iii) no prior periodontal treatment; (iv) no use of any immunosuppressive agents; (v) no antibiotics or anti-inflammatory drugs taken within the preceding 6 months. All the subjects were examined at a screening session for checking their suitability for the study. Then they received baseline examination and a course of non-surgical periodontal therapy including oral hygiene instructions, scaling and root planing, and follow-up monitoring of treatment responses for at least 6 months with routine prophylaxis at interval of 3 months. At the subsequent re-examination session for evaluation of treatment outcomes, all the subjects exhibited unresolved periodontitis with remaining probing depth ≥ 6.0 mm and bleeding on probing at least in one quadrant of their dentitions and they required periodontal surgery as an essential part of the definitive treatment plan (16).

Fifteen systemically and periodontally healthy subjects with a mean age of 23.4 ± 3.6 years who required tooth extraction for orthodontic treatment purposes were selected as control subjects. They were all non-smokers. Inclusion criteria were: (i) systemically healthy condition; (ii) no sites with probing depth > 4 mm or clinical attachment loss > 1 mm in the whole dentition; (iii) no radiographic evidence of periodontal bone loss after a full-mouth radiographic examination; (iv) a full-mouth score of bleeding on probing < 15% of sites; (v) no antibiotics or anti-inflammatory drugs taken within the preceding 6 months (16). The purposes and procedures of the study were explained and informed consents were obtained from all recruits, and the study protocol was approved by the Ethics Committee, Faculty of Dentistry, the University of Hong Kong.

Collection of samples

All patients were screened for their suitability and selection of sampling sites, 1 week prior to collection of samples. Gingival biopsies were collected through internal beveled incision during periodontal surgery in unresolved periodontitis sites with probing depth ≥ 6 mm and significant loss of alveolar bone following non-surgical treatment, consisting of (i) periodontal pocket tissues (PoTs); (ii) clinically healthy tissues (HT-Ps) adjacent to the pocket sites with probing depth ≤ 3 mm, absence of bleeding on probing and clinical attachment loss ≤ 1 mm. Fifteen gingival biopsies were obtained from the 15 periodontally healthy subjects as healthy controls (HT-Cs) during tooth extraction for orthodontic reasons. These sites sampled met the following criteria: (i) probing depth not exceeding 3 mm; (ii) absence of bleeding on probing; (iii) clinical attachment loss not exceeding 1 mm; and (iv) no radiographic evidence of alveolar bone loss.

Immunohistochemistry

Serial paraffin sections of biopsies were cut 4 μm thick, mounted onto slides and stained with hematoxylin and eosin using standard procedures or used for immunohistochemistry procedure. Immunoperoxidase staining procedures were performed as described below. Briefly, after deparaffin, slides were treated with high pressure for 3–4 min and then immersed in phosphate-buffered saline (PBS) containing 3% H_2O_2 for 20 min to quench endogenous perioxidase activity. Non-specific binding was blocked for 30 min with 3% bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) in Tris-buffered saline (TBS) for 30 min. Mouse anti-human LBP monoclonal antibody (biG 42, Biometec Ltd, Greifswald, Germany) was placed on tissue sections for 2 h at 37°C. Slides were then washed with PBS and incubated for 30 min with biotinylated horse anti-mouse IgG antibody. The slides were washed with PBS again and incubated for 30 min at 37°C with avidin-conjugated peroxidase. Finally, slides were incubated for 1–2 min with the chromogen 3,3-diaminobenzidine and counterstained with hematoxylin. After desiccating in graded ethanol (75%, 95% and 100%) and xylene, slides were permanently mounted. Negative control experiments were performed by incubation with secondary antibody alone or TBS instead of the primary antibody. Mouse anti-rabbit Collagen X monoclonal

antibody was used as the primary antibody (negative control) to demonstrate the specificity of the LBP primary antibody.

Image analysis

The LBP expression was evaluated and quantitatively analysed by a single examiner (LR) according to the positive 3,3-diaminobenzidine staining which was analyzed by a true-colour computerized image system with a digital camera (Leica DC 300 V2.0, Leica, Wetzlar, Germany) and software (Qwin version 2.4, Leica, Cambridge, UK). The proportion of positively stained area over the total area of the specimen was calculated and presented as area% ($\times 10^2$). Hematoxylin and eosin stained sections were used for morphometric analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

Isolation and extraction of total RNA — Total RNA in gingival tissues was isolated by using the Qiagen Viral RNA mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. Briefly, the fresh gingival tissue was homogenized with 350 μl of Buffer RLT for 20–40 s, until a complete lysate was obtained. The lysate was centrifuged for 3 min at 10,000 *g* and the supernatant transferred to a fresh tube. The supernatant was mixed with 350 μl of 70% ethanol and then 700 μl of sample was transferred into an RNeasy mini spin column and centrifuged for 15 s at 8000 *g*. Then the column was washed with Buffer RPE twice, 30–50 μl of RNase-free water was pipetted onto the RNeasy membrane, then centrifuged for 1 min at 8000 *g* to elute RNA.

cDNA synthesis — Total RNA was reversed transcribed by using the SuperScript™ First-strand synthesis system (Invitrogen Inc, Carlsbad, CA, USA). Briefly, the isolated RNA (1 μg) was pre-incubated with 0.4 μg of 12–18 oligo (dT) and 10 mM dNTP at 65°C for 5 min. The mixture was then incubated with 25 mM $MgCl_2$, 0.1 M dithiothreitol, 1 μl of RNase inhibitor and 1 μl of Superscript II reverse transcriptase at 42°C for 50 min. All the reversed transcription procedure stopped at 75°C for 15 min.

PCR — The synthesized cDNA was then amplified by the PCR method. A total volume of 50 μl containing 0.4 U *Taq* polymerase and specific primers derived from the human LBP sequence, 1.5 mM $MgCl_2$, 100 μM dNTP, and reaction buffer was used. The primers used to amplify the human LBP gene were 5′-AGGGCCTGAGTCTCAGCATCT-3′ (sense) and 5′-CAGGCTGGCCGTGTTGAAGAC-3′ (antisense) (17). β-actin was used as an internal standard or housekeeping gene. The cycling conditions used were initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s; and a final extension at 72°C for 10 min. Ten-microliter aliquots of final PCR products were analyzed by electrophoresis with 1.5% agarose gels and ethidium bromide. The bands were visualized under UV transillumination.

Statistical analysis

Chi-square test was performed to determine the significance of the differences in LBP expression frequency between healthy controls and patients under various conditions. Mann–Whitney and Kruskal–Wallis tests were used to determine the significance of the differences in LBP expression levels (mean ± SE) among various categories of gingival tissues. The differences between data sets with a probability of less than 0.05 were regarded as statistically significant.

Results

Sample collection

Gingival biopsies consisted of 42 PoTs (probing depth of 7.5 ± 1.3 mm) and 33 HT-Ps from 44 subjects with chronic periodontitis. Of them, 24 patients contributed 22 PoTs and 13 HT-Ps for detection of LBP peptide, and 20 patients contributed 20 PoTs and 20 HT-Ps for detection of LBP mRNA. Fifteen periodontally healthy tissues were obtained from 15 healthy subjects as controls. Among them, seven samples were used to detect LBP peptides and eight samples were used to detect LBP mRNA.

Immunohistochemistry

LBP peptide was detected in all healthy control tissues, 11 of 13 clinically healthy tissues (85%) and 20 of 22 pocket tissues (91%). LBP expression was mainly observed in the epithelium spanning from the oral sulcular epithelium to the coronal portion of oral epithelium as seen in healthy controls (Fig. 1A). The expression density was variable with a lower expression at the apical portion of oral sulcular epithelium (Fig. 1B). Within the epithelium, the expression of LBP was mainly confined to the cytoplasm of granular and keratinized layers (Fig. 1C). In oral epithelium, the expression of LBP decreased gradually from coronal portion to apical portion (Figs 1D and E). Low expression of LBP was noted in connective tissue beneath the epithelium (Fig. 1F).

In patients, LBP expression pattern was similar to that in healthy controls (Figs 2A and B). In connective tissue beneath the epithelium, LBP was mainly detected on the surface of vascular endothelial cells and/or inside the lumens of blood vessels (Figs 2C and D). It was detected in six of eight healthy control tissues (75%), seven of 12 clinically healthy tissues (58%) and 10 of 22 pocket tissues (45%). No significant difference was found in the LBP expression frequency among them.

The mean expression levels of LBP peptides in HT-Cs (101.4 ± 38.9) were significantly higher than those in HT-Ps (23.0 ± 9.4, $p < 0.01$) and PoTs (35.4 ± 8.8, $p < 0.05$). No significant difference was found between HT-Ps and PoTs.

RT-PCR

The expression of LBP and β-actin mRNA in various categories of gingival tissues was presented in Fig. 3. LBP message was detected in over 50% of the samples, i.e. six of eight HT-Cs

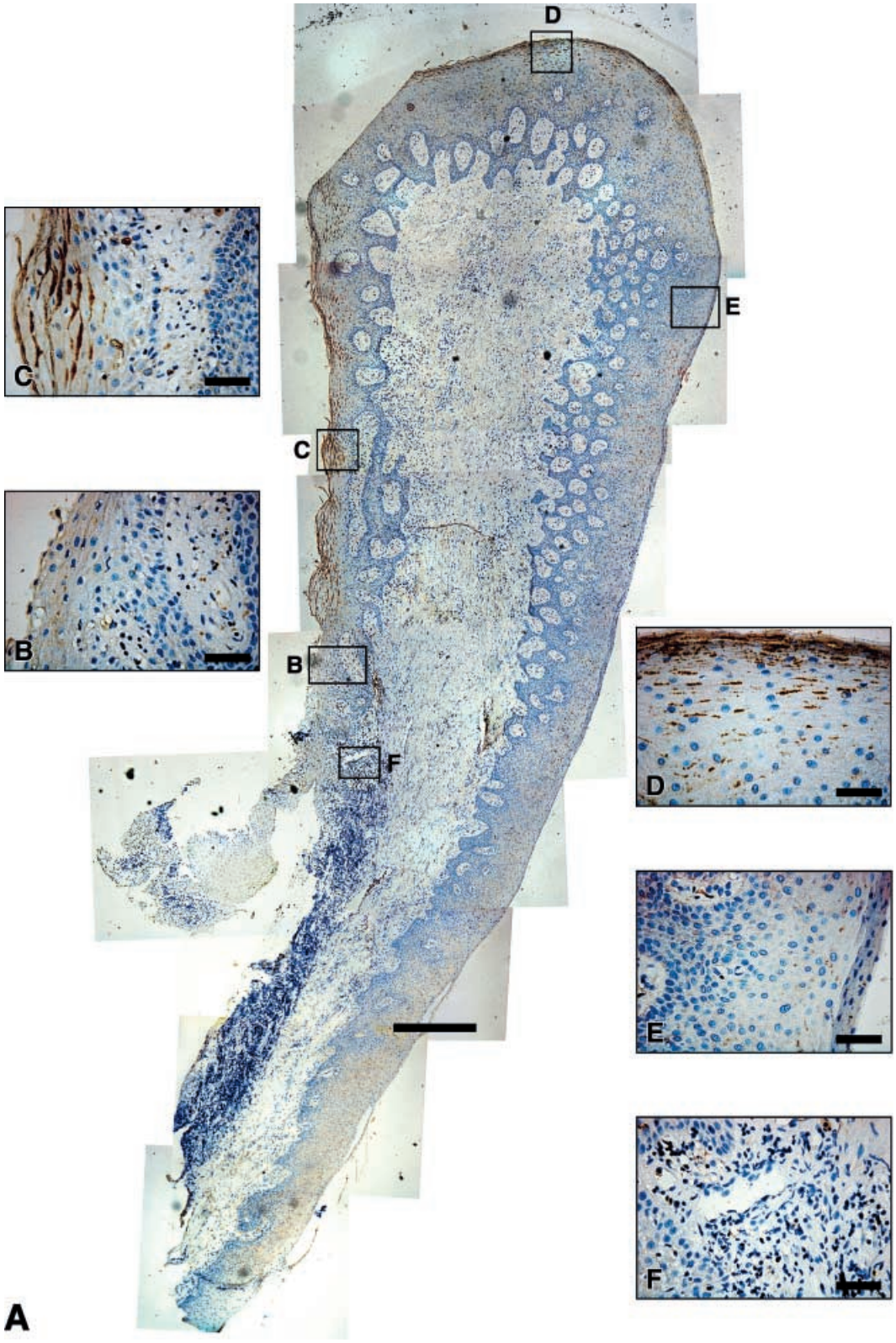

Fig. 1. (A) Lipopolysaccharide-binding protein (LBP) expression in a human healthy gingival biopsy. Significant expression of LBP is spanning from the oral sulcular epithelium to the coronal portion of oral epithelium. (B) Low expression at the apical portion of oral sulcular epithelium. (C) Expression in oral sulcular epithelium, which is mainly confined to the cytoplasm of granular and keratinized layers. (D) Expression in oral epithelium around the gingival margin. (E) Expression in oral epithelium, which gradually decreases from coronal to apical portion. (F) Low expression in connective tissue beneath the epithelium. Bar = 400 μm (A); Bars = 50 μm (B–F).

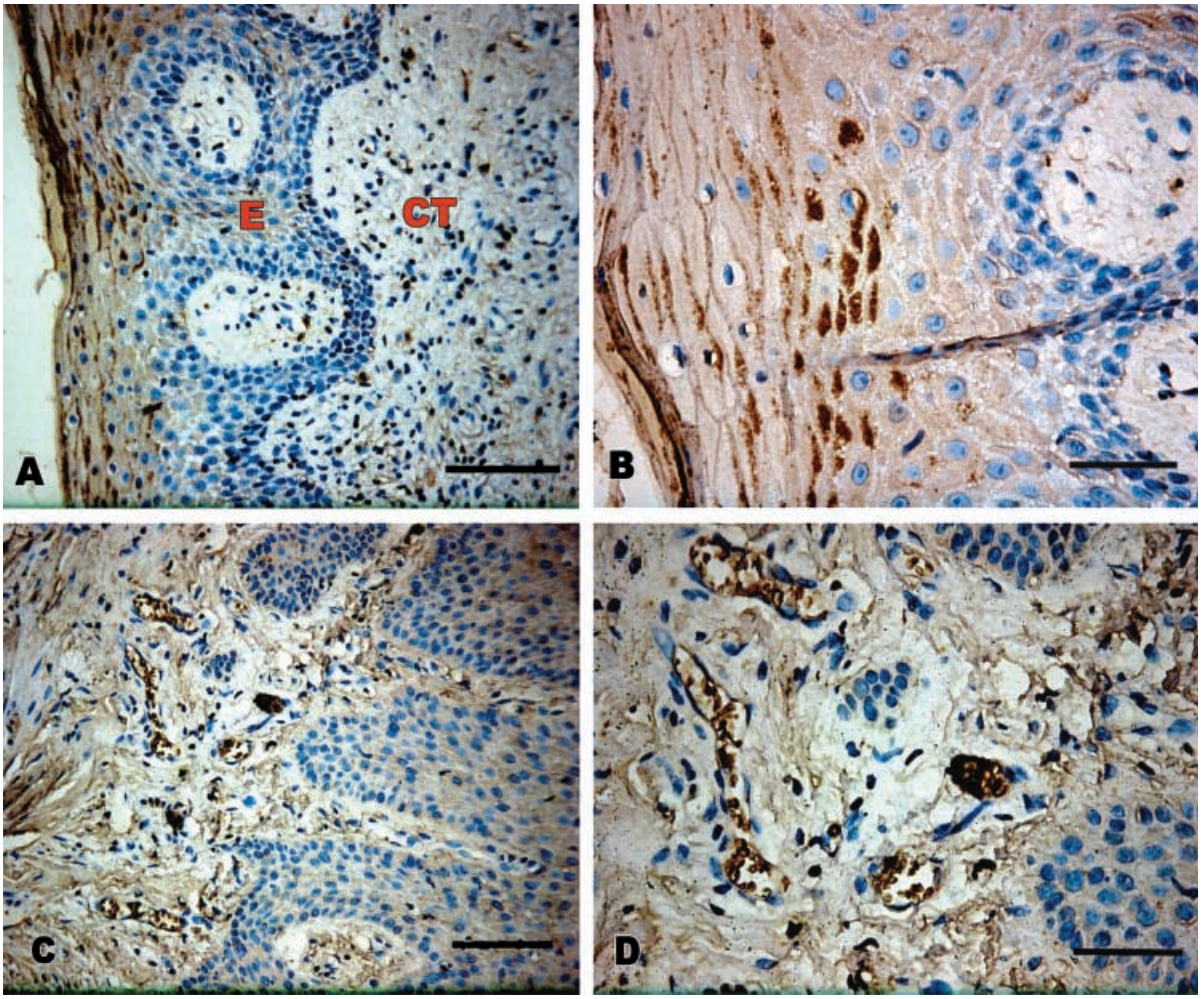

Fig. 2. (A) Significant expression of lipopolysaccharide-binding protein (LBP) in the gingival biopsies of subjects with chronic periodontitis. E: epithelium; CT: connective tissue. (B) Expression in cytoplasm of granular and keratinized layers. (C and D) Expression at the blood vessels of subepithelial connective tissue. Bars = 100 μm (A and C); bars = 50 μm (B and D).

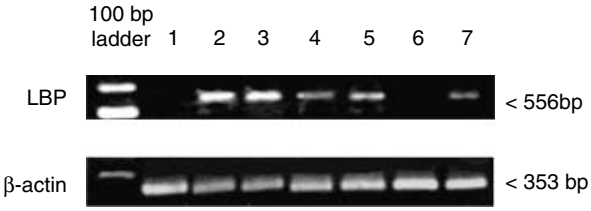

Fig. 3. Lipopolysaccharide-binding protein (LBP) mRNA expression in various categories of gingival tissues. Representative gel showing bands with amplified cDNA. Lanes 1 and 4: PoTs; lanes 3 and 6: HT-Ps; lanes 2, 5 and 7: HT-Cs. The size marker is 100 bp ladder. No mRNA expression in Lanes 1 and 6. PoTs, periodontal pocket tissues; HT-Ps, healthy tissues; HT-Cs, healthy controls.

(75%), 11 of 20 HT-Ps (55%) and 11 of 20 PoTs (55%). No significant difference was found among various categories of gingival tissues.

Discussion

LBP is a serum protein that strongly modulates the host response to LPS. It can enhance the sensitivity or recognition of the immune system to bacterial endotoxin by catalyzing its binding to macrophages (13, 18). LBP mediated the neutralization of LPS by lipoproteins (19) and acted in the cellular clearance of endotoxin from body fluid as an opsonin (20). An *in vitro* study showed that high concentrations of murine LBP blocked LPS-mediated secretion of TNF-α from a murine macrophage cell line (21), whereas *in vivo* studies demonstrated that murine LBP could transfer LPS into high-density lipoprotein and result in a protective effect towards LPS toxicity without cell stimulation in the circulation (21, 22). Moreover, recombinant

human LBP suppressed LPS-induced TNF production in a dose-dependent manner on mouse peritoneal exudates macrophages (23, 24). It has been suggested that the modulatory properties of LBP varied with target cells, the source of LBP or the mediators under investigation (1).

Periodontal pathogenesis is characterized by bacterial LPS activation of series pro-inflammatory cytokines and mediators from various host cells through a key pathway of cell stimulation, LPS, LBP, CD14 and IL-1 receptor/toll-like receptor (TLR) superfamily, induced transduction of transmembrane signalling cascades, and activation of transcription factors for gene expression (16, 25–27). It has become increasingly clear that innate immune system has a much more important and fundamental role in host defense (28). Innate host responses are triggered by TLRs that recognize a variety of microbial products like LPS (3). Stimulation of TLRs by LPS elicits activation of host cells to produce inflammatory cytokines and mediators. It is known that the quality of the host immuno-inflammatory response appears to ultimately determine the severity and extent of disease expression (29). The gingival epithelial cells are the first host cells to come in contact with periodontopathogens and they are in a unique position to function as an early signalling system to the immune cells (30). Although LBP was extensively studied in various bacteria-induced diseases in human (5, 6), the study on its roles in local immune system was limited. An *in vitro* study demonstrated that human intestinal epithelial cells could express LBP in response to pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α instead of LPS even in the presence of CD14 (18). To our knowledge, this was the first evidence to demonstrate the production of LBP by human local cells other than hepatocytes, implying the active role of LBP in local defense mechanisms. Currently, no information is available on the local expression of LBP in gingival tissues and its association with periodontal conditions.

In the present study, a specific monoclonal antibody was used to detect the expression of LBP peptides in human gingival tissues. LBP peptides were detected in both gingival epithelium and the lumen of blood vessels in gingival connective tissues in periodontally healthy controls as well as in periodontitis patients. It was interesting to note a specific pattern of LBP expression in gingival epithelium that spans from the oral sulcular epithelium to the coronal portion of oral epithelium with the expression density decreasing gradually from coronal portion to apical portion. To our knowledge, this was the first immunohistochemical evidence of the LBP expression by human gingiva.

LBP peptides were detected in cytoplasm of granular and keratinized layers of gingival epithelium but not in intercellular space, which implied that LBP was likely produced by gingival epithelium. In terms of this observation, it was known that the basal layer cells were mainly responsible for division, whereas the role of spinous cells was to traverse the epithelium as a keratinocyte. Once the keratinocyte left the basement membrane, it could no longer divide but maintain a capacity for protein production (31). Our findings were consistent with this notion.

The local expression of LBP peptide in human gingival tissues was confirmed by detection of LBP mRNA accordingly. In the past decades, extensive studies have revealed that LBP is mainly produced by hepatocytes. Grace *et al.* (17) have demonstrated the extrahepatic production of LBP mRNA in rat lung, kidney and heart. They also found LBP mRNA could only be produced by rabbit liver but not rabbit lung. Recently, it was also shown that LBP mRNA can be produced by human respiratory type II epithelial cells (32) and human intestinal epithelial cells (18). Taken together, our current observation on LBP expression in human gingival tissues supports that LBP can be expressed by non-hepatocytes in human as well.

The gingival epithelium as the first line of innate immunity plays a crucial role in containing microbial challenge. The local expression of LBP in epithelial cells might play a role in limiting the penetration of LPS to periodontal tissues by binding and immobilizing LPS, probably through the exfoliation of LPS–LBP complex. Besides its role in neutralization and clearance of LPS, a basal concentration of LBP may enhance the sensitivity of the immune system to LPS by catalyzing the LPS binding to host cells (11). However, high concentration of LBP could decrease LPS activity (21). It is speculated that local production of LBP by gingival epithelial cells might contribute to neutralization of bacterial endotoxin and thus enhance the host immune defense mechanisms. The present study showed that the mean LBP expression levels in gingival tissues of periodontally healthy subjects were significantly higher than those of periodontitis patients. These results suggest that LBP might play a role in the local defense to bacterial endotoxin and maintenance of periodontal homeostasis. The expression profile of LBP in gingival epithelium, to some extent, was similar to that of antimicrobial peptides like human beta-defensins (33). We suspected that LBP might function as an antimicrobial peptides-like protein and further study is warranted to confirm this hypothesis.

In addition, it was not surprising to note the expression of LBP peptides on the surface of endothelial cells and/or inside the lumen of blood vessels of gingival connective tissues. An *in vitro* study showed that LBP was not detected in the culture fluid of human umbilical vein endothelial cells even in the presence of various forms of stimulation, which implied that these endothelia cells unlikely produce LBP (18). In this regard, we postulated the detected LBP peptides in gingival connective tissues were derived from serum. Further investigation was needed to elaborate the relevant mechanisms involved in the local expression of LBP and the significance of LBP in the pathogenesis of periodontal diseases.

In conclusion, we for the first time found the expression of LBP peptide and mRNA in human gingival tissues. Local expression of LBP in gingival

tissues might play a role in enhancement of innate immunity and contribute to periodontal homeostasis.

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