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Lipopolysaccharide-binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast

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Background: Lipopolysaccharide-binding protein (LBP) participates in the interaction of lipopolysacchaide (LPS) with CD14 to modulate the expression of cytokines. Human gingival fibroblast may actively participate in LPS-induced immuno-inflammatory responses through CD14, toll-like receptor (TLR) superfamily, MD-2 and related adaptive proteins, leading to the expression of cytokines.

Objectives: The present *in vitro* study aimed to investigate the possible effect of LBP and *E. coli* LPS interaction on the expression of cellular LPS receptors and IL-6 by human gingival fibroblast.

Methods: The mRNA expression of CD14, LBP, TLR-2, TLR-4, MD-2 and IL-6 in human gingival fibroblast explants was detected by reverse transcription–polymerase chain reaction (RT–PCR) in the presence or absence of *E. coli* LPS and recombinant human LBP (rhLBP), while IL-6 peptides were analyzed by ELISA and immunohistochemistry, respectively.

Results: Human gingival fibroblast could constitutively express CD14, MD-2 and IL-6 mRNAs, but not TLR-2, TLR-4 and LBP mRNAs. *E. coli* LPS induced the messages expression of MD-2, TLR-2 and −4. The expression of both IL-6 message and peptide was up-regulated by *E. coli* LPS in a dose dependent manner. Whereas rhLBP could significantly down-regulate the expression of both mRNAs and peptides of CD14 and IL-6 but not MD-2 signals in the presence or absence of *E. coli* LPS. The up-regulated expression of TLR-2 and −4 by *E. coli* LPS no longer existed in the presence of rhLBP.

Conclusions: This study suggests that LBP may down-regulate the expression of IL-6 by human gingival fibroblast. Further studies are warranted to clarify the molecular mechanisms of LBP in regulation of cytokine expression by host cells and to elaborate the relevant clinical implications.

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Human periodontitis is a chronic inflammatory disease characterized by the destruction of tooth-supporting periodontal tissues. It is initiated and perpetuated by a group of predominantly Gram-negative bacteria (1). Lipopolysaccharide (LPS) has been shown to play an important role in the pathogenesis of various infections caused by gram-negative bacteria (2). Human gingival fibroblast is the most abundant structural cell in gingival

tissues and responsible for the synthesis and degradation of connective tissue, which maintains tissue integrity and homeostasis (3–6). Human gingival fibroblast may also play an important role in immune regulation through the following pathways. It has been reported that human gingival fibroblast can express various pattern recognition receptors, such as CD14 (7, 8), toll-like receptors (TLRs) (9) and a costimulatory molecule of CD40 (10, 11). Under the stimulations of LPS through these receptors, human gingival fibroblast produces various cytokines, such as interleukin (IL)-1, IL-6, IL-8, IL-10 and monocyte chemoattractant protein-1 (7–9, 12, 13). It was demonstrated that LPS from periodontal pathogens can penetrate gingival tissues and up- or down-regulate the production of pro-inflammatory cytokines by human gingival fibroblast (9, 14); in turn, these cytokines exert their effects in a paracrine and autocrine fashion to modulate inflammatory and immune responses (15). Subsequently, human gingival fibroblast intervenes in controlling the balance between extracellular matrix synthesis and degradation in response to various pro- and anti-inflammatory cytokines and growth factors (16–18).

Cytokines are thought to play a central role in the modulation of immuno-inflammatory systems. They are produced not only by immune cells, but also by epithelial cells, endothelial cells and fibroblasts. In inflammatory lesions, these cytokines are produced locally and participate in both local inflammatory tissue destruction and systemic effects (19). Various *in vitro* studies showed that the responses of human gingival fibroblast to different bacterial LPSs remained controversial. Some investigations showed that *Escherichia coli* LPS and *Porphromonas gingivilis* LPS exhibited a similar effect on the IL-6 production by human gingival fibroblast (20), whereas others found that *E. coli* LPS did not directly induce IL-6 production (13). As human gingival fibroblasts constitute a remarkably diverse group with respect to form, proliferation rate, expression of membrane markers, function and other characteristics (21–23), the results of human gingival fibroblast and cytokines interaction varied with the properties and source of cells or the stimulators under investigation. Further investigations are necessary to clarify LPS-induced IL-6 production by human gingival fibroblast.

Recent data showed that human gingival fibroblast could express membrane-bound CD14 (mCD14), which mediated the LPS-induced signal-transducing pathway, leading to the production of cytokines (7). Wang *et al*. (24) even demonstrated that mCD14 could be constitutively expressed by fibroblasts derived from gingival connective tissues but not those from other connective tissues. Recently, it was suggested that human gingival fibroblast consisted of cell populations that were heterogeneous in terms of the expression of mCD14 and CD14 mRNA, and that they could respond to the stimulation of LPS in different manners by producing various levels of IL-8 or interferon-γ (8).

Lipopolysaccharide-binding protein (LBP) is a glycosylated protein synthesized by hepatocytes and it is released into bloodstream upon acute-phase stimulation (25). It has been reported that LBP can also be produced by other human host cells, such as respiratory type II epithelial cells (26) and intestinal epithelial cells (27). Our recent *in vivo* study showed that LBP could be expressed by gingival epithelial cells and that the expression levels of LBP peptides in periodontally healthy subjects were significantly higher than in patients with chronic periodontitis (28). Furthermore, we also detected mCD14 on the fibroblastic cells (1).

In the past decade, both *in vivo* and *in vitro* studies elaborated the dual roles of LBP in LPS–LBP interactions. In *in vivo* studies, as a lipid transfer protein, high concentrations of LBP can neutralize LPS by transferring it to high density lipoproteins and result in detoxification of LPS (29). In contrast, LBP, CD14 and TLRs enhanced the sensitivity of the host to LPS-induced uncontrolled, acute inflammatory response that resulted in animal death (30). Some *in vitro* studies indicated that LBP participated in the interaction of LPS with CD14 to up-regulate the secretion of IL-6, IL-8 and tumor-necrosis factor-α (TNF-α) (31), via TLR-4–MD-2 complex (30). Other studies also found that LBP-oponized gram-negative bacteria or LPS–LBP complex could bind to mCD14 on macrophages or Chinese hamster ovary cells, and the complex was subsequently internalized, which resulted in phagocytosis or degradation of LPS (32, 33). These contrary results indicated that the dual roles of LBP might be related to various factors, such as concentrations of LPS, presence of other costimulators and the cytokines or mediators investigated as well as outcome measurements.

Although various studies explored the mechanisms of CD14 and LBP interactions on monocytes or macrophages and the resultant expression profiles of cytokines in response to LPS, no similar study was conducted to explore the effect of LPS on the expression of cytokines by human gingival fibroblast in relation to CD14 and LBP. Therefore, the present study investigated the effect of LBP on the *E. coli* LPS-induced expression of IL-6 by human gingival fibroblast and the concurrent expression profiles of CD14, TLR-2, TLR-4 and MD-2.

Materials and methods

Preparation of human gingival fibroblast and cell culture

Human gingival fibroblast was prepared as reported previously with modifications (13, 34). Human gingival fibroblast was prepared from the explants of normal gingival tissues in 15 periodontally healthy subjects (aged 18–25 years) with informed consent. They were systemically and periodontally healthy and the gingival biopsies were collected during the tooth extraction for orthodontic treatment purposes. The explants were minced finely into small pieces ($\leq$ 1 mm^3) by two sterile scalpels. They were then distributed into a plastic 75-cm^2 cell culture flask (Corning Glass Works, Corning, NY, USA) with Dulbecco's modified Eagle's medium (DMEM/

F-12) that contained 15% fetal bovine serum, 15 mM HEPES buffer, L-glutamine, pyridoxine hydrochloride, penicillin (100 U/ml), streptomycin (100 μg/ml) and insulin (4 μg/ml) (Invitrogen Corporation, Carlsbad, CA, USA). The culture flask was incubated at 37°C in 95% air and 5% CO_2. All tissue culture medium was removed and replaced with fresh medium every 7 days for 15–20 days. When the confluent human gingival fibroblast cell monolayers were formed, the cells were detached with 0.25% trypsin, washed with phosphate-buffered saline and subcultured in 75-cm^2 plastic flasks with DMEM/F-12 supplemented with 10% fetal bovine serum. The medium was changed every 3 days for 3–9 days. After two to three subcultures by trypsinization, homogeneous, slim, spindle-shaped cells growing in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels 4 through 7.

The harvested human gingival fibroblasts ($1–5 \times 10^5$ cell/ml) were seeded into four-well plates or 75-cm^2 culture flasks and grown until confluent. The medium was replaced with fresh DMEM/F-12 supplemented with 1% fetal bovine serum, followed by incubation for 24 h with various concentrations of recombinant human LBP (rhLBP: 0.01–10 μg/ml, R & D Systems Inc. Minneapolis, MN, USA) and/or *E. coli* LPS (0.01–100 μg/ml, Sigma-Aldrich Co., St. Louis, MO, USA). At the end of incubation, the cell culture supernatants of nine subjects were collected and stored for assay of cytokines. The cells in the four-well plates were fixed with 4% polyformaldehyde and 70% ethanol for 24 h, respectively, for detection of mCD14 by immunohistochemical staining. The cells of six subjects in the 75-cm^2 flask were used for mRNA detection.

Immunohistochemical staining

Human gingival fibroblasts immunoperoxidase staining procedures were performed as described previously (35). Briefly, the fixed human gingival fibroblasts were blocked in phosphate-buffered saline containing 0.3% H_2O_2 for 20 min at room temperature. After the block of non-specific binding in 10% normal horse serum, mouse anti-human mCD14 monoclonal antibody (NCL-CD14-223, Novocastra Ltd, Newcastle upon Tyne, UK) was placed on cells for 2 h at 37°C. Then cells were washed with phosphate-buffered saline and biotinylated horse anti-mouse IgG antibody was placed in each well and incubated for 30 min. The cells were incubated with avidin-conjugated peroxidase for 30 min at 37°C. Finally, the cells were incubated for 1–2 min with the diaminobenzidine (DAB) and counterstained with hematoxylin. After desiccation in graded ethanol (75%, 95% and 100%) and xylene, slides were mounted. Negative control was performed by incubating with secondary antibody alone or Tris-buffered saline instead of the primary antibody.

Sandwich enzyme-linked immunosorbent assay

The quantikine human IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Ltd., Mannheim, Germany) was used to evaluate the levels of IL-6 in the human gingival fibroblast culture supernatant, in accordance with the manufacturer's instructions. In brief, 100 μl of standard IL-6 and sample supernatants were placed, respectively, in the 96-well polystyrene microplate precoated with anti-IL-6 antibody. IL-6 was detected by a horseradish peroxidase-labelled monoclonal antibody to IL-6. Then 200 μl of anti-IL-6 conjugates were placed in each well and incubated for 2 h at room temperature. The microplate was washed to remove unbound enzyme-labelled antibodies. The amount of horseradish peroxidase bound to each well was determined by the addition of 200 μl of chromogenic solution. The reaction was stopped by the addition of 1 M sulfuric acid. The plates were read at 450 nm. The concentrations of IL-6 were determined by interpolation from a standard curve and presented as pg/ml. The detection sensitivity was determined as 10 pg/ml.

Extraction of total RNA from human gingival fibroblast

The lysis of human gingival fibroblast was performed by using streptavidin total RNA isolation system (Promega Co., Madison, WI, USA). The cells at the density of $1–5 \times 10^6$ were collected by centrifugation at 300 *g* for 5 min. The cell pellets were washed with 15 ml of ice-cold, sterile phosphate-buffered saline and centrifuged at 300 *g* for 5 min to obtain the cells. After the supernatant was removed, the human gingival fibroblasts were lysed by 175 μl of SV RNA lysis buffer. Then 350 μl of SV RNA dilution buffer were added to the 175 μl of lysate and mixed by inverting the tube three to four times. The tube was placed in a water bath at 70°C for 3 min and centrifuged at 12,000–14,000 *g* for 10 min at room temperature. Finally, the cleared lysate solution was transferred to a fresh microcentrifuge tube. Then 200 μl of 95% ethanol were added to the cleared lysate for precipitation of the total RNA. The mixture was transferred to the Spin Column and it was centrifuged at 12,000–14,000 *g* for 1 min. Then 600 μl of SV RNA were added to the RNA and centrifuged at 12,000–14,000 *g* for 1 min. Then the RNA was treated with 50 μl of DNase incubation mixture (40 μl of yellow core buffer, 5 μl of 0.09 M $MnCl_2$ and 5 μl of DNase I enzyme per sample) for 15 min at room temperature. The reaction was stopped by adding 200 μl of SV DNase stop solution. The RNA was washed twice and 100 μl of nuclease-free water was applied to the centre of the membrane, which contained the total RNA. The purified RNA was centrifuged at 12,000–14,000 *g* for 1 min and stored in −70°C.

Reverse transcription–polymerase chain reaction

cDNA was synthesized by using the SuperScript™ First-strand synthesis system (Invitrogen), according to the manufacturer's instructions. Briefly, the isolated RNA (1 μg) was preincubated 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 III reverse transcriptase at 50°C for 50 min. The reversed transcription procedure was stopped at 85°C for 5 min. The synthesized cDNA was then amplified by the polymerase chain reaction (PCR) method. β-actin was used as an internal standard. The primers used for the PCR are shown in Table 1. The cycling conditions used were: initial denaturation at 94°C for 5 min; 30–40 cycles at 94°C for 1 min, 65°C for 1 min (IL-6)/57°C for 1 min (CD14)/55°C for 1 min (LBP)/54°C for 1 min (MD-2)/55°C for 1 min (TLR-2)/52°C for 1 min (TLR-4), 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

All experiments were performed at least twice to confirm the reproducibility of the results. IL-6 concentrations were presented as means ± standard deviations (SD). The significance of the differences among the groups was analyzed by ANOVA. Differences between data sets with a probability of < 0.05 were regarded as statistically significant.

Results

Expression of interleukin-6 mRNA and peptides

IL-6 mRNA could be basally produced by human gingival fibroblast, whereas the message expression was markedly suppressed in the presence of rhLBP (0.1 μg/ml). Furthermore, the expression of IL-6 mRNA by human gingival fibroblast was significantly up-regulated in response to *E. coli* LPS (5, 10 μg/ml), whereas in the presence of *E. coli* LPS (5, 10 μg/ml) and rhLBP (0.1 μg/ml), the expression of IL-6 message was down-regulated markedly compared with that induced by *E. coli* LPS (5, 10 μg/ml) alone (Fig. 1A).

Overall, IL-6 peptide levels increased in a time-dependent manner (Fig. 1B). Within the initial 3 h, the IL-6 production by human gingival fibroblast remained at the basal levels with or without the stimulation of *E. coli* LPS. From 3 to 12 h, the basal expression levels of IL-6 doubled and increased by threefold at 24 h. When human gingival fibroblast was primed by rhLBP at the concentration of 0.1 μg/ml, the production of IL-6 by human gingival fibroblast was not significantly affected. In the presence of *E. coli* LPS at the concentration of 5 μg/ml and 10 μg/ml, human gingival fibroblast produced over sixfold the amounts of IL-6 from 3 to 24 h, compared with the basal expression levels. In the exposure of both *E. coli* LPS and rhLBP (0.1 μg/ml), the expression of IL-6 by human gingival fibroblast was markedly down-regulated from 9 to 24 h.

Figure 1(C) shows the IL-6 levels in the supernatants of human gingival fibroblast culture fluid after 24-h incubation with various doses of rhLBP alone, *E. coli* LPS alone or combinations of *E. coli* LPS and rhLBP. IL-6 levels markedly increased in the presence of *E. coli* LPS as compared with the basal expression levels ($p < 0.01$). The IL-6 levels were significantly higher in the presence of *E. coli* LPS 10 μg/ml than those of *E. coli* LPS 5 μg/ml ($p < 0.05$). When human gingival fibroblast was treated by rhLBP alone (0.01–10 μg/ml), IL-6 levels were slightly suppressed, whereas no significant difference was found as compared with the basal expression levels (rhLBP 0 μg/ml). In the presence of *E. coli* LPS (5, 10 μg/ml) and rhLBP (0.01−10 μg/ml), overall the IL-6 production significantly decreased about 50–60% as compared to that of *E. coli* LPS alone ($p < 0.01$).

Expression of membrane-bound CD14

Weak expression of mCD14 peptide could be detected on human gingival fibroblasts without any stimulation (Fig. 2A). In rhLBP (0.1 μg/ml)-primed human gingival fibroblasts, the number and the morphology of gingival fibroblasts appeared to maintain that of no stimulation, but the mCD14 expression was undetectable (Fig. 2B). When human gingival fibroblasts were stimulated by various concentrations of *E. coli* LPS (1, 5, 10, 50 and 100 μg/ml) for 24 h, the total human gingival fibroblast density and the number of mCD14-positive cells seemed to increase, reaching the peak at the concentration of 50 μg/ml. Furthermore, stronger mCD14 staining was also found in the human gingival fibroblast compared with that of no stimulation (Fig. 2C). In the concentration of LPS 100 μg/ml, human gingival fibroblast number and mCD14 expression decreased compared to that at the peak point. In the presence of various concentrations of LPS (1–100 μg/ml) and rhLBP (0.1 μg/ml), the cell density and morphology remained as that of no stimulation,

Table 1 . Polymerase chain reaction (PCR) primer sets for detection of mRNAs of CD14, interleukin-6, lipopolysaccharide-binding protein, toll-like receptor-2 and -4 and MD-2

Molecules	Sense	Anti-sense	References
CD14	5′-CAACTTCTCCGAACCTCAGC-3′	5′-TAGGTCCTCGAGCGTCAGTT-3′	36
IL-6	5′-ATGAACTCCTTCTCCACAAGCGC-3′	5′-GAAGAGCCCTCAGGCTGGACTG-3′	36
LBP	5′-AGGGCCTGAGTCTCAGCATCT-3′	5′-CAGGCTGGCCGTGTTGAAGAC-3′	37
TLR-2	5′-TTAGCAACAGTGACCTACAGAG-3′	5′-CAAATCAGTATCTCGCAGTTCC-3′	38
TLR-4	5′-TGGATACGTTTCCTTATAAG-3′	5′-GAAATGGAGGCACCCCTTC-3′	39
MD-2	5′-GCACATTTTCTACATTCC-3′	5′-CACAGTCTCTCCCTTCAG-3′	39

IL-6, interleukin-6; LBP, lipopolysaccharide-binding protein; TLR, toll-like receptor.

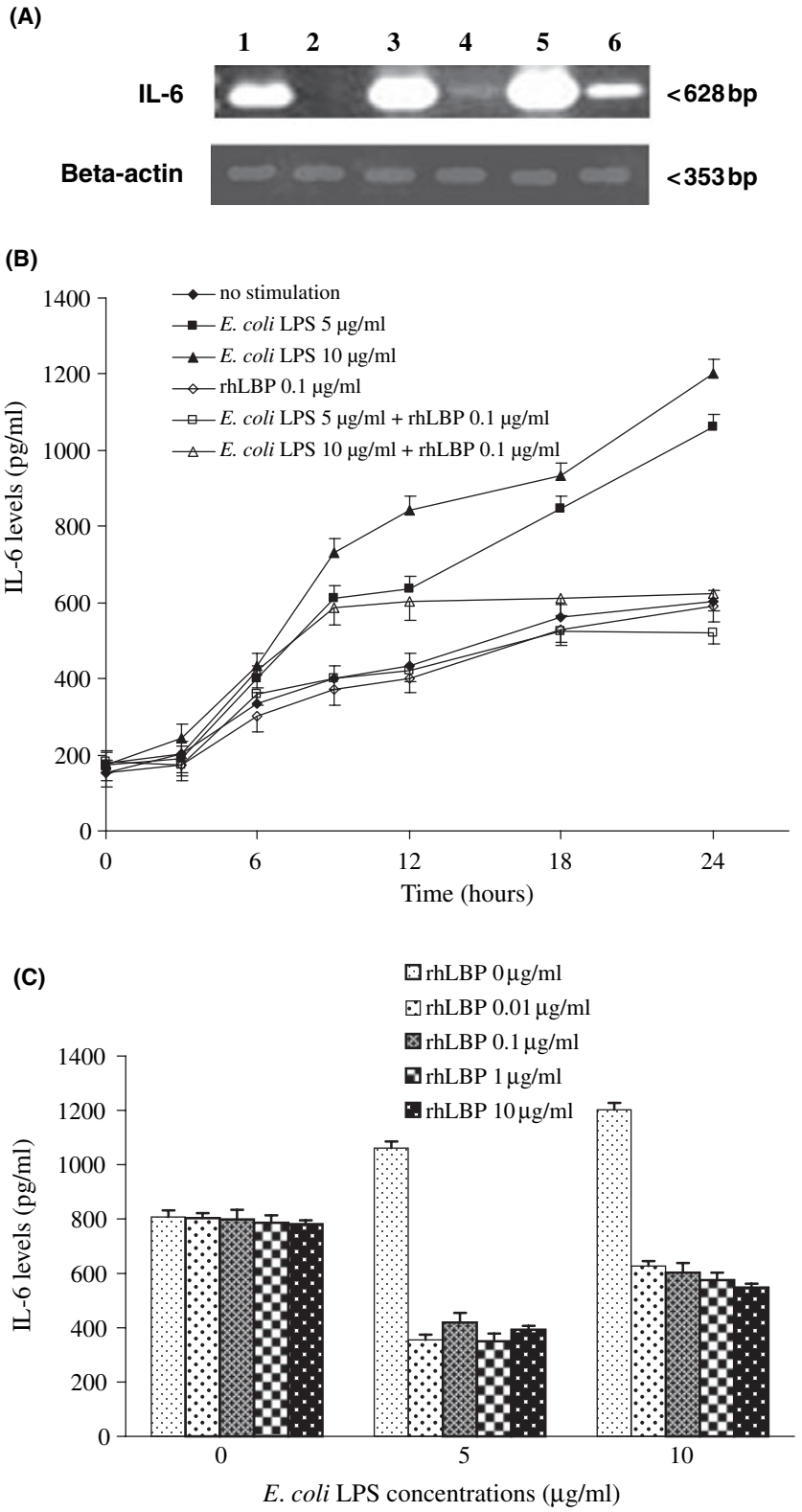

Fig. 1. (A) Expression of interleukin-6 (IL-6) mRNA by human gingival fibroblast after 24-h incubation with recombinant human lipopolysaccharide-binding protein (rhLBP) alone, *Escherichia coli* lipopolysaccharide (LPS) alone or combinations of *E. coli* LPS and rhLBP. It was found that human gingival fibroblast could express IL-6 message without any stimulation and *E. coli* LPS up-regulated its production, whereas when rhLBP was added, the IL-6 expression was significantly down-regulated. Lane 1: no stimulations; lane 2: rhLBP (0.1 μg/ml); lane 3: *E. coli* LPS (5 μg/ml); lane 4: *E. coli* LPS (5 μg/ml) + rhLBP (0.1 μg/ml); lane 5: *E. coli* LPS (10 μg/ml); lane 6: *E. coli* LPS (10 μg/ml) + rhLBP (0.1 μg/ml). (B) IL-6 levels in the supernatants of human gingival fibroblast culture fluid after incubation with rhLBP alone, *E. coli* LPS alone or combinations of *E. coli* LPS and rhLBP for 3, 6, 9, 12, 18 and 24 h. Overall, IL-6 peptide levels increased in a time-dependent manner. *Escherichia coli* LPS enhanced the IL-6 production by human gingival fibroblast, whereas rhLBP suppressed the IL-6 production from 9 h to 24 h. Similar experiments were repeated for three times. (C) IL-6 levels in the supernatants of human gingival fibroblast culture fluid after 24-h incubation with various doses of rhLBP alone, *E. coli* LPS alone or combinations of *E. coli* LPS and rhLBP. When human gingival fibroblast was treated by various concentrations of LBP alone, no significant reduction of IL-6 levels was noted, whereas in the presence of *E. coli* LPS and rhLBP, the IL-6 levels were down-regulated, despite of the doses of rhLBP applied.

whereas markedly reduced mCD14 expression was found on human gingival fibroblasts (Fig. 2D) compared with that of LPS alone. Besides, when a range of concentrations of rhLBP (0.01, 1, 10 μg/ml) and/or LPS (1–100 μg/ml) was applied, the mCD14 expression pattern remained similar to that of rhLBP (0.1 μg/ml) and/or LPS (1–100 μg/ml) and no significant difference was detected among them.

Expression of CD14, lipopolysaccharide-binding protein, toll-like receptor-2, toll-like receptor-4 and MD-2 mRNAs

In the human gingival fibroblasts without any stimulation, CD14 and MD-2 mRNAs were detected in all of the explants (6/6), whereas no mRNA expression of TLR-2, TLR-4 and LBP was detected in all of the explants (0/6). In *E. coli* LPS (5, 10 μg/ml)-primed human gingival fibroblast, CD14 mRNA remained at basal levels, whereas MD-2 mRNA was

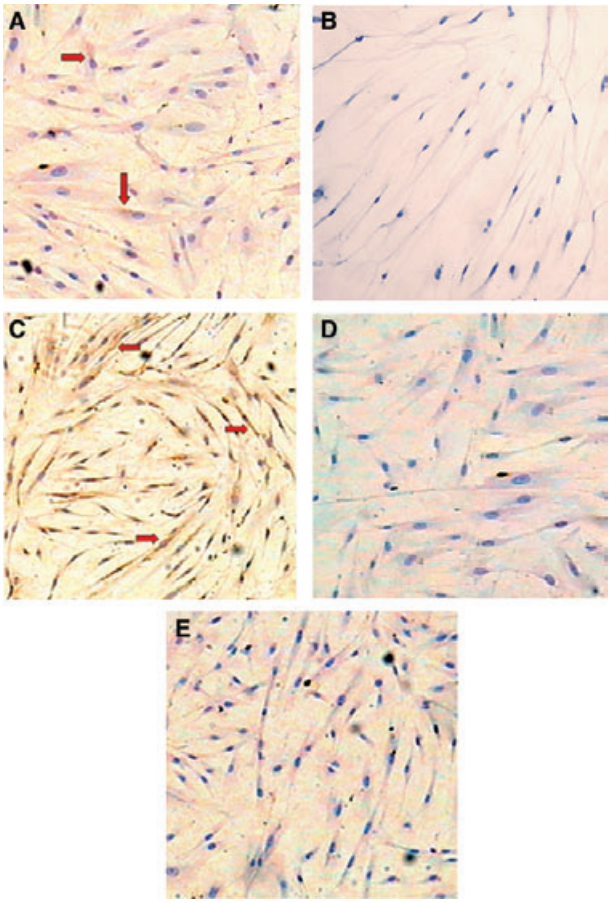

Fig. 2. Membrane-bound CD14 (mCD14) peptide expression by human gingival fibroblast. (A) mCD14 peptide could be detected on human gingival fibroblasts without any stimulation. (B) mCD14 expression was suppressed by recombinant human lipopolysaccharide-binding protein (rhLBP: 0.1 μg/ml) alone. (C) When human gingival fibroblasts were stimulated by *Escherichia coli* lipopolysaccharide (LPS: 10 μg/ml), the cell density increased and stronger mCD14 expression was found, compared to that of no stimulation. (D) Significantly reduced expression of mCD14 peptide was detected on human gingival fibroblasts under the stimulations of *E. coli* LPS (10 μg/ml) and rhLBP (0.1 μg/ml), compared to that of *E. coli* LPS alone. (E) Negative control.

up-regulated with reference to that of no stimulation; although no significant difference was noted between that induced by *E. coli* LPS at 5 μg/ml and LPS at 10 μg/ml. Furthermore, *E. coli* LPS could induce the expression of TLR-2 and -4 messages by human gingival fibroblast in a dose-dependent manner (Fig. 3).

When human gingival fibroblast was treated by rhLBP at the concentration of 0.1 μg/ml for 24 h, the basal expression of CD14 mRNA was significantly down-regulated, whereas MD-2 mRNA expression was not significantly affected and the TLR-2 and -4 messages remained undetectable. When human gingival fibroblasts were exposed to *E. coli* LPS (5, 10 μg/ml) and rhLBP (0.1 μg/ml), the expression of CD14, TLR-2 and -4 mRNAs in human gingival fibroblast was markedly suppressed, whereas the expression of MD-2 mRNA appeared to be independent of the presence of LBP. No detectable expression of LBP messages by human gingival fibroblast *per se* was observed (Fig. 3).

Discussion

LPS may challenge the attempt of host to maintain an effective and appropriate defense under conditions of clinical health and this process represents a highly evolved interaction between bacteria and host (40). The co-evolutionary process of bacteria and host is believed to induce production of a variety of host pattern recognition receptors to cope with the bacterial challenge, such as LBP, CD14 and TLR family. This bacteria–host interaction is ultimately regulated and controlled by a finely tuned feedback, so that the synthesis and inhibition of various cytokines and other mediators would undergo dynamic changes in response to bacterial challenge, hence limit tissue destruction (41). However, excessive stimulation of immune system by infectious agents may be detrimental to the host. This has prompted much research into the molecular and cellular events involved in the initiation and regulation of immunological mechanisms that control various infectious diseases.

In recent years, many studies were conducted to elucidate the mechanisms of LBP–CD14 interactions in LPS-induced cellular activation pathways, especially on human and mouse monocytes or macrophages (42). It has been shown that LBP plays a dual role in both enabling and inhibiting cellular responses to LPS depending upon the concentrations of LBP (43). LBP may inhibit LPS-induced synthesis of TNF-α, IL-6 and IL-8 by mouse macrophages or human alveolar macrophage, but not inducible nitric oxide synthase (42, 44–46). In contrast, LBP-treated mouse macrophages could induce rapid expression of TNF and IL-1α by increasing cytokine mRNA stability (47, 48). However, a recent study indicated that autocrine stimulation of TNF-α on mouse macrophages contributed to the signaling pathways initiated by LPS in the absence of LBP (49), which suggested that LBP may not be absolutely necessary for the activation of CD14-bearing cells with LPS (50).

Escherichia coli LPS is the most commonly used LPS for *in vitro* studies and it was therefore employed in the present study to lay down basal information and reference for our further studies on the potential effect of LBP on periodontopathogenic LPS-induced

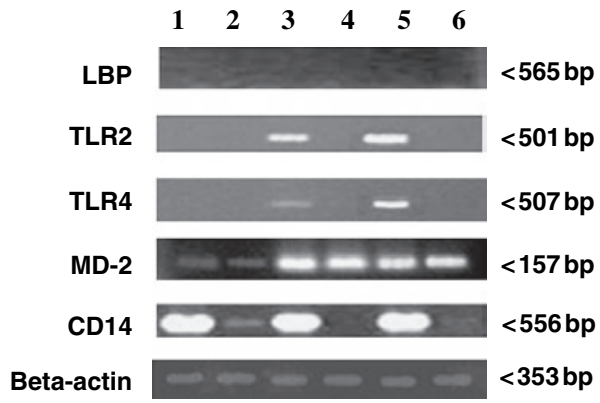

Fig. 3. Expression of CD14, MD-2, toll-like receptor 2 (TLR-2), TLR-4 and lipopolysaccharide-binding protein (LBP) mRNAs by human gingival fibroblast after 24-h incubation with recombinant human lipopolysaccharide-binding protein (rhLBP) alone, *Escherichia coli* lipopolysaccharide (LPS) alone or combinations of *E. coli* LPS and rhLBP. Human gingival fibroblast could express CD14 and MD-2 mRNAs but not TLR-2 and TLR-4 mRNAs without any stimulation. *Escherichia coli* LPS had no effect on the expression of CD14 message, but enhanced MD-2 mRNA expression and induced the expression of TLR-2 and TLR-4 messages as well. When human gingival fibroblast was primed by rhLBP, suppression of CD14, TLR-2 and TLR-4 mRNAs was found, whereas MD-2 mRNA expression was not affected. Lane 1: no stimulations; lane 2: rhLBP (0.1 μg/ml); lane 3: *E. coli* LPS (5 μg/ml); lane 4: *E. coli* LPS (5 μg/ml) + rhLBP (0.1 μg/ml); lane 5: *E. coli* LPS (10 μg/ml); lane 6: *E. coli* LPS (10 μg/ml) + rhLBP (0.1 μg/ml). The experiments were repeated for two times.

expression of cytokines and related pattern recognition receptors by human gingival fibroblasts. Our study for the first time found that rhLBP could significantly suppress both the IL-6 message and peptide production by human gingival fibroblast within 24 h in the presence of *E. coli* LPS. These results were in line with recent studies on LPS-dependent activation of monocytes (46, 51). To our surprise, when human gingival fibroblast was exposed to rhLBP alone for 24 h, the concentrations of IL-6 peptide remained elevated, whereas no concurrent message was detected. It is speculated that the down regulation of IL-6 mRNA expression by rhLBP might be a time-dependent process and the remaining levels of IL-6 peptides may be attributable to the basal mRNA expression of IL-6 gene. Furthermore, in an additional test we compared the IL-6 expression levels in the condition with 1% fetal bovine serum to those without 1% fetal bovine serum and no significant difference was found between the human gingival fibroblasts with or without the presence of 1% fetal bovine serum, which implied that in the present study the IL-6 expression levels may not be significantly affected by the addition of 1% fetal bovine serum in the culture medium.

Currently, the ability of human gingival fibroblast on CD14 expression remains controversial. Several studies reported that human gingival fibroblast could express mCD14 and the LPS-induced signal-transducing pathway was mediated by this LPS receptor (7). In contrast, Hayashi *et al.* (52) found that neither mCD14 peptides nor CD14 message was expressed by human gingival fibroblast, but human gingival fibroblast could be activated by LPS and sCD14. In the present study, *E. coli* LPS did not appear to significantly stimulate CD14 mRNA expression, which was consistent with a recent study (53), whereas rhLBP could markedly down-regulate the basal expression of CD14 message by human gingival fibroblast. In the presence of *E. coli* LPS, rhLBP remained able to inhibit the CD14 expression. These results were confirmed in the present study by immunohistochamical staining on the mCD14 expression by human gingival fibroblast under various conditions. Furthermore, rhLBP could significantly down-regulate the *E. coli* LPS-induced expression of IL-6 peptides and mRNA. It was interesting to note that although CD14 mRNA can be inhibited by rhLBP in the presence of a relatively high dose of LPS, IL-6 mRNA production was only partly inhibited. These findings implied that *E. coli* LPS-induced IL-6 production by human gingival fibroblast might be related to CD14. Recently, a study demonstrated that constitutive expression of CD40 contributed to the increased secretion of IL-6 by human gingival fibroblast (54). Moreover, it is speculated that the down-regulation of IL-6 and CD14 by rhLBP may be related to the roles of neutralization and clearance of bacterial LPS by LBP. This hypothesis was supported by a recent study (55). Additionally, the present study confirmed that no significant expression of LBP by human gingival fibroblast was observed with or without the stimulation of *E. coli* LPS, which implied that there was a neglectful autocrine effect of LBP on human gingival fibroblast *per se*. To our knowledge, this was the first report to show the inhibitory effect of LBP on the expression of CD14 and IL-6 by human gingival fibroblast.

As CD14 lacks a transmembrane domain and the LPS-induced intracellular signal transduction is mediated by CD14-TLRs-MD-2 complex, the present study extended to study the expression of TLR-2, TLR-4 and MD-2 mRNAs by human gingival fibroblast. It was found that in the absence of *E. coli* LPS, no messages of TLR-2 and -4 were detected, which was different from previous studies, although the LPS-induced expression of TLR-2 and -4 message was diminished by the presence of LBP. So far, it is well-documented that human gingival fibroblasts are heterogeneous in the expression of CD14. Whether TLRs expression by human gingival fibroblast is heterogeneous remains unknown. The present observations on TLR-2 and TLR-4 message expression were different from the previous studies and this discrepancy could probably

be due to the relatively small size of samples in the present study. In future studies, it is worthwhile to assess the expression of pattern recognition receptors by human gingival fibroblast in relation to genetic background and environmental factors. Furthermore, it is worthy of noting that LBP had no significant effect on MD-2 mRNA expression. A recent study offered the evidence that LBP had no effect on the expression of nuclear factor-κB mRNA in mouse macrophages, although it was found that LBP suppressed TNF-α production in the same cells (42), implying that LBP might not affect the signal molecules inside the cells. Furthermore, the ability of LBP to inhibit cellular responses could not be simply explained by its effect on LPS internalization, because LBP did not significantly increase the internalization of the cell-bound LPS (56). These studies suggest that LBP may modulate the expression of host pattern recognition receptors by human gingival fibroblast in the absence or presence of LPS likely through different regulation mechanisms. Further studies are needed to clarify this hypothesis.

On the other hand, the effect of LBP on the activation of cells or neutralization of LPS remained unclear. It is generally agreed that low concentrations of LBP may facilitate the activation of cells, whereas in high concentrations it enables the neutralization of LPS (57). A recent study found that 1 μg/ml of rhLBP in the presence of LPS increased the secretion of IL-6 by human monocytes (58). Furthermore, rhLBP at the concentrations of 0.1–1.0 μg/ml could enhance the LPS-induced TNF-α production by mouse microglial cell line in a concentration-dependent manner (59). In contrast, the addition of up to 0.1 μg/ml rhLBP markedly inhibited TNF-α production on mouse macrophages (42, 44), whereas the same concentrations of rhLBP have no effect on the production of IL-6 mRNA (42). In the present study, rhLBP at the concentrations of 0.01–10 μg/ml markedly down-regulated relatively high concentrations (5, 10 μg/ml) of *E. coli* LPS-induced production of IL-6 by human gingival fibroblast. Even in the low concentrations of *E. coli* LPS, the IL-6 peptide levels were also down-regulated by rhLBP. Therefore, it seems that the modulatory effects of LBP on the LPS-induced cellular responses may vary with different types of cells, the target mediators released by the cells concerned, as well as the stimulators under investigations.

Our recent *in vivo* study for the first time reported the local expression of LBP in human gingiva and the expression levels were significantly higher in periodontally healthy subjects than that in patients with chronic periodontitis, which suggested that LBP might play an active role in enhancement of innate immunity and contribute to periodontal homeostasis (28). Additionally, in the present study the inhibitory effect of LBP on *E. coli* LPS-induced IL-6 expression by human gingival fibroblast suggests that the appropriate expression of LBP may have a beneficial effect on innate host defense, thereby keeping the host protected from further insult of periodontal pathogens. The present study suggests that LBP may down-regulate the expression of IL-6 by human gingival fibroblast. Further studies are warranted to clarify the molecular mechanisms of LBP in regulation of cytokine expression by host cells and to elaborate the relevant clinical implications.

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