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Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease

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Background and Objective: A major factor in the pathogenesis of periodontal disease, which is one of the biofilm infectious diseases, is thought to be lipopolysaccharide (LPS), owing to its ability to cause inflammation and promote tissue destruction. Moreover, the elimination of pathogens and their component LPSs is essential for the successful treatment of periodontal disease. Lipopolysaccharide tolerance is a mechanism that prevents excessive and prolonged responses of monocytes and macrophages to LPS. Since persistence of inflammation is necessary for inflammatory cytokine production, cells other than monocytes and macrophages are thought to maintain the production of cytokines in the presence of LPS. In this study, we investigated whether human gingival fibroblasts (HGFs), the most abundant structural cell in periodontal tissue, might be able to maintain inflammatory cytokine production in the presence of LPS by not displaying LPS tolerance.

Material and Methods: Human gingival fibroblasts were pretreated with LPS (from *Porphyromonas gingivalis* and *Escherichia coli*) and then treated with LPS, and the amounts of interleukin (IL)-6 and IL-8 in the cell culture supernatants were measured. The expression of negative regulators of LPS signalling (suppressor of cytokine signalling-1, interleukin-1 receptor-associated-kinase M and SH2 domain-containing inositol-5-phosphatase-1) was also examined in LPS-treated HGFs.

Results: Human gingival fibroblasts did not display LPS tolerance but maintained production of IL-6 and IL-8 when pretreated with LPS, followed by secondary LPS treatment. Lipopolysaccharide-treated HGFs did not express negative regulators.

Conclusion: These results demonstrate that HGFs do not show LPS tolerance and suggest that this characteristic of HGFs sustains the inflammatory response in the presence of virulence factors.

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Caries and periodontal disease are two major oral diseases, and both are considered to be infections caused by biofilms (1). In particular, periodontal disease is highly prevalent and can affect most of the world population. Periodontal disease is accompanied by inflammation of the gingiva and destruction of periodontal tissues, leading to alveolar bone loss in severe clinical cases. *Porphyromonas gingivalis* (*P. gingivalis*), a gram-negative bacterial species with black pigmentation, is one of the suspected periodontopathic bacteria and is frequently isolated from

the periodontal pockets of patients with chronic periodontal disease (2,3). Periodontopathic bacteria produce many virulence factors, such as lipopolysaccharide (LPS) and peptidoglycan, and these bacterial factors induce host responses, including the production of pro-inflammatory cytokines. These immune responses lead to the destruction of periodontal tissue. For instance, interleukin (IL)-6 induces osteoclastogenesis (4) and results in alveolar bone loss. Clinically, the IL-6 concentration in gingival crevicular fluid is higher in patients with periodontal disease than in healthy control subjects (5). Therefore, excessive production of inflammatory cytokines appears to be important for the pathogenesis of periodontitis. Moreover, it is clinically known that removal of the bacterial biofilm is necessary for the improvement of periodontal inflammation, hence it is an effective treatment for periodontal disease (6).

Lipopolysaccharide tolerance is known to be a mechanism that prevents excessive and prolonged responses of the host or cultured monocytes and macrophages to LPS (7). Several factors are involved in LPS tolerance, and they include downregulation of Toll-like receptor 4 (TLR4) (8,9) and induction of the suppressor of cytokine signalling-1 (SOCS-1 10,11), interleukin-1 receptor-associated kinase M (IRAK-M) (12) and SH2 domain-containing inositol-5-phosphatase (SHIP-1) (13).

Human gingival fibroblasts (HGFs) are the most abundant resident cells in periodontal tissue. Human gingival fibroblasts express cell surface CD14 (14), TLR4 and MD-2 (15,16) and produce pro-inflammatory cytokines, such as IL-6 and IL-8, upon LPS stimulation (17–19). Therefore, HGFs may play an important role in modulating host defense against putative gram-negative periodontopathogens in the oral cavity.

To date, monocytes/macrophages, as well as lymphocytes and neutrophils, are considered to be the major cell population that elicits inflammatory response in periodontal disease. However, these cells are known to develop LPS tolerance, and therefore should produce inflammatory cytokines only transiently. Yet gingival inflammation persists as long as biofilms are present. Based on these observations, we hypothesized that inflammatory cytokine production by HGFs may be important in sustaining gingival inflammation even after prolonged exposure to LPS. Therefore, we investigated whether HGFs display LPS tolerance because, if our hypothesis is true, HGFs would be unlikely to develop LPS tolerance.

Material and methods

Reagents

Lipopolysaccharide from *P. gingivalis* 381 (PgLPS) was provided by Drs Tatsuji Nishihara and Nobuhiro Hanada (National Institutes of Public Health, Wako, Saitama, Japan). Lipopolysaccharide of *Escherichia coli* serotype O128:B12 (EcLPS) was purchased from Sigma (St Louis, MO, USA). Antibodies against SOCS-1 (sc-7005), SHIP-1 (sc-8425) and actin (sc-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibody against IRAK-M was from Chemicon International (Temecula, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Zymed Laboratory Inc. (South San Francisco, CA, USA). Transforming growth factor (TGF)-β_1 (supplied as an active form) was purchased from R&D Systems (Minneapolis, MN, USA), and recombinant human IL-10 from PeproTec (Rocky Hill, NJ, USA).

Cells

Human gingival fibroblasts were prepared as described previously (14). Human skin fibroblasts, cell line TIG-103, were obtained from JCRB cell bank (JCRB0528; Ibaraki, Osaka, Japan). Human gingival fibroblasts and TIG-103 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere of 5% CO_2/95% air. Human gingival fibroblasts were used between the seventh and 13th passages. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by the two-step procedure to enrich monocytes (20). A human monocytic cell line, THP-1, was obtained from RIKEN Cell Bank (RCB1189; Tsukuba, Ibaraki, Japan). Peripheral blood mononuclear cells and THP-1 cells were cultured in RPMI1640 (Sigma) containing 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin, and 10% FCS. This study was approved by the Ethical Committee of our institution. Informed written consent was obtained from each subject.

Cytokine measurement

Human gingival fibroblasts, TIG-103 or PBMC were seeded in 96-well plates (Asahi Techno Glass Corp., Tokyo, Japan) at 1×10^4 cells per well. Then, the cells were pretreated with medium only or indicated concentrations of PgLPS (10 ng/mL), EcLPS (10 ng/mL), TGF-β_1 (0.01–1000 ng/mL) or IL-10 (1–100 ng/mL) for 24 h. After washing with PBS, the cells were further incubated with LPS for an additional 24 h (200 μL per well). The culture supernatants were collected, and the concentrations of IL-6, IL-8 and TGF-β_1 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Biosource International Inc., Camarillo, CA, USA). Alternatively, HGFs were treated with 10 ng/mL of PgLPS for 7 days, the culture supernatants collected once every 24 h, and the concentrations of IL-6 and IL-8 measured. In the measurement of the active form of TGF-β_1, culture supernatants were not acidified because the acidification process converts latent TGF-β_1 into the active form.

Western blotting

After treating cells with LPS (0, 10, 100 and 1000 ng/mL) for between 0 and 24 h, cells were treated with LPS at the indicated concentration. At the indicated time, cells were washed twice

with PBS and lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin and 1 μg/mL pepstatin) for 30 min at 4°C. The lysed cells were collected into microcentrifuge tubes and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were collected, and protein concentrations were measured using BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, USA). Western blotting was performed as described previously (16).

Statistics

Data are presented as means ± SD. Differences between control and experimental groups were evaluated using Williams (Figs 1A and 3) or Dunnett method (Fig. 1B). All computations were performed with the statistical program R (http://www.r-project.org/). Values with $P < 0.05$ were considered significantly different.

Results

Lipopolysaccharide tolerance is not observed in HGFs

All experiments were performed using PBMC (positive control) in addition to HGFs because LPS tolerance has been well documented in PBMC (21,22) monocytes (9,23) and macrophages (24,25). Human gingival fibroblasts and PBMC were pretreated with various concentrations of *P. gingivalis* LPS (PgLPS), washed and again treated with PgLPS. Both cell types produced a low baseline level of IL-6, but PgLPS-treated cells produced high levels (Fig. 1A). While the production of IL-6 from PBMC was significantly reduced by PgLPS pretreatment at all concentrations tested, production of IL-6 by HGFs was not affected (Fig. 1A). Similar results were found for IL-8 (data not shown). These results indicate that HGFs do not display LPS tolerance to PgLPS in terms of IL-6 and IL-8 production.

Next we examined whether structurally different LPSs from *P. gingivalis* and *E. coli* could induce LPS tolerance in HGFs. Treatment with PgLPS or EcLPS induced production of IL-6 from medium-pretreated HGFs and PBMC (Fig. 1B). Upon treatment with PgLPS, both PgLPS- and EcLPS-pretreated HGFs produced a similar amount of IL-6 to that produced by medium-pretreated HGFs. Similar results were obtained when HGFs were treated with EcLPS. By contrast, LPS-induced IL-6 production by PBMC decreased significantly when PBMC were pretreated with either PgLPS or EcLPS (Fig. 1B). Similar results were found for IL-8 (data not shown).These results indicate that HGFs do not develop tolerance to structurally different LPSs.

We did the same experiments using human skin fibroblast TIG-103 cells. Secondary PgLPS-induced IL-6 production was decreased to approximately 30% of PgLPS pretreatment values in a dose-dependent manner (data not shown). In addition, upon treatment with PgLPS or EcLPS, TIG-103 cells pretreated with PgLPS or EcLPS produced approximately 30% of IL-6 compared with cells that had not been pretreated (data not shown). In contrast, IL-8 production was only slightly decreased in both experiments. These results indicate that skin fibroblasts display partial LPS tolerance.

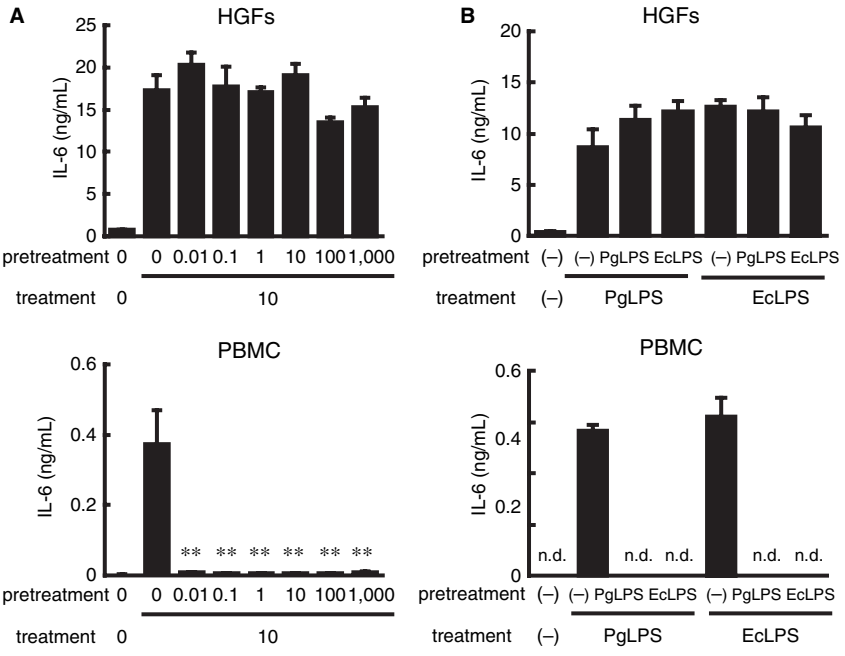

Fig. 1. Effects of pretreatment with LPS on inflammatory cytokine production. (A) HGFs or PBMC were pretreated with various concentrations (0–1000 ng/mL) of *P. gingivalis* LPS (PgLPS) for 24 h, washed and treated with 10 ng/mL of PgLPS for and additional 24 h. The levels of IL-6 in the culture supernatants were measured by ELISA. (B) Cells were pretreated with medium only (–) or with 10 ng/mL of PgLPS or EcLPS for 24 h, washed, and then treated with medium or 10 ng/mL of PgLPS or EcLPS for an additional 24 h. The levels of IL-6 in the culture supernatants were measured by ELISA. Results are the means ± SD of triplicate samples. Experiments were performed at least three times, and representative results are presented. $^{**}p < 0.01$ compared with medium-pretreated (–) and PgLPS-treated cells; n.d. not detected.

Lipopolysaccharide-treated HGFs do not express negative regulators

Since several negative regulators of TLR signalling, such as SOCS-1 (10,11), IRAK-M (12) and SHIP-1 (13), are involved in LPS tolerance, we examined whether LPS-treated HGFs express these molecules. Human gingival fibroblasts, TIG-103 and THP-1 cells were treated with 10 ng/mL of

PgLPS for 0 (unstimulated), 4, 8 and 24 h, and expression of SOCS-1, IRAK-M and SHIP-1 at the protein level was analysed. Human gingival fibroblasts treated with PgLPS did not express any of these molecules at any of the time points examined, nor did TIG-103 cells (data not shown). This was also the case for HGFs after treatment with 1000 ng/mL PgLPS (Fig. 2). In contrast, THP-1 cells expressed all three negative regulators of TLR signalling irrespective of PgLPS treatment (Fig. 2).

Transforming growth factor-β_1 partly suppressed LPS-induced IL-6 and IL-8 production by HGFs

Anti-inflammatory cytokines (TGF-β_1 and IL-10) are known to suppress the production of LPS-induced pro-inflammatory cytokines by PBMC (26–29). We therefore examined the effects of TGF-β_1 and IL-10 on cytokine production by HGFs. Human gingival fibroblasts and PBMC were pretreated with various concentrations of TGF-β_1 and IL-10 and then treated with PgLPS. When HGFs were pretreated with 1 or 0.1 ng/mL of TGF-β_1, IL-6 production decreased significantly to approximately 50% (Fig. 3A). However, further reduction of IL-6 production was not observed even though HGFs were pretreated with up to 100 ng/mL of TGF-β_1 (data not shown). In contrast, IL-8 production was not altered (Fig. 3A and data not shown). Moreover, pretreatment of HGFs with up to 100 ng/mL of IL-10 did not significantly alter the PgLPS-induced IL-6 and IL-8 production (Fig. 3A), indicating that HGFs did not respond to IL-10. In contrast to HGFs, when PBMC were pretreated with TGF-β_1 or IL-10, PgLPS-induced IL-6 and IL-8 production was decreased at the highest concentrations tested (Fig. 3A). The PgLPS-induced IL-6 production by TIG-103 cells was decreased by pretreatment with a lower concentration of TGF-β_1 compared with HGFs (data not shown). In contrast, IL-8 production was only slightly decreased by pretreatment with TGF-β_1. When TIG-103 cells were pretreated with IL-10, PgLPS-induced IL-6 and IL-8 production were also altered.

Since TGF-β_1 produced by LPS-treated macrophages induces LPS hyporesponsiveness (13), we examined the levels of active TGF-β_1 produced by PgLPS-treated HGFs and PBMC. Upon stimulation with PgLPS, the concentration of active TGF-β_1 increased in a dose-dependent manner in PBMC and TIG-103 cells, but did not change in HGFs (data not shown). These results indicate that LPS-treated HGFs did not produce active TGF-β_1 adequate to decrease IL-6 production.

Cytokine production after prolonged exposure to LPS

To investigate whether prolonged exposure to LPS affects the production of inflammatory cytokine, HGFs were treated with 10 ng/mL of PgLPS for 7 days. The levels of IL-6 and IL-8 in culture supernatants collected every 24 h were measured by ELISA (Fig. 4). Even at day 7 HGFs produced a large amount of IL-6 compared to that released from unstimulated HGFs (see Fig. 1) although the level was decreased compared with day 1 (Fig. 4). Similar results were observed for IL-8 production (Fig. 4). These results indicate that HGFs sustain production of IL-6 and IL-8 for at least 1 week after exposure to PgLPS.

Discussion

In this study, we demonstrated that HGFs do not develop LPS tolerance, unlike PBMC, in which tolerance is thought to be mediated via several inhibitory factors. The negative regulator of TLR signalling SOCS-1 is upregulated by LPS stimulation in macrophages (10,11). The regulator IRAK-M is also upregulated by LPS stimulation and suppresses Myeloid differentiation factor 88 (MyD88)-dependent TLR signalling (12). The regulator SHIP-1 is upregulated by LPS stimulation indirectly through the TGF-β_1-dependent autocrine/paracrine pathway (13) and suppresses TLR signalling through a phosphatidylinositol 3-kinase-dependent (30) and/or -independent mechanism (31).

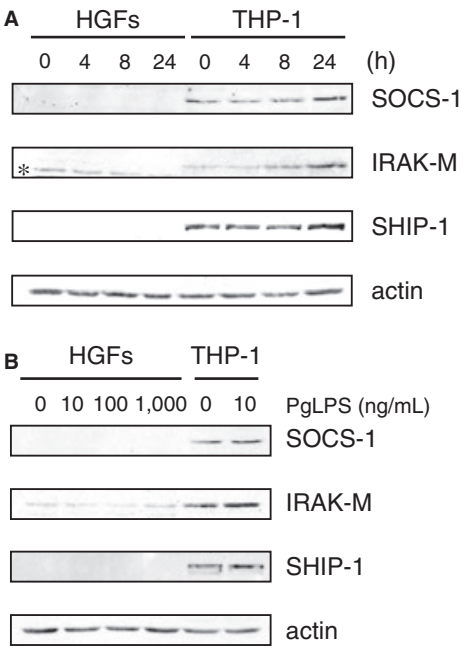

Fig. 2. Expression of negative regulators in HGFs and human monocytic cell line THP-1 treated with 10 ng/mL of PgLPS for the indicated times (A) or with various concentrations of PgLPS for 24 h(B). Cell lysates were prepared and subjected to Western blotting using antibodies against SOCS-1, IRAK-M, SHIP-1 and actin. Asterisk indicates non-specific band.

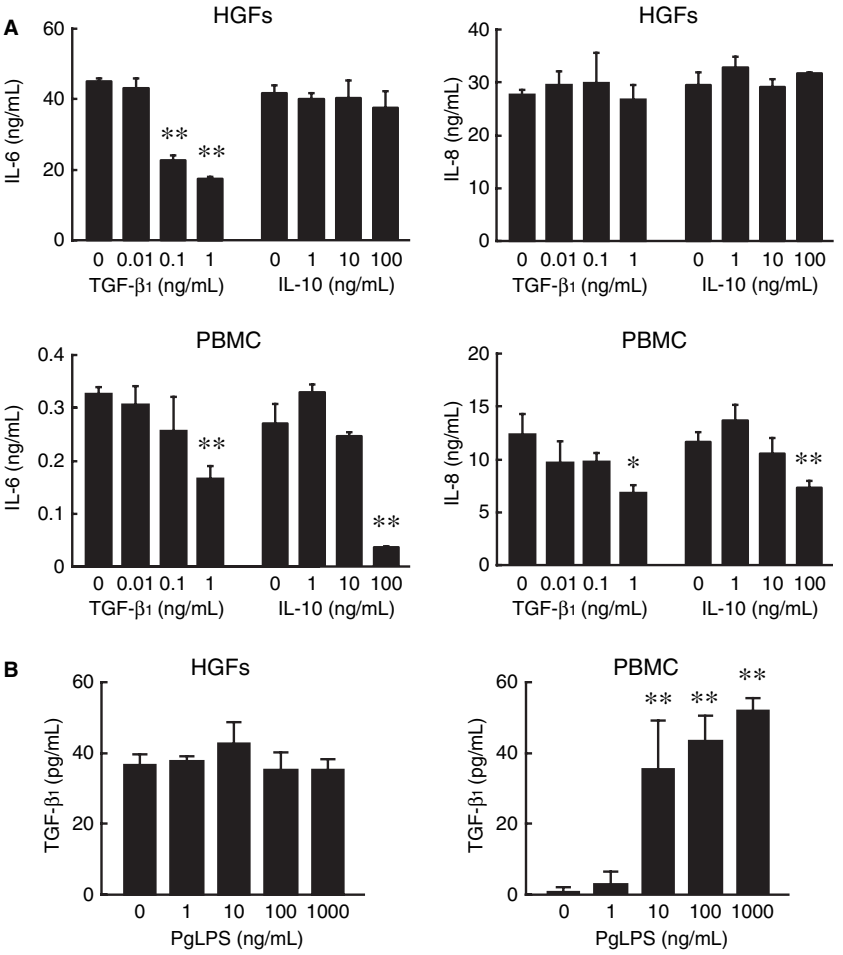

Fig. 3. (A) Effects of anti-inflammatory cytokines (TGF-β_1 and IL-10) on inflammatory cytokine production (IL-6 and IL-8) by HGFs and PBMC. Cells were pretreated with various concentrations of TGF-β_1 and IL-10 for 24 h, washed, and treated with 10 ng/mL of PgLPS for another 24 h. The levels of IL-6 and IL-8 in the culture supernatants were measured by ELISA. Results are the means ± SD of triplicate samples. Experiments were performed at least three times, and representative results are presented. $*p < 0.05$ and $**p < 0.01$ compared with cells without pretreatment. (B) Production of TGF-β_1 by HGFs and PBMC upon treatment with PgLPS. Cells were treated with indicated concentrations of PgLPS, and the levels of active TGF-β_1 in the culture supernatants were measured by ELISA. Results are the means ± SD of triplicate samples. Experiments were performed three times, and representative results are presented. $**p < 0.01$ compared with cells without PgLPS treatment.

Our results show that LPS-treated HGFs did not express SOCS-1, IRAK-M or SHIP-1, which explains why they did not demonstrate LPS-induced tolerance and supports our hypothesis that HGFs persistently produce inflammatory cytokines in the presence of LPS.

We also demonstrated that LPS-induced IL-6 production from HGFs was reduced by pretreatment with 0.1–1 ng/mL of TGF-β_1. However, unlike PBMC, the amount of active TGF-β_1 produced by HGFs did not increase after LPS treatment, suggesting that autocrine/paracrine anti-inflammatory effects of TGF-β_1 on HGFs are unlikely to reduce their response to LPS. In contrast, it is suggested that the TGF-β_1-dependent autocrine/paracrine pathway is at least partly involved in LPS tolerance in TIG-103 cells because: (1) IL-6 production was decreased by a lower concentration of pretreatment than in HGFs; (2) TGF-β_1 production was induced in PgLPS-treated TIG-103 cells; and (3) the changes in IL-6 and IL-8 production induced by pretreatment with TGF-β_1 were similar to those induced by LPS pretreatment.

Our results show that IL-10, another anti-inflammatory cytokine, downregulates LPS-stimulated IL-6 and IL-8 production by PBMC and agree with published data on monocytes, macrophages and PBMC (26,28,29). However, LPS-induced cytokine production by HGFs was not reduced even when HGFs were pretreated with high concentrations of IL-10 (100 ng/mL), suggesting that the anti-inflammatory effect of IL-10 would not affect LPS-induced pro-inflammatory cytokine production by HGFs *in vivo*.

It is well known that downregulation of cell surface TLR4 expression is involved in LPS tolerance (8,9). However, we previously reported that HGFs treated with LPS for 24 h expressed a similar level of TLR4 to untreated HGFs (32).

Taken together, our results strongly suggest that HGFs do not develop LPS tolerance by the following mechanisms. (1) Lipopolysaccharide-treated HGFs do not express negative regulators of TLR signalling. (2) Lipopolysaccharide-treated HGFs did not produce active TGF-β_1 adequate to decrease cytokine production, and LPS-induced cytokine production by HGFs is not affected by IL-10. (3) Human gingival fibroblasts do not downregulate cell surface expression of TLR4 in response to LPS.

Although various bacterial virulence factors other than LPS play important roles in periodontal disease, host immune responses are also significant determinants of tissue destruction. Since HGFs continuously produce inflammatory cytokines in the presence of LPS for at least 7 days, we consider that HGFs rather than monocytes/macrophages are the primary cells responsible for the production of inflammatory cytokines in periodontal disease. These results suggest that inflammatory responses persist in the presence of biofilm, which is associated with the characteristics of HGFs in producing inflammatory cytokines upon exposure to LPS, without

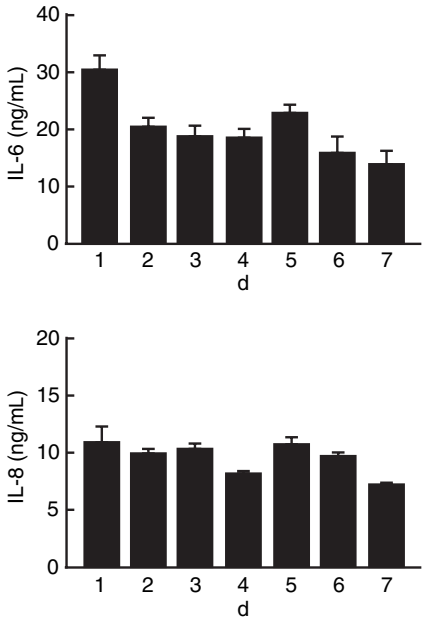

Fig. 4. Cytokine production during prolonged exposure to LPS. Human gingival fibroblasts were treated with 10 ng/mL of PgLPS for 7 days. Culture supernatants were collected every 24 h. Every day, cells were washed with PBS, and culture medium with PgLPS was added. The levels of IL-6 and IL-8 in culture supernatants at from day 1 to day 7 were measured by ELISA. Results are the means ± SD of triplicate samples. Experiments were performed three times, and representative results are presented.

developing tolerance to LPS. Moreover, partial LPS tolerance is observed in skin fibroblasts (concerning IL-6 production but not IL-8), suggesting that our findings are characteristic of HGFs.

In summary, we demonstrated that HGFs do not exhibit LPS tolerance and that negative regulators involved in LPS tolerance are not induced in HGFs. From these findings, we suggest that HGFs are critical in the pathology of periodontal disease. Our findings may provide the evidence that this characteristic of HGFs sustains the inflammatory response in the presence of virulence factors.

Acknowledgements

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