



Research Article

Enhancement of *Porphyromonas gingivalis*-Lipopolysaccharide Induced MCP-1 Expression by High Glucose in Human Endothelial Cells

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ABSTRACT

To investigate the synergistic effects of periodontal disease and diabetes mellitus on atherosclerosis, we evaluated the monocyte chemoattractant protein-1 (MCP-1) expression of endothelial cells induced by *Porphyromonas gingivalis*-lipopolysaccharide (LPS) and high glucose. We also tested whether antioxidants could inhibit the MCP-1 expression induced by the simultaneous stimulation of LPS and high glucose. Human umbilical vein endothelial cells (HUVECs) were stimulated with various concentrations of *P. gingivalis*-LPS (0.1, 1.0, and 10 µg/mL) in normal glucose (5.5 mM), with high glucose (10 mM and 20 mM), and with 0.1 µg/mL *P. gingivalis*-LPS in high glucose. MCP-1 expressions were measured by real-time RT-PCR and ELISA. Vitamin C (100 µM) and vitamin E (50 µM) were administered before simultaneous stimulation with 0.1 µg/mL *P. gingivalis*-LPS and 20 mM glucose. LPS ≥ 1.0 µg/mL increased the expression of MCP-1 mRNA and protein compared to unstimulated HUVECs. High glucose in the culture medium increased the MCP-1 mRNA expression slightly but significantly at 2 hr of incubation, but the MCP-1 protein level was not increased. Simultaneous stimulation with 0.1 µg/mL LPS and high glucose increased the expression of MCP-1 mRNA and protein compared to unstimulated HUVECs. By contrast, pre-incubation of vitamin C or E inhibited the increase of MCP-1 mRNA expression induced by 0.1 µg/mL LPS and 20 mM glucose. Our finding that high glucose enhanced the MCP-1 expression with even a low level of LPS suggests that caution is advisable regarding the atherogenicity of diabetic patients with periodontal disease.

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1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arteries that is characterized by the activation of endothelial cells, the recruitment of monocytes into the vessel wall, and the differentiation of recruited macrophages into cholesterol-laden foam cells [1]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is considered a key player in monocyte recruitment into the arterial wall, which is thought to be an essential step in the development and progression of atherosclerosis [2]. We demonstrated that low-density lipoprotein (LDL) obtained from diabetic patients was oxidized and enhanced the expression of MCP-1 mRNA in human umbilical vein endothelial cells (HUVECs), and we thus speculated that the LDL of diabetic patients accelerates their atherosclerosis [3].

Porphyromonas gingivalis-derived lipopolysaccharide (LPS) upregulates the expression of adhesion molecules, growth factors, and

MCP-1 in arterial wall cells [4]. In addition, high glucose induces monocyte-endothelial cells' adhesion and transmigration by increasing the expressions of MCP-1 and vascular cell adhesion protein 1 (VCAM-1) in human aortic endothelial cells [5]. However, synergistic effects of LPS and high glucose on MCP-1 expression in HUVECs have not yet been investigated, to the best of our knowledge. We have, therefore, investigated whether a high glucose concentration in a culture medium enhances the *P. gingivalis*-LPS induced MCP-1 expression in HUVECs compared to a normal glucose concentration in the culture medium.

In atherosclerosis, oxidative stress has been implicated as an important etiologic factor and in vascular function [6]. Antioxidants such as vitamin C and E may thus inhibit atherogenesis and improve vascular function by decreasing the cellular production and the release of reactive oxygen species (ROS) and inhibiting endothelial activation (i.e., expression of adhesion molecules and monocyte chemoattractants) [6]. In the present study, therefore, we used vitamins C and E to test whether

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they could suppress the expression of MCP-1 induced by *P. gingivalis*-LPS and high glucose.

2. Materials and Methods

2.1. Cell Culture

HUVECs were harvested from umbilical cord veins and cultured in M199 medium supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), 20 µg/mL endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO), 100 µg/mL heparin (Sigma-Aldrich), and 5 µL/mL penicillin-streptomycin liquid (Sigma-Aldrich). This cell culture medium contains a concentration of 5.5 mM glucose. Cells were used within six passages.

2.2. Measurement of MCP-1 mRNA Expression by Real-Time Reverse Transcriptase-Polymerase Chain Reaction Analysis

The expressions of MCP-1 mRNA in HUVECs were measured by a real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR). For the quantitation of MCP-1 mRNA expression induced by LPS, HUVECs were grown to confluence in 35-mm gelatin-coated dishes in the cell culture medium. The cell culture medium was replaced with a new 0.5 mL of the cell culture medium and LPS at 0.1, 1.0, and 10 µg/mL was added. After each incubation time of 1, 2, 4, 6, and 12 hr with LPS, total RNA was extracted using TRIzol (Invitrogen), and a real-time RT-PCR for MCP-1 mRNA was performed with the same primers used in our previous study [7]. The standard curve of MCP-1 mRNA was composed by using the total RNA of the unstimulated HUVECs, and the expression of MCP-1 mRNA is given relative to that in the unstimulated HUVECs.

To examine the MCP-1 mRNA expression induced by high glucose, 2.3 µL or 7.3 µL of 1 M glucose was added to 0.5 mL of the cell culture medium for the concentration of 10 mM glucose or 20 mM glucose, respectively, and HUVECs were incubated for 1, 2, 4, 6, and 12 hr. Then, total RNA was extracted, and a real-time RT-PCR for MCP-1 mRNA was performed. In the case of simultaneous stimulation with LPS and high glucose, 0.1 µg/mL LPS plus 10 mM glucose or 20 mM glucose as final concentrations were added together to 0.5 mL of cell culture medium and HUVECs were incubated for 2, 4, 6, and 12 hr. The total RNA was then extracted, and a real-time RT-PCR for MCP-1 mRNA was performed.

To examine the inhibitory effect of vitamin C or E on the enhancement of MCP-1 mRNA expression induced by 0.1 µg/mL LPS and 20 mM glucose, 1 hr before the administration of 0.1 µg/mL LPS + 20 mM glucose, 100 µM vitamin C or 50 µM vitamin E was added to 0.5 mL of the cell culture medium. After 2-hr stimulation by 0.1 µg/mL LPS + 20 mM glucose with or without Vitamin C or E, total RNA was extracted, and real-time RT-PCR analyses of MCP-1 mRNA were performed. In addition, to test the suppressive effect of vitamin E against 0.1 µg/mL LPS + 20 mM glucose, we conducted the same experiment using a 24-hr pre-incubation with 50 µM vitamin E in place of the 1-hr pre-incubation time.

2.3. Measurement of MCP-1 Protein Expression in the Cell Culture Medium by Enzyme-Linked Immunosorbent Assay

The concentrations of MCP-1 protein in the cell culture medium produced by HUVECs were measured by enzyme-linked immunosorbent assay (ELISA) using the MCP-1 ELISA kit (R&D Systems, Minneapolis, MN). For the quantitation of MCP-1 protein expression induced by LPS, HUVECs were grown to confluence in 96-well gelatin-coated dishes in the cell culture medium. The cell culture medium was replaced with new 0.5 mL of the cell culture medium, and LPS at 0.1, 1.0, or 10 µg/mL was added. After incubation times of 2, 6, 12, and 24 hr with LPS, supernatants were extracted, and the measurement of the concentrations of MCP-1 protein was performed according to the manufacturer's instructions.

For the quantitation of MCP-1 protein expression induced by LPS and high glucose, 0.1 µg/mL LPS plus 10 mM glucose or 20 mM glucose were added together to 0.5 mL of cell culture medium in the 96-well gelatin-coated dishes, then incubated for 2, 4, 6, or 12 hr. We then measured the concentrations of MCP-1 protein in the supernatants. The concentrations of MCP-1 protein were divided by that of unstimulated HUVECs and are given relative to that in the unstimulated HUVECs.

2.4. Statistical Analysis

All data are presented as the mean ± SEM. The statistical analysis was performed by a one-way analysis of variance (ANOVA) followed by Fisher post-hoc tests for comparisons of multiple groups. Results were considered significant when the *p*-value was <0.05.

3. Results

3.1. Expression of MCP-1 mRNA Induced by LPS

Figure 1 shows the expression of MCP-1 mRNA induced by LPS in HUVECs. HUVECs were stimulated by LPS at 0.1, 1.0, and 10 µg/mL and incubated for 1, 2, 4, 6, or 12 hr under a normal glucose concentration (5.5 mM) in the culture medium. LPS over the concentration of 1.0 µg/mL increased the MCP-1 mRNA expression dose-dependently at the 2-, 4-, and 6-hr incubation times. At 12 hr of incubation, only 10 µg/mL LPS increased the MCP-1 mRNA expression compared to that of the unstimulated HUVECs.

3.2. Expression of MCP-1 Protein Induced by LPS

Figure 2 shows the ratio of MCP-1 protein concentration in the medium induced by LPS in HUVECs. HUVECs were stimulated by LPS at varying doses (0.1, 1.0, 10 µg/mL) and incubation times (2, 6, 12, 24 hr) under a normal glucose concentration (5.5 mM) in the culture medium. At the 6-hr and 12-hr incubation times, 10 µg/mL LPS increased the MCP-1 protein to approximately threefold higher than that of the unstimulated HUVECs, and at the 12-hr incubation, 1.0 µg/mL LPS also increased the MCP-1 protein to 2.2-fold higher than that of the unstimulated HUVECs.

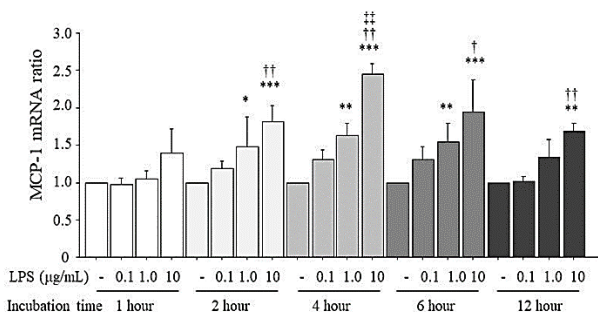


FIGURE 1: The expression of MCP-1 mRNA induced by LPS in HUVECs. HUVECs were stimulated with 0.1, 1.0, or 10 µg/mL LPS and incubated for 1, 2, 4, 6, or 12 hr under a normal glucose (5.5 mM) in the culture medium. The total RNA was then extracted and the MCP-1 mRNA expression was measured by real-time RT-PCR. The expression of MCP-1 mRNA is given relative to that in the unstimulated HUVECs. Data are means±SEM of 4–6 experiments. *p<0.05, **p<0.01, ***p<0.001 vs. unstimulated HUVECs, †p<0.05, ††p<0.01 vs. 0.1 µg/mL LPS-stimulated HUVECs, †††p<0.001 vs. 1.0 µg/mL LPS-stimulated HUVECs.

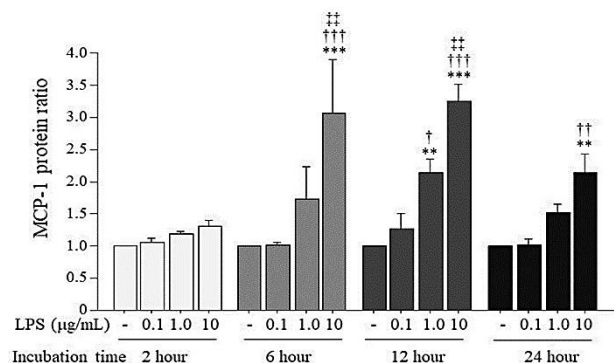


FIGURE 2: The expression of MCP-1 protein induced by LPS in HUVECs. HUVECs were stimulated with 0.1, 1.0, or 10 µg/mL LPS and incubated for 2, 6, 12 or 24 hr under normal glucose (5.5 mM) in the culture medium. The MCP-1 protein in the culture medium was then measured by ELISA. The expression of MCP-1 protein is given relative to that in the unstimulated HUVECs. Data are means±SEM of four experiments. **p<0.01, ***p<0.001 vs. unstimulated HUVECs, †p<0.05, ††p<0.01, †††p<0.001 vs. 0.1 µg/mL LPS-stimulated HUVECs, ††††p<0.001 vs. 1.0 µg/mL LPS-stimulated HUVECs.

3.3. Expression of MCP-1 mRNA and Protein Induced by High Glucose

Figure 3 shows the expression of MCP-1 mRNA produced by HUVECs cultured in normal glucose (5.5 mM) or high glucose (10 mM or 20 mM). At the 1-hr incubation, 20 mM glucose in the culture medium and at the 2-hr incubation, both 10 mM and 20 mM glucose in the culture medium increased the MCP-1 mRNA expression slightly but significantly. From 4-hr incubation to 12 hr incubation, no increase in the MCP-1 mRNA expression by high glucose was seen.

Regarding the production of MCP-1 protein, from 2- to 24-hr incubation, there was no increase of the MCP-1 protein in the medium by high glucose, and the levels of MCP-1 protein in the medium were equal to those observed with 5.5 mM glucose (data not shown).

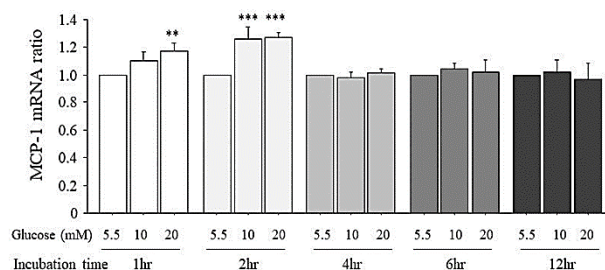


FIGURE 3: The expression of MCP-1 mRNA produced by HUVECs cultured in normal glucose 5.5 mM and high glucose (10 mM or 20 mM). HUVECs were incubated under normal glucose (5.5 mM) or high glucose (10 mM, 20 mM) in the culture medium for 1, 2, 4, 6, or 12 hr. The total RNA was then extracted, and the MCP-1 mRNA expression was measured by real-time RT-PCR. The expression of MCP-1 mRNA is given relative to that in the unstimulated HUVECs. Data are means±SEM of seven experiments. **p<0.01, ***p<0.001 vs. 5.5 mM glucose.

3.4. Enhancing Effects of High Glucose on the MCP-1 mRNA Expression Induced by LPS

To investigate the effects of high glucose on LPS-induced MCP-1 mRNA expression in HUVECs, we employed the LPS concentration of 0.1 µg/mL, which did not increase the MCP-1 mRNA expression at any incubation time. As shown in (Figure 4), at the 2-, 4-, 6-, and 12-hr incubations, 0.1 µg/mL LPS did not increase the MCP-1 mRNA expression compared to that of the unstimulated HUVECs, but high glucose at 10 mM and 20 mM added to 0.1 µg/mL LPS increased the MCP-1 mRNA expression compared to that of the unstimulated HUVECs.

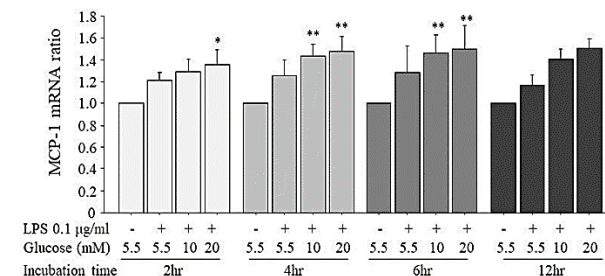


FIGURE 4: Enhancing effects of high glucose on the MCP-1 mRNA expression induced by LPS. HUVECs were stimulated by 0.1 µg/mL LPS under normal glucose (5.5 mM) or high glucose (10 mM, 20 mM) in the culture medium for 2, 4, 6, or 12 hr. The total RNA was then extracted, and the MCP-1 mRNA expression was measured by real-time RT-PCR. The expression of MCP-1 mRNA is given relative to that in the unstimulated HUVECs. Data are means±SEM of 6-9 experiments. *p<0.05, **p<0.01 vs. unstimulated HUVECs.

At the 2-hr incubation, 0.1 µg/mL LPS + 20 mM glucose increased the MCP-1 mRNA expression to 1.36-fold that of the unstimulated HUVECs, and at 4 hr and 6 hr of incubation, 0.1 µg/mL LPS + 10 mM or 20 mM glucose increased the MCP-1 mRNA expression compared to that of the unstimulated HUVECs, but the expressions of MCP-1 mRNA in 0.1 µg/mL LPS + 10 mM or 20 mM glucose were not significantly different from those in 0.1 µg/mL LPS + 5.5 mM glucose. At 12 hr of incubation, the MCP-1 mRNA expression induced by LPS 0.1

µg/mL + 10 mM or 20 mM glucose tended to be increased compared to that of the unstimulated HUVECs, but the difference was not significant.

3.5. Enhancing Effects of High Glucose on MCP-1 Protein Expression Induced by LPS

As in the case of MCP-1 mRNA expression (Figure 4), high glucose (10 mM and 20 mM) added to 0.1 µg/mL LPS increased the MCP-1 protein production compared to that of the unstimulated HUVECs (Figure 5). In particular, at 12-hr incubation, the MCP-1 protein expression induced by 0.1 µg/mL LPS + 10 mM or 20 mM glucose was higher than that induced by 0.1 µg/mL LPS + 5.5 mM glucose and at 24-hr incubation, the MCP-1 protein expression induced by 0.1 µg/mL LPS + 20 mM glucose was higher than that induced by 0.1 µg/mL LPS + 5.5 mM glucose.

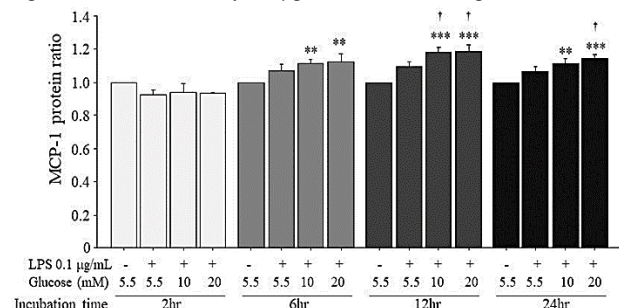


FIGURE 5: Enhancing effects of high glucose on the MCP-1 protein production induced by LPS. HUVECs were stimulated by 0.1 µg/mL LPS under normal glucose (5.5 mM) or high glucose (10 mM, 20 mM) in the culture medium for 2, 6, 12 or 24 hr. The MCP-1 protein in the culture medium was then measured by ELISA. The expression of MCP-1 protein production is given relative to that in the unstimulated HUVECs. Data are means±SEM of eight experiments. **p<0.01, ***p<0.001 vs. unstimulated HUVECs, †p<0.05 vs. 0.1 µg/mL LPS-stimulated HUVECs.

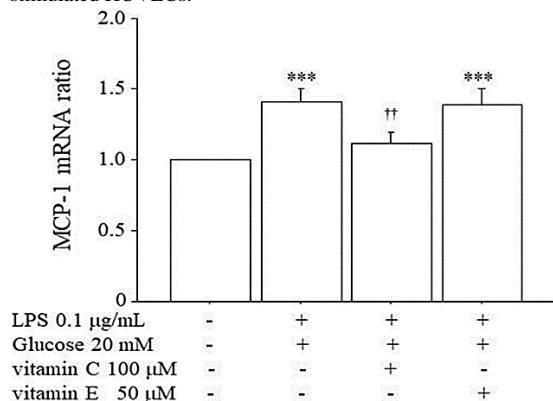


FIGURE 6: Inhibitory effects of vitamins C and E on the MCP-1 mRNA expression induced by LPS high glucose. Vitamin C (100 µM) or vitamin E (50 µM) was administered 1 hr before the simultaneous stimulation of 0.1 µg/mL LPS + 20 mM glucose for 2 hr. The total RNA was then extracted from the HUVECs, and the MCP-1 mRNA expression was measured by real-time RT-PCR. The expression of MCP-1 mRNA is given relative to that in the unstimulated HUVECs. Data are means±SEM of seven experiments. ***p<0.001 vs. unstimulated HUVECs, ††p<0.01 vs. 0.1 µg/mL LPS and 20 mM glucose-stimulated HUVECs.

3.6. Inhibitory Effects of Vitamin C and E on the MCP-1 mRNA Expression Induced by High Glucose + LPS

Because vitamins C and E are known as antioxidants, and since they are known to have inhibitory effects on MCP-1 expression, we investigated whether vitamin C or E can suppress the increase of MCP-1 mRNA expression induced by LPS + high glucose. Vitamin C at 100 µM or vitamin E at 50 µM was administered 1 hr before the simultaneous stimulation by 0.1 µg/mL LPS + 20 mM glucose. Figure 6 shows that vitamin C, but not vitamin E, significantly inhibited the increase of MCP-1 mRNA expression induced by 0.1 µg/mL LPS + 20 mM glucose. In the case of 24-hr pre-incubation with vitamin E before the stimulation by LPS + 20 mM glucose, the 50 µM vitamin E significantly suppressed the MCP-1 mRNA expression induced by LPS + 20 mM glucose (LPS + 20 mM glucose: 1.35 ± 0.16 vs. LPS + 20 mM glucose with vitamin E: 1.12 ± 0.03, p=0.0496, n=12).

4. Discussion

In a study using a human macrophage cell line, U937, it was reported that high glucose (25 mM) enhanced the secretion of inflammatory mediators such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α) and prostaglandin E₂ (PGE₂) induced by 0.1, 1.0 or 10 µg/mL of *P. gingivalis*-LPS, and the study's authors concluded that hyperglycemia compromises the periodontal health of patients with diabetes mellitus [9]. In addition, Iwata *et al.* demonstrated that in THP-1 monocytes, high glucose upregulated the *Escherichia coli*-LPS-stimulated production of inflammatory cytokines such as TNF-α and MCP-1 through the activation of c-jun N-terminal kinase (JNK), and the study's authors suggested that these results may be one of the mechanisms by which subclinical infections by Gram-negative bacteria promote atherosclerosis in diabetic subjects [10].

In view of this, our results obtained using endothelial cells (HUVECs) stimulated simultaneously with *P. gingivalis*-LPS and high glucose are along this line of evidence of synergistic effects of periodontal disease and diabetes mellitus on atherosclerosis, although the detailed mechanisms are unknown except for that of oxidative stress because vitamins C and E suppressed the enhancement of MCP-1 expression induced by simultaneous stimulation with *P. gingivalis*-LPS and high glucose.

Vitamins C and E are thought to reduce the risk of cardiovascular disease due to their antioxidant effects. Vitamin C at 50–200 µM suppressed the MCP-1 expression induced by lysophosphatidylcholine, an oxidized component of LDL in HUVECs [11] and 20–60 µM vitamin E also suppressed IL-1β-induced MCP-1 production dose-dependently in human aortic endothelial cells (HAECs) [12]. We, therefore, used 100 µM vitamin C and 50 µM vitamin E in the present study. Our results demonstrated that both of these vitamins at those concentrations suppressed the enhancement of MCP-1 mRNA expression induced by the simultaneous stimulation with LPS + high glucose with a 1-hr pre-incubation for vitamin C and 24-hr pre-incubation for vitamin E.

LPS is known to stimulate endothelial cells such as HUVECs through Toll-like receptor 4 complex (which consists of soluble CD14 and MD2), and then NF-κB (which is the transcription factor of MCP-1) is

activated through two signaling pathways: the PI3 kinase/Akt pathway and the I κ B kinase (IKK) pathway [13]. On the other hand, it was reported that high glucose (25 mM and 35 mM) accelerates MCP-1 production via p38 MAPK in HUVECs with induced Jun N-terminal kinase (JNK) activity [14]. However, this phenomenon was seen after a 7-day incubation. By contrast, regarding short-term high-glucose exposure, Piga *et al.* reported that in HAECs, 25 mM glucose significantly increased the MCP-1 mRNA expression at a 3-hr incubation, as is the case in the present study [5]. In addition, Piga *et al.* suggested that the short-term high-glucose exposure induced NF- κ B activation via the production of intracellular reactive oxygen species (ROS), as determined using the fluorescence probe DCFH-DA. Therefore, in the case of simultaneous stimulation by LPS and high glucose in HUVECs, we speculate that oxidative stress evoked by high glucose enhanced the LPS-induced MCP-1 expression through additional NF- κ B activation; however, we do not yet know the detailed signaling pathway.

Periodontitis is reported to be associated with endothelial dysfunction [15, 16], which is an initial step of atherosclerosis due to the subsequent overexpression of adhesion molecules and chemokines, including MCP-1 [17]. Hence, our present observation that high glucose enhanced the MCP-1 expression stimulated by even a low level of LPS (which alone did not increase the MCP-1 expression) compared with unstimulated HUVECs indicates that the atherogenicity of diabetic patients with periodontal disease should be monitored carefully, although it is not yet known whether diabetic patients with periodontal disease are more likely to be affected by atherogenicity compared to diabetic patients without periodontal disease. On the other hand, although it is reported that the use of an antioxidant with periodontal treatment has the potential to improve periodontal clinical parameters [18], the combination of periodontal therapy with antioxidants for diabetic patients with periodontal disease may be effective against atherogenicity.

5. Conclusion

Our present findings confirmed that high glucose concentrations of culture medium enhanced the MCP-1 expression induced by 0.1 μ g/mL of *P. gingivalis*-derived LPS, a concentration which alone was not great enough to strengthen the expression of MCP-1 mRNA and protein in HUVECs. This means that glucose has a synergistic effect with LPS on the induction of MCP-1 expression in HUVECs, and it indicates that in diabetic patients with periodontal disease, atherosclerosis may be accelerated compared to that of diabetic patients without periodontal disease because MCP-1 has a central role in the formation of atherosclerotic lesions. Our results thus suggest the need to determine whether the simultaneous presence of diabetes mellitus and periodontal disease promotes atherosclerosis synergistically in humans, and if so, how this occurs.

Acknowledgments

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Conflicts of Interest

None.

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