High Glucose Enhances Interleukin-6-induced Vascular Endothelial Growth Factor 165 Expression via Activation of Gp130-mediated p44/42 MAPK-CCAAT/Enhancer Binding Protein Signaling in Gingival Fibroblasts*

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Diabetic patients are susceptible to severe inflammatory periodontal disease manifested as swollen gingiva with bleeding, but the underlying mechanism is not well understood. Our purpose was to determine the effect of a high glucose (HG) condition on the interleukin-6/soluble interleukin-6 receptor (IL-6/sIL-6R)-induced activation of signaling and vascular endothelial growth factor (VEGF) expression in human gingival fibroblasts (HGFs). In this study, HGFs were cultured for at least two passages under a normal glucose (NG; 5.5 mM) condition or high glucose (25 mM) condition. Importantly, the HG condition significantly induced expression of gp130 mRNA in HGFs compared with levels in control cells. Consistent with the expression of its mRNA, the HG condition also increased the expression of gp130 protein, and phosphorylation of the tyrosine residue by gp130 was enhanced significantly by IL-6/sIL-6R stimulation. Furthermore, the HG condition enhanced the IL-6/sIL-6R-induced phosphorylation of p44/42 MAPK and led to subsequent activation of CCAAT/enhancer binding protein in nuclei. In contrast, there was no significant difference in phosphorylation of JNK between the HG and NG condition. Interestingly, HGFs increased IL-6/sIL-6R-induced VEGF165 mRNA expression and VEGF165 secretion under the HG condition compared with levels under the NG condition. In contrast, the induction of VEGF165 secretion was partially inhibited by PD98059 (selective p44/42 MAPK inhibitor) under the HG condition. In addition, the VEGF165 secretion was completely inhibited by the combination of PD98059 and SP600125 (JNK inhibitor). Our findings suggest that the HG condition indirectly increases VEGF expression via activation of gp130-mediated p44/42 MAPK-CCAAT/enhancer binding protein signaling in HGFs. Thus, elevated VEGF secretion in HGFs under the HG condition may play a role in the development of the severe periodontitis observed in diabetic patients.

Diabetes mellitus is a systemic disease with several major complications such as retinopathy, nephropathy, and neuropathy (1, 2). Several studies have reported that hyperglycemia and subsequent biochemical events were correlated with the development of their diseases, although the precise mechanism of them remains unclear (3, 4). It has been generally accepted that bacterial infection-induced inflammatory periodontal disease, so-called periodontitis, is one of the diabetic complications (5). Poor metabolic control of diabetes mellitus has often been associated with the severity of periodontitis (6).

Interleukin-6 (IL-6), a central proinflammatory cytokine, has been implicated in the progression of periodontitis, although its role remains elusive (7, 8). We reported previously that IL-6 signals in the presence of the soluble form of IL-6 receptor (sIL-6R) are initiated in the phosphorylation of gp130 in human gingival fibroblasts (HGFs) (9). At least two distinct signaling cascades, the signal transducer and activator of transcription 3 (STAT3) pathway and the mitogen-activated protein kinase (MAPK) pathway, have been activated in cytoplasm and lead to various responses in HGFs (9).

Vascular endothelial growth factor (VEGF) is a potent antiangiogenic factor that acts as a specific mitogen for vascular endothelial cells through specific cell surface receptors (10, 11). Molecular cloning of cDNAs for the VEGF family has revealed the existence of at least four isoforms of 206, 189, 165, and 121 amino acids produced as a result of alternative splicing (12). Molecular cloning of cDNAs for the VEGF family has revealed the existence of at least four isoforms of 206, 189, 165, and 121 amino acids produced as a result of alternative splicing (12). VEGF165 is the most abundant isoform expressed in the majority of normal human tissues (12). VEGF121 is also well expressed in many normal tissues, however its biological activity has been shown to be 10–100-fold weaker than that of VEGF165 (12). VEGF is also one of the major factors promoting diabetic complications and is implicated in the development of neovascularization and endothelial dysfunction in diabetic vascular complications (13, 14). Interestingly, Unlu et al. (15) reported that VEGF is increased in gingival tissues of diabetic patients, especially those with periodontitis. Therefore, VEGF could be as an important mediator in the severe periodontitis seen in diabetic patients, although the precise mechanism remains unknown.

HGFs are the most abundant cells in gingival connective

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The abbreviations used are: IL-6, interleukin-6; AP-1, activator protein-1; C/EBP, CCAAT/enhancer binding protein; ELISA, enzyme-linked immunosorbent assay; HG, high glucose; HGF, human gingival fibroblast; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NG, normal glucose; sIL-6R, soluble form of IL-6 receptor; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

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tissues and play an important role in the control of inflammation in inflamed gingiva (9). Recently, we demonstrated that VEGF165 expression is significantly induced by IL-6 in the presence of sIL-6R in HGFs (16). Our finding suggests that IL-6 promotes angiogenesis in inflamed gingiva via VEGF expression by HGFs, resulting in progression of periodontitis. However, little has been known about the effects of the high glucose (HG) condition on VEGF expression in HGFs. Unveiling this precise mechanism may contribute to clarifying the pathogenesis of severe periodontitis in diabetic patients. In the present study, we have examined the regulation of VEGF expression by HGFs under the HG condition and further evaluated the effects of the HG condition on the IL-6-mediated signaling pathway involved in VEGF expression in HGFs.

EXPERIMENTAL PROCEDURES

Cell Culture—HGFs were prepared as reported previously (9) and were divided into the normal glucose (NG) group, incubated in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum containing 5.5 mM glucose, or the HG group, incubated in Dulbecco’s modified Eagle’s medium containing 25 mM glucose. HGFs were cultured under the 25 mM glucose (HG) condition and were stimulated with a combination of 50 nM PD98059 and specific JNK inhibitor SP600125 before and during stimulations. To examine the effect of the c-Jun NH₂-terminal kinases (JNK) pathway on IL-6-induced activator protein-1 (AP-1) activation or VEGF165 secretion, 50 nM PD98059 was added 60 min before and during stimulations. Western blotting using antibody to gp130 as described under “Experimental Procedures.” The sets of PCR primers used were as follows: gp130, 5′-TCT CCT TGA GCA AAC TTT GGG G-3′ for forward and 5′-ACA TGC TTT GGG TGT AAT GG-3′ for reverse (17); VEGF, 5′-TGC CCT GCT GCT CTA CCT CC-3′ (on exon 1) for forward and 5′-TCA CCG CCT GGT CTT GTC AC-3′ (on exon 8) for reverse (18); β-actin, 5′-AGC ACC ATG ATT TTT GAC-3′ for forward and 5′-AGG AGC AAT GAT CCT GAT CAT CA-3′ for reverse (17). The thermal cycle (25, 30 cycles) contained the following profile: denaturation, 95 °C for 1 min; annealing, 55 °C for gp130, 64 °C for VEGF and 60 °C for β-actin for 1 min; and extension, 72 °C for 1 min. The PCR products were electrophoresed on agarose gel and stained with ethidium bromide. For semiquantification, the amounts of mRNAs encoding both gp130 and VEGF were estimated by the relative intensity against the intensity of β-actin control.

Western Blot Analysis—Total cellular protein was extracted with lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4, and protease inhibitor mix (Complete™; Roche Applied Science). The lysates (10 μg each) were separated in a denaturing 7.5–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were then blocked with 6% skim milk in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and subsequently incubated with antibodies against gp130, phosphotyrosine, STAT3, and each phospho-MAPK protein. Antibody against actin was used as an internal control of loading proteins. Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared using NE-PER™ Reagent (Pierce) according to the manufacturer’s instructions. The electrophoretic mobility shift assay was performed using Gel Shift Assay System (Promega) according to the manufacturer’s instructions. Consensus double strand oligonucleotides for C/EBP and AP-1 were end labeled with 3,000 Ci/mmol [γ-32P]ATP (Amersham Biosciences). Nuclear extracts (10 μg) and 0.5 ng of labeled probe were incubated at room temperature for 30 min and were electrophoresed on a 6% polyacrylamide gel in 0.2 × TGE (0.25 × Tris-HCl, pH 8.5, 2 mM glycine, 0.01 M EDTA) at 80 V. The gels were transferred to filter paper, vacuum dried, and autoradiographed at −80 °C for 48 h.

Immunoblot—After cells were cultured under the indicated conditions, supernatants were collected and stocked at −80 °C until use. The amount of VEGF165 was measured using sandwich ELISA kits with antibody specific for human VEGF165 (R&D Systems) according to the manufacturer’s instructions.

Statistical Analysis—Statistical evaluations were performed by Student’s t test using a statistical program, StatView software (Abacus Concepts, Berkeley, CA).
RESULTS

Induction of Gp130 mRNA and Its Protein Expression in the HG Condition—To examine the effects of the HG condition on gp130 expression, HGFs were cultured under the HG or NG condition and stimulated by IL-6 and sIL-6R (50 ng/ml each) for the indicated periods of time. Whole cell lysates were analyzed by Western blotting using antibodies to the phosphotyrosine residue of cellular proteins as described under “Experimental Procedures.” The same membrane was reprobed using antibodies to gp130 and STAT3 to show gp130 and STAT3 proteins. The density of each protein band was analyzed by a densitometry measurement, and relative levels of each protein were expressed as a percentage of control, normalized by the corresponding actin level. As shown in Fig. 1, we demonstrated that accumulation of gp130 mRNA was enhanced significantly under the HG condition (25 cycle: p < 0.0001, versus NG, NG + mannitol; 30 cycle: p = 0.0023, versus NG, p = 0.0020, versus NG + mannitol). Also, there was no significant difference in gp130 mRNA levels between the NG alone and the NG + mannitol group. Consistent with the expression of gp130 mRNA, the HG condition also significantly increased expression of gp130 protein in HGFs, resulting in 1.8-fold higher than that under the NG condition (p < 0.001) (Fig. 2). An equivalent concentration of mannitol had no significant effect on either gp130 mRNA or its protein expression, suggesting that glucose-induced up-regulation of gp130 was independent of osmolality.

Effects of the HG Condition on Activation of IL-6/sIL-6R-induced Signals—As shown in Fig. 3, phosphorylation of gp130 by IL-6/sIL-6R under the HG condition was enhanced significantly after 10 min compared with that under the NG condition (p < 0.001) and returned to the basal level after 60 min, whereas the HG condition without IL-6/sIL-6R had no effect on phosphorylation of gp130. On the other hand, there was no significant difference in the amount of IL-6/sIL-6R-induced STAT3 phosphorylation between the HG and NG conditions (Fig. 3). The signals downstream of gp130 phosphorylation were examined further. The phosphorylation of p44/42 MAPK was enhanced significantly by IL-6/sIL-6R under the HG condition, whereas there was no significant difference in the amount of IL-6/sIL-6R-induced JNK phosphorylation between the HG and NG conditions (Fig. 4). In contrast, IL-6/sIL-6R did not activate the phosphorylation of p38 MAPK at all. To determine whether the HG condition enhances IL-6/sIL-6R-induced DNA binding activity of C/EBP or AP-1, we examined the kinetics of their binding activity in IL-6/sIL-6R-treated cells cultured under the HG condition. As shown in Fig. 5, IL-6/sIL-6R-induced C/EBP expression was higher significantly under the HG condition (25 cycle: p < 0.001, versus NG, NG + mannitol; 30 cycle: p = 0.0023, versus NG, p = 0.0020, versus NG + mannitol).
binding activity increased 3.4-fold over the control levels at 4 h, whereas IL-6/sIL-6R had no significant effect on the AP-1 binding to activity at 4 h despite the stimulation. These bindings were competed efficiently with 200-fold molar excess of the same unlabeled oligonucleotides.

Effects of the HG Condition on IL-6/sIL-6R-induced VEGF mRNA Expression—As shown in Fig. 6, IL-6/sIL-6R induced an obvious increase of VEGF165 mRNA accumulation after 12 h under the HG condition compared with its accumulation in the NG condition, although IL-6/sIL-6R stimulation increased its mRNA accumulation in the NG condition. In addition, accumulation of VEGF121 mRNA also increased similarly. The HG condition without IL-6/sIL-6R stimulation had no effect on the induction of VEGF mRNA accumulation.

Increase of IL-6/sIL-6R-induced VEGF165 Secretion in the HG Condition and Effects of p44/42 MAPK and JNK Inhibitors—To determine the effects of the HG condition on VEGF165 secretion in HGFs, the levels of VEGF165 secreted into the culture medium were measured using specific ELISA methods. As shown in Fig. 7, IL-6/sIL-6R significantly increased VEGF165 secretion in HGFs, even in the NG condition (24, 48 h, p < 0.001 versus untreated cells). Furthermore, IL-6/sIL-6R dramatically increased VEGF165 secretion under the HG condition (24 h, p < 0.01; 48 h, p < 0.001 versus NG). To confirm the effects of the p44/42 MAPK pathway on IL-6/sIL-6R-induced VEGF165 secretion, we examined whether the selective p44/42 MAPK inhibitor PD98059 inhibits VEGF165 secretion. PD98059 partially inhibited VEGF165 secretion in HGFs. Furthermore, to confirm the effects of the JNK pathway on IL-6/sIL-6R-induced VEGF165 secretion, we examined whether the JNK inhibitor SP600125 inhibits VEGF165 secretion. SP600125 also partially inhibited VEGF165 secretion in HGFs. In addition, VEGF165 secretion was inhibited completely by the combination of PD98059 and SP600125 to the base-line level.

No Effect of Glucose Stimulation on VEGF165 Secretion—VEGF165 secretion by HGFs was observed constitutively under different concentration of glucose. As shown in Fig. 8, in the absence of added IL-6/sIL-6R, the amount of secreted VEGF165 remained base line at any concentration of glucose tested at 24

Fig. 6. HG enhances IL-6/sIL-6R-induced VEGF mRNA expression. HGFs were cultured under the HG or NG condition, and then cells were stimulated by IL-6 and sIL-6R (50 ng/ml each) for 0–24 h. Total RNA was prepared as described under “Experimental Procedures” and analyzed by semiquantitative reverse transcription-PCR analysis using specific primer for VEGF. A, the two major products correspond in size to the products expected for the 165-amino acid isoform of VEGF (VEGF165; upper bright band) and the 121-amino acid isoform of VEGF (VEGF121; lower bright band). The relative densities of amplified cDNA encoding VEGF165 (B) and VEGF121 (C) were calculated and expressed as a ratio to that encoding β-actin. M, 100-bp DNA marker.
and 48 h. However, especially at 48 h, the addition of either 16.5 or 25 mM glucose to IL-6/sIL-6R resulted in a significantly greater degree of VEGF165 secretion compared with lower concentrations of added glucose.

**DISCUSSION**

Cellular responses to elevated extracellular glucose are thought to contribute to the development of diabetic complications (19), including severe periodontitis (15), although the precise mechanism remains elusive. In the present study, we demonstrated for the first time that chronic HG exposure significantly increased gp130 mRNA as well as its protein expression in HGFs (Figs. 1 and 2). This overexpression of gp130 is probably not related to the osmotic effects of HG because the same concentration of mannitol had no effect on gp130 expression. It has been widely accepted that gp130 is a common receptor for the IL-6 family such as IL-11, leukemia inhibitory factor (20). Thus, we examined whether activation of gp130 could be enhanced by elevated glucose in HGFs. As shown in Fig. 3, HG significantly enhanced tyrosine phosphorylation of gp130 by IL-6/sIL-6R. The intracellular signaling pathways evoked by gp130 activation include the Janus kinase/STAT pathway and the Ras-MAPKs pathway (20). Funamoto et al. (21) reported that gp130-mediated signals increase VEGF expression via the Janus kinase/STAT3 pathway in cardiac myocytes, whereas we demonstrated that HG showed no increase in IL-6/sIL-6R-induced phosphorylation of STAT3 in HGFs (Fig. 3). This discrepancy might be caused by the difference in cell types used in experiments. Next, we examined the possibility that activation of MAPK cascades is enhanced by IL-6/sIL-6R in HGFs cultured under the HG condition. There are at least three distinct MAPK families: 1) p44/42 MAPK or extracellular-regulated kinases, 2) p38 MAPK or the stress/cytokine-activated kinases, and 3) JNK/stress-activated protein kinases known as the JNKs/SAPKs (22). As shown in Fig. 4, phosphorylation of p44/42 MAPK was enhanced significantly by IL-6/sIL-6R in HGs cultured under the HG condition. Phosphorylation of JNK by IL-6/sIL-6R was almost same level between NG and HG. In contrast, IL-6/sIL-6R had no effect on phosphorylation of p38 MAPK. These findings suggest that HG dramatically enhances the gp130-mediated p44/42 MAPK pathway in IL-6/sIL-6R-treated HGFs.

We have found recently that IL-6/sIL-6R increases VEGF expression in HGFs via the JNK pathway (16). AP-1 is one of the JNK-mediated transcription factors involved in the regulation of VEGF expression (23). Importantly, as shown in Fig. 5, we demonstrated that HG significantly enhanced C/EBP-DNA binding rather than AP-1-DNA binding at 4 h after IL-6/sIL-6R stimulation. In addition, we obtained promoter sequences of VEGF gene from GenBank (accession no. AF095785) and searched possible binding sequences of C/EBP on the promoter using SIGSCAN version 4.05 (24). As expected, several consensus sequences, including a binding site for C/EBP, could be found in the promoter of the VEGF gene. This results...
support that overexpression of gp130 caused by HG results in the specific activation of p44/42 MAPK and its downstream transcription factor C/EBP, leading to VEGF expression in HGFs. The p44/42 MAPK is activated by phosphorylation on threonine and tyrosine residues by dual specificity kinase MEK1, which induces their translocation into the nucleus where they activate or suppress a variety of transcription factors involved in growth and differentiation (25). To evaluate whether the gp130-mediated p44/42 MAPK-C/EBP pathway we identified is involved in the regulation of VEGF expression, we examined the effect of HG on IL-6/sIL-6R-induced VEGF mRNA expression and the amount of VEGF secretion in HGFs cultured with or without PD98059. As expected, HG enhanced IL-6/sIL-6R-induced VEGF165 mRNA expression in HGFs (Fig. 6). Furthermore, we found that HG also significantly enhanced IL-6/sIL-6R-induced VEGF165 secretion and demonstrated that PD98059 partially inhibits the enhancement of VEGF165 secretion (Fig. 7). Also, we demonstrated that PD98059 completely inhibits the C/EBP-DNA binding (Fig. 5). These results suggest that induction of VEGF165 is dependent on the activation of the gp130-mediated p44/42 MAPK-C/EBP signaling pathway in HGFs cultured under the HG condition. Several transcription factors of the C/EBP family have been identified so far in different tissues, and the C/EBP family regulates a variety of cellular phenotypes in a wide range of cell types (26). There are at least six isoforms of this family, α, β, γ, δ, ε, and ζ, which can both homodimerize and heterodimerize with each other and bind to the same C/EBP regulatory element in the promoters of many different genes (27). To clarify the isoforms of C/EBP activated by IL-6/sIL-6R under the HG condition, further elucidation is needed using supershift analysis.

In addition, we found that the specific JNK inhibitor SP600125 also partially inhibits the enhancement of VEGF165 secretion (Fig. 7). Furthermore, we demonstrated that VEGF165 secretion is completely inhibited in HGFs cultured with a combination of PD98059 and SP600125. PD98059 especially is known as a highly selective inhibitor of p44/42 MAPK activation and does not inhibit activation of other protein kinases (28). However, this regulation would be subject to further experiments using dominant negative form of each MAPK because these pharmacological selective inhibitors may not fully reflect the outcome in an intracellular pathway. Interestingly, it was reported that the JNKAP-1 pathway might be responsible for the greater induction and activation of C/EBP in lipopolysaccharide-treated macrophages (29). Therefore, we speculate that VEGF expression by HGFs may be also mediated, directly or indirectly, at least in part, via the JNK/AP-1 signaling pathway, although IL-6/sIL-6R had no significant effect on the AP-1 binding to DNA under the HG condition.

VEGF165 secretion remained at base-line level at any concentration of glucose at the 24-h time point, although it was slightly up-regulated at 16.5 mM glucose. However, it was up-regulated by IL-6/sIL-6R stimulation at 25 mM glucose at this time point. The IL-6 signaling pathway was also strongly activated under this condition. This tendency turned remarkable at the 48-h time point (Fig. 8). Although it is possible that the events observed in HGFs may occur in humans, direct experimental confirmation will be required. Furthermore, it has been reported that HG alone induces VEGF expression in vascular muscle cells (30) and glomerular podocytes (31). In our study, we found that HG alone had no significant effect on the VEGF165 secretion in HGFs (Fig. 8). The distinctive feature of HGFs in VEGF expression might explain the difference of clinical observation in various tissues and organs in diabetic patients.

VEGF is known as a vascular permeability factor based on its ability to induce vascular leakage (32). In fact, VEGF-induced vascular permeability may be critical for its angiogenic action, as it provides a provision fibrin matrix essential for endothelial cell spouting and migration. Furthermore, in addition to its angiogenic activity and vascular permeable property, VEGF has an important role in indirect monocyte chemotraction through the up-regulation of chemokines including MCP-1 and IL-8 (33). Thus, VEGF could be a key regulator of inflammatory responses, leading to severe periodontitis. Clinically in diabetic patients, we often observe severe gingiva interrupted in the circulation such as ischemia rather than angiogenic. It is difficult to explain this discrepancy based on the roles of VEGF in hyperglycemic gingiva. However, HGFs stimulated by IL-6/sIL-6R might play an important role in this discrepancy of pathogenesis. It might be assumed that HGFs overexpressing gp130 under the HG condition enhance the VEGF secretion by IL-6/sIL-6R and lead to angiogenic and then severe destruction in inflamed gingiva. Therefore, IL-6 might be an important mediator in the pathogenesis of severe periodontitis in diabetic patients.

Funatsu et al. (34) reported that aqueous levels of VEGF and IL-6 were significantly correlated with the severity of diabetic retinopathy, although the role of IL-6 in diabetic retinopathy is unclear. Recent studies suggested that oral infections such as periodontitis increase the risk of various systemic diseases after inflammatory cytokines including VEGF entered the bloodstream. Thus, VEGF produced by HGFs may affect the progression of nearby diabetic retinopathy. Appropriate periodontal therapy might exert beneficial effects on VEGF-mediated diabetic complications such as retinopathy. In summary, we demonstrated that HG indirectly increases VEGF expression via a gp130-mediated signaling pathway in HGFs (Fig. 9). This pathway could be an attractive target for clarifying the pathophysiology of severe periodontitis in diabetic patients.

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