High glucose enhances the gene expression of interleukin-8 in human endothelial cells, but not in smooth muscle cells: possible role of interleukin-8 in diabetic macroangiopathy

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Summary We examined the effect of high glucose concentrations on the production of interleukin(IL)-8, which seems to be important for the development of atherosclerosis, in cultured human aortic endothelial cells (AoEC) and smooth muscle cells (AoSMC). After incubating these cells with various concentrations of glucose for 2 days or 7 days, the IL-8 concentration in cell lysates was measured by enzyme-linked immunosorbent assay and the IL-8 mRNA expression was examined by Northern analysis. After 2 days' culture, 42.5 mmol/l glucose enhanced IL-8 mRNA expression in AoEC, but not in AoSMC, compared to 4 mmol/l glucose. After 7 days' culture, the IL-8 concentration in AoEC lysate and the expression of IL-8 mRNA were significantly increased

by 20.5 mmol/l glucose, or 42.5 mmol/l glucose compared to 4 mmol/l glucose. On the other hand, the IL-8 concentration in AoSMC lysate was not affected by any glucose concentration and the expression of IL-8 mRNA in AoSMC was diminished by high glucose. These results suggest that the chemotactic gradient by IL-8 is established between arterial intima and media in response to high glucose levels in diabetic patients, and that it may be one of the key factors for SMC migration to the intima leading to diabetic macroangiopathy. [Diabetologia (1997) 40: 610–613]

Keywords Diabetic angiopathy, IL-8, high glucose, endothelial cells, smooth muscle cells.

Several large prospective epidemiological investigations have disclosed that coronary artery disease is the main cause of morbidity and death in patients with diabetes mellitus. The Multiple Risk Factor Intervention Trial [1] revealed that the mortality rate for coronary artery disease and cardiovascular disease such as cerebral infarction and arteriosclerosis obliterans was three times higher in diabetic men than in non-diabetic men.

However, the exact cellular and molecular mechanism for increased risk of these atherosclerotic

vascular diseases in diabetic patients remains unclear. Atherosclerosis is described as a response of the artery wall to initiating agents followed by the formation of the atherosclerotic plaque by multiple pathogenic mechanisms [2]. One critical pathological event in the atherosclerotic lesion is the migration of medial smooth muscle cells (SMC) to the intima where these cells proliferate. Various molecules regulating SMC growth are secreted from platelets, monocyte/macrophages, lymphocytes, endothelial cells, and SMC themselves. These cells, in concert with a damaged and/or "activated" endothelium, synthesize and release many kinds of growth factors and cytokines, which lead to the migration and proliferation of SMC, the recruitment of additional blood cells, and the accumulation of lipids. Among proinflammatory cytokines, tumour necrosis factor(TNF)- α , interleukin(IL)-1 β , IL-6, and IL-8 are reported to be involved in the pathogenesis of atherosclerosis [3].

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Abbreviations: AoEC, Aortic endothelial cells; AoSMC, Aortic smooth muscle cells; TNF, tumour necrosis factor; IL, interleukin; PBS, phosphate buffered saline; BCA, bicinchoninic acid; PKC, protein kinase C.

On the other hand, diabetic metabolic changes including hyperglycaemia, generation of free radicals, glycosylation and oxidation of lipoproteins, compositional changes of lipoproteins, and platelet aggregation may contribute to the diabetic angiopathies. However, there has been no report regarding the influence of high glucose levels on the production of proinflammatory cytokines in vascular cells except our previous report [4], in which we reported that high glucose stimulated the production of IL-8 in human umbilical venous endothelial cells (HUVEC) and aortic endothelial cells.

In this paper, we examined the effects of high glucose levels on both IL-8 protein synthesis and the steady-state level of IL-8 mRNA expression in human endothelial cells and smooth muscle cells.

Materials and methods

Cell culture. Human aortic endothelial cells (AoEC; Sanko Junyaku, Tokyo, Japan) were cultured in 10-cm culture dish coated with type-1 collagen (Iwaki, Chiba, Japan) in MCDB 107 (Kyokutou, Tokyo, Japan) supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 20 mmol/l HEPES, 60 mg/ml heparin, 20 mg/ml bovine pituitary extracts (Kyokutou), 1.25 mg/ml fungizone, 100 U/ml penicillin, and 100 mg/ml streptomycin. Human aortic smooth muscle cells (AoSMC, Sanko Junyaku) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with the same components as those used in MCDB 107 except that heparin and bovine pituitary extracts were not included. The medium was replaced twice weekly.

Cytokine measurements in the cell lysate. For the measurement of cytokines in the cell lysate, AoEC and AoSMC were plated onto a 24-well tissue culture plate coated with type-I collagen. The media were then changed to the various glucose concentrations in the semiconfluent state for 2 days' culture, and at the half-confluent state for 7 days' culture. The cell lysate samples were prepared using lysis buffer containing 0.1% Triton-X and 1% Nonidet P-40 in phosphate-buffered saline (PBS). Before adding the lysis buffer, the media was discarded, and cells were washed twice with PBS. The concentrations of TNF- α , IL-1 β , IL-6 and IL-8 in the cell lysate were measured by enzyme linked immunosorbent assay (ELISA) using a commercial kit. The total protein concentration in cell lysate was also measured using bicinchoninic acid (BCA protein assay, Pierce, Rockford, Ill., USA). In our preliminary experiments, we confirmed that lysis buffer per se does not interfere with the ELISA or the BCA protein assay.

Northern blot analysis. AoEC and AoSMC in 6-cm culture dishes coated with type-I collagen were cultured with the same medium as described above. RNA extraction was done using guanidine thiocyanate solution. Twenty micrograms of total RNA was electrophoresed at 45 V in a 1% agarose gel containing 6.6% formaldehyde. RNA was blotted onto a nylon filter membrane (Amersham, Buckinghamshire, UK) and analysed by the recommended method of the manufacturer. The plasmids containing the full-length cDNA of human IL-8 [5] were kindly donated by K. Matsushima (University of Kanazawa, Japan), and IL-8 cDNA was labelled with digoxigenin

Fig. 1. IL-8 concentrations in cell lysates of cultured human aortic endothelial cells (AoEC, left) and smooth muscle cells (AoSMC, right) cultured in the media with various glucose concentrations for 7 days. Cell lysates were prepared using lysis buffer and assayed for IL-8 by enzyme-linked immunosorbent assay. The results were corrected by the total protein concentration. The data are shown as the ratio over the control (4 mmol/l). The data are representative of three different experiments in triplicate

dUTP (Boehringer Mannheim, Mannheim, Germany) by the random priming method. Expression of GAPDH mRNA was quantified by using a human GAPDH cDNA probe and used to correct for variations in RNA loading. The intensity of the band was analysed by NIH National Institute of Health Image.

Statistical analysis. Data are presented as the mean $\pm$ SD. Statistical analysis was performed by unpaired *t*-test.

Results

We first examined the effects of high glucose levels on IL-8 production in the cell lysate of AoEC and AoSMC. After 2 days' culture, high glucose did not affect the IL-8 concentration in either AoEC lysate or AoSMC lysate (data not shown). After 7 days' culture, the amount of IL-8 in AoEC lysate cultured at both 20.5 and 42.5 mmol/l glucose is significantly greater than that cultured at 4 mmol/l glucose (Fig. 1, left). On the other hand, the level of the IL-8 concentration in AoSMC lysate was not affected by any glucose concentrations (Fig. 1, right).

We next examined the effects of high glucose levels on the gene expression of IL-8 in AoEC and AoSMC. The steady-state level of IL-8 mRNA expression in AoEC was enhanced by high glucose. After 2 days' culture, there was a significant increase in the IL-8 mRNA expression in AoEC cultured at 42.5 mmol/l glucose compared to that cultured at 4 mmol/l glucose (Fig. 2a, left). After 7 days' culture, IL-8 mRNA expression was significantly increased in AoEC cultured at 20.5 and 42.5 mmol/l glucose compared to that at 4 mmol/l (Fig. 2b, left). On the other hand, the IL-8 mRNA expression in AoSMC was diminished by

Fig 2. (a, b) Northern blot analysis of IL-8 mRNA expression in cultured human aortic endothelial cells (AoEC) and smooth muscle cells (AoSMC) cultured in media with various glucose concentrations. Total RNA was extracted by guanidine thiocyanate solution, and 20 μ g of total RNA per lane except that 10 μ g RNA from AoEC after 2 days' culture was electrophoresed and transferred to a nylon membrane. The blot was sequentially hybridized with cDNA probes for IL-8 and GAPDH. Then, the bands were analysed by NIH image, and the intensity of the IL-8 mRNA band was corrected by that of the GAPDH mRNA band. The data are representative of three different experiments. **(a)** AoEC (left) and AoSMC (right) were cultured for 2 days at various glucose concentrations. **(b)** AoEC (left) and AoSMC (right) were cultured for 7 days at various glucose concentrations

high glucose. After 2 days' culture, there was a significant decrease in IL-8 mRNA expression in AoSMC cultured at 42.5 mmol/l glucose compared to that cultured at 4 mmol/l glucose (Fig. 2a, right). After 7 days' culture, the IL-8 mRNA expression was significantly decreased in AoSMC cultured at 20.5 and 42.5 mmol/l glucose, compared to that at 4 mmol/l glucose (Fig. 2b, right).

The concentrations of TNF- α , IL-1 β , and IL-6 in the cell lysates of AoEC and AoSMC were not affected by any glucose concentrations (data not shown).

Discussion

IL-8 was originally found as a chemokine for neutrophils, and has been shown to have a chemotactic activity even for SMC [6]. Furthermore, Huber et al. [7] have reported that IL-8 is secreted preferentially into the vessel wall by the endothelial cells and is deposited in the subendothelial matrix, giving rise to a transmural chemotactic gradient. These reports suggest that the transmural chemotactic gradient by IL-8 enhances the migration of SMC to the subendothelial space. We also reported in our previous study that IL-8 had the chemotactic activity for AoSMC which was assessed by the migration assay using a Millipore filter membrane [4].

So far, there has been no evidence to prove that IL-8 exists in the atherosclerotic lesions in vivo. However, Wang et al. [8] have recently found IL-8 to be expressed in a macrophage-rich area in specimens of human coronary atheromas by immunohistochemistry and in situ hybridization analysis. This finding also indicates an atherogenic role of IL-8 in vivo.

In the pathogenesis of diabetic macroangiopathy, cytokines, growth factors and other undefined factors may be involved. We report here that the high glucose level only enhanced IL-8 production in AoEC at both protein and mRNA levels, but not in AoSMC. The increased production of IL-8 by high glucose levels in AoEC was specific because no change in other cytokines including TNF- α , IL-1 β and IL-6 was found.

The mechanism by which glucose specifically stimulates IL-8 production in human endothelial cells is unclear. The osmolar effect may be considered, since it has been reported that osmotic stress increases IL-8 mRNA levels in human peripheral blood monocytes, which were exposed to 40 mmol/l of NaCl [9]. However, it is not able to explain our results, because the osmolarity of 40 mmol/l NaCl is extremely high compared to that of 42.5 mmol/l glucose, and they also reported that 20 mmol/l NaCl, of which osmolarity is nearly equal to that of 42.5 mmol/l glucose, did not enhance the level of IL-8 mRNA [9]. Besides, we already reported that 4 mmol/l glucose plus 38.5 mmol/l mannitol did not affect the release of IL-8 from human umbilical vein endothelial cells (HUVEC) [4]. Therefore, high glucose itself, but not high osmolarity, regulates IL-8 production in endothelial cells.

Several investigators have shown that hyperglycaemia and the diabetic state induce a persistent activation of protein kinase C (PKC) in vascular tissues and in cultured vascular cells [10] and suggest that the activation of PKC may alter vascular functions in macro- and micro-vessels. As it has been reported that IL-8 gene expression is regulated by the PKC-dependent pathway [5], the persistent PKC activation by high glucose level may contribute to the increase

in the steady-state level of IL-8 mRNA in AoEC. The induction of IL-8 mRNA by high glucose level was not observed in AoSMC. Therefore, there seem to be cell specific regulations in the IL-8 mRNA expression in response to high glucose. Further study on the mechanism of IL-8 mRNA regulation in response to high glucose is underway at present.

In conclusion, our results suggest that in hyperglycaemic states, the chemotactic gradient between arterial intima and media is enhanced by the increased IL-8 production in endothelial cells, not in smooth muscle cells, compared to that in normoglycaemic states, and that it may be one of the key factors for SMC migration to the intima leading to diabetic macroangiopathy.

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