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Long-term exposure to high glucose up-regulates VCAM-induced endothelial cell adhesiveness to PBMC

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Long-term exposure to high glucose up-regulates VCAM-induced endothelial cell adhesiveness to PBMC.

Background. The changes induced on endothelial cells by a long-term exposure to high glucose, a situation that mimics the hyperglycemia of diabetics, have not yet been determined. We compared short- and long-term effects of elevated glucose on macrovascular and microvascular endothelial cells.

Methods. Endothelial cells were grown in high-glucose media for 24 hours and for 8 weeks. Cell proliferation was evaluated by cell counting, apoptosis and expression of adhesion molecules by flow cytometry; nitric oxide (NO) by measuring the concentration of nitrite/nitrate in the cell supernatant; α2(IV) collagen mRNA and protein by reverse transcriptase-polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. The adhesion of peripheral blood mononuclear cells (PBMCs) to endothelial cells was evaluated by adhesion assay. In some experiments, endothelial cells were preincubated with anti-vascular cell adhesion molecule-1 (VCAM-1) and anti-receptor for advanced glycation end product (RAGE) blocking antibodies.

Results. At 24 hours, but not at 8 weeks, high glucose increased endothelial cell proliferation and apoptosis. High glucose did not modify NO synthesis at 24 hours and 8 weeks. Collagen production and expression were increased only after eight weeks. VCAM-1 but not intercellular adhesion molecule-1 was up-regulated after 8 weeks, a change not observed after 24 hours. The adhesion of PBMCs was significantly increased at eight weeks and was completely abrogated by anti–VCAM-1 and by anti-RAGE antibodies. After 24 hours, there was a modest increase of PBMC adhesion that was not blunted by anti-RAGE antibodies.

Conclusions. Increased adhesion of PBMCs, caused by up-regulation of VCAM-1 with a mechanism involving advanced glycation end product (AGE) adducts, and augmented collagen deposition are critical effects of long-term high glucose on endothelial cells, and may eventually promote the atherosclerotic process.

Key words: diabetes, atherosclerosis, adhesion molecules, cell proliferation, vascular disease, blood-glucose control, hyperglycemia.

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Microvascular and macrovascular diseases are very common in diabetics [1, 2] and are the leading cause of death in these patients [3]. Although the pathogenesis of diabetic vasculopathy is still not clear, hyperglycemia is recognized as essential for the development of vascular disease since a better blood glucose control reduces the progression and long-term complications of diabetes mellitus [4, 5]. High glucose has been shown to exert multiple effects at cell level either directly [6, 7] or through the formation of intermediate products such as advanced glycation end product (AGE) adducts [8], sorbitol [9], or the induction of growth factors and hormones [10, 11]. Endothelial cells, which form the luminal vascular surface, are among the cell types that may be affected in diabetes particularly because they are directly exposed to high glucose. High-glucose–induced endothelial dysfunction is characterized by impaired endothelium-dependent relaxation caused by changes in nitric oxide (NO) production [12], increased endothelial permeability [13], changes in the expression of adhesion molecules [14], induction of procoagulant activity [8], and increased production of extracellular matrix proteins [15]. These and other several phenotypic changes have been observed mainly in in vitro experiments usually performed after short-term incubation of endothelial cells with glucose. However, because vascular complications are common in patients with a poor glycemic control over a long period of time, we thought it compelling to evaluate the long-term effects of high glucose and to differentiate them from the acute effects. The majority of the long-term effects of high glucose on endothelial cells investigated in the present study were different from those induced after short-term incubation; furthermore, when the effects of long- and short-term incubation were similar, they were mediated by distinct mechanisms.

METHODS

Reagents

Antibodies to factor VIII and cytokeratin were from Dako (Milan, Italy). Antibodies to human type IV collagen were kindly donated by Dr. Liliane Striker (Univer-

sity of Miami, FL, USA). Antibodies to vascular cell adhesion molecule-1 (VCAM-1) were from R&D Systems (Minneapolis, MN, USA). TRITC-labeled phalloidin was from Sigma (Milan, Italy). Antibodies to receptor for advanced glycation end products (RAGE) were kindly donated by Professor David Stern (Columbia University, NY, USA). All tissue culture media and reagents were supplied by Sigma-Aldrich (Milan, Italy). All molecular biology reagents were from Boehringer-Mannheim-Roche (Munich, Germany). AGE-albumin was prepared by incubating bovine serum albumin (BSA; fatty acid free; Sigma) with glucose (0.5 mol/L) at 37°C for six weeks as previously described [8].

Cell isolation and culture

Bovine aortic and human microvascular endothelial cells were obtained from aortas and skin, respectively, after collagenase (1%) digestion as previously described [16, 17]. They were characterized as endothelial cells based on their positivity for anti-factor VIII and antithrombomodulin antibodies and by their negativity for anticytokeratin antibodies using indirect immunofluorescence. The cells were differentiated from smooth muscle cells and fibroblasts according to their morphology and to the arrangement of F-actin filaments by phalloidin staining. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics. For the experiments, cells were cultured with medium containing 5 or 30 mmol/L glucose for 24 hours or for 8 weeks. In some experiments, medium containing 25 mmol/L mannitol was added to some wells. Both bovine aortic and human microvascular endothelial cells were used for all the experiments, and no differences were observed in the responses of the two cell types.

Cell proliferation

Cells were seeded in 24-well plates at a concentration of 50×10^3 cells/well and incubated with medium containing 30 mmol/L glucose. Control cells were incubated with 5 mmol/L glucose. At variable intervals of time, cells were washed with phosphate-buffered saline (PBS), trypsinized [trypsin-ethylenediaminetetraacetic acid (EDTA)], and counted with a NeuBauer chamber.

Cell morphology

After 24-hour and 8-week incubations with 30 mmol/L glucose media, cells were washed and fixed with 2% paraformaldehyde/PBS, pH 7.2. Cell morphology was evaluated by phase-contrast microscopy.

Apoptosis

Apoptosis was determined by a sandwich-enzyme-linked immunosorbent assay (ELISA) method, which employs mouse monoclonal antibodies directed against DNA and histones, respectively, as previously described [17].

Nitric oxide production

Nitric oxide production was measured as nitrite/nitrate using a two-step chromogenic assay. Briefly, the supernatant from endothelial cells treated with glucose (5 and 30 mmol/L for 24 hours and 8 weeks) was collected and the cells were counted. Cell supernatant (100 μL) was added to a 96-well plate with nitrate reductase (50 μL) in assay buffer. After two hours of incubation at room temperature, Griess reagents (100 μL) were added to each well to convert nitrite into a deep blue azo compound [18]. The concentration of nitrite was calculated from a standard curve made with known amount of nitrite.

ELISA for collagen type IV

Endothelial cells were cultured with medium containing glucose at the concentration of 5 and 30 mmol/L for 24 hours and 8 weeks. To perform the assay with cells in the same growing conditions, they were seeded into six-well plates keeping them in low- and high-glucose media. At confluence, cells were washed and incubated with fresh medium containing 0.1% FCS. After 24 hours, incubation medium was collected, and cells were counted with a NeuBauer chamber. ELISA was carried out as previously described [17].

Expression of adhesion molecules

The expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), E-selectin, and VCAM-1 was evaluated by flow cytometry. Briefly, after 24-hour and 8-week incubations with glucose, endothelial cells were treated with 10 μg/200 μL of mouse anti–VCAM-1, anti–ICAM-1, and anti–E-selectin and nonimmune serum for 30 minutes. Cells were washed with PBS (pH 7.2) and incubated with a FITC-conjugated goat anti-mouse IgG (1:10,000) for a further 30 minutes. After three washes, cells were analyzed by flow cytometry using a Becton Dickinson FACScan.

Immunoblotting of VCAM-1

Cell membranes were solubilized using lysis buffer (Tris 20 mmol/L, pH 7.4, NaCl 0.1 mol/L, IgePal CA-630, 1%; Sigma). Forty micrograms of solubilized cell membrane protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. VCAM-1 (20 ng) was used as a positive control. Rabbit anti-human VCAM-1 antibody was used as the primary antibody. The nitrocellulose membrane was then incubated with biotin-conjugated anti-Ig antibody, followed by alkaline phosphatase-conjugated streptavidine and substrate. The reaction was stopped by extensive washing with distilled water.

Peripheral blood mononuclear cell adhesion assay

Endothelial cells cultured with 30 mmol/L glucose media for 24 hours or 8 weeks were grown at confluence

in 12.5 cm^2 wells, washed with culture medium, and exposed to freshly isolated peripheral blood mononuclear cells (PBMCs; 1.5×10^6 cells/well) for 2 hours at 37°C. Nonadherent PBMCs were removed by extensive washing with cold saline solution, and adherent cells were counted on four consecutive microscopic fields. Where indicated, ECs were pretreated with one of the following: cycloheximide (1 mmol/L for 24 hours), AGE-albumin (100 μg/mL, for 24 h), anti–VCAM-1 antibodies (10 μ/mL for 30 min), anti-RAGE $F(ab')_2$ (2 μg/mL for 3 hours), and nonimmune $F(ab')_2$ (2 μg/mL for 3 hours). PBMCs were isolated from fresh collected human venous blood from healthy volunteers on EDTA, diluted with an equal volume of saline solution, stratified on a Ficoll-Hypaque gradient, and centrifuged at 2×10^3 r.p.m. for 20 minutes. The mononuclear cell fraction with a purity of 99% was obtained, washed twice in Hanks balanced salt solution (HBSS), and resuspended in RPMI 1640 [19].

Statistical analysis

Each experiment was performed twice in duplicate or triplicate wells. Data are expressed as mean ± SD. The two-tailed unpaired Student *t* test or one-way analysis of variance (ANOVA) were used to measure differences between short- and long-term high-glucose–treated and control cells. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of high glucose on cell proliferation and morphology

Figure 1 shows the endothelial cell growth curve after 24-hour and 8-week exposures to high-glucose–containing media. Short-term exposure to high glucose induced a significant increase in cell number after 24 hours. This effect, however, was not detectable at 48 hours. Long-term exposure to high glucose did not modify the growth curve that was comparable to that of cells grown in low-glucose–containing media. High glucose induced a change in cell morphology after eight weeks (Fig. 2A) compared with low-glucose–treated cells (Fig. 2B). Treated cells were elongated with loss of their cobblestone appearance and of cell–cell contacts.

Effect of high glucose on endothelial cell apoptosis

Apoptosis is a finely regulated process that plays an important role in regulating cell number [20]. The different effect of high glucose on endothelial cell proliferation at 24 hours and 8 weeks led us to examine the induction of apoptosis on endothelial cells by glucose. At 24-hours, high glucose induced a significant increase of apoptotic cells (3.82 ± 0.26 vs. 2.68 ± 0.19 arbitrary units, $P < 0.05$), as determined by ELISA. However, there was no

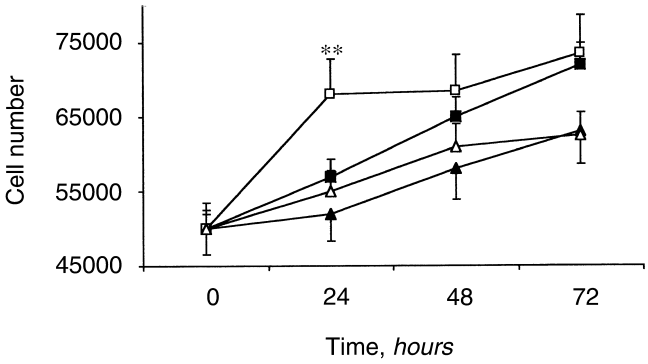

Fig. 1. Effect of short- and long-term high glucose on endothelial cell proliferation. Endothelial cells exposed to high glucose for 24 hours and 8 weeks were plated in a 24-well plate (5×10^4 cell/well) and allowed to attach. At the indicated times cells were counted. Low-glucose cultured cells were used as controls. Symbols are: (□) high-glucose and low-glucose (■) 24-hour exposure; (△) high-glucose and low-glucose (▲) eight-week exposure. The results are expressed as means ± SD. $^{**}P < 0.01$ vs. short-term high glucose.

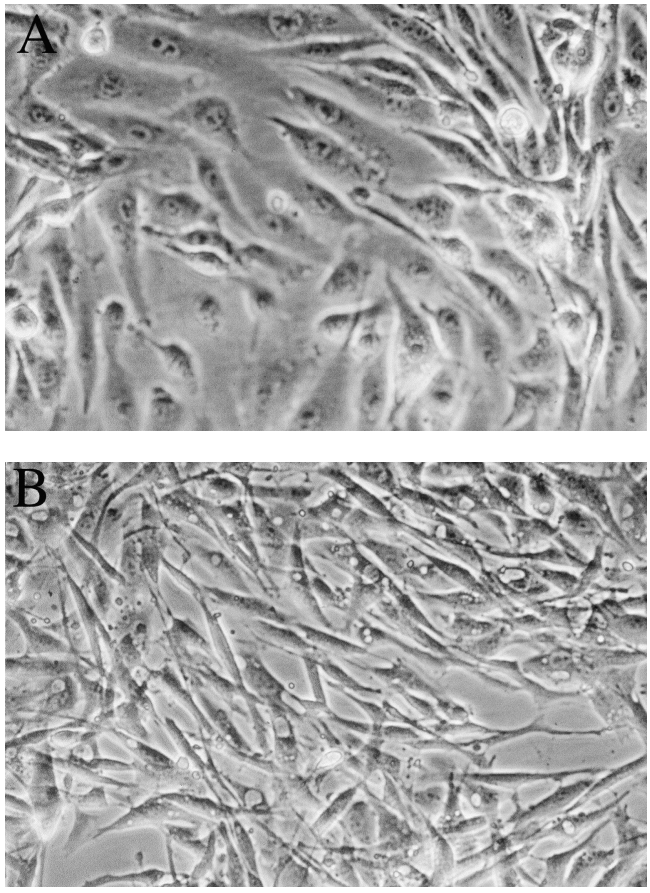

Fig. 2. Effect of high-glucose, long-term exposure on endothelial cell morphology. Endothelial cells were cultured with high-glucose–containing media for eight weeks. They were fixed with paraformaldehyde (2%), and cell morphology was evaluated by phase-contrast microscopy (×40). (*A*) Low-glucose–treated cells. (*B*) High-glucose–treated cells.

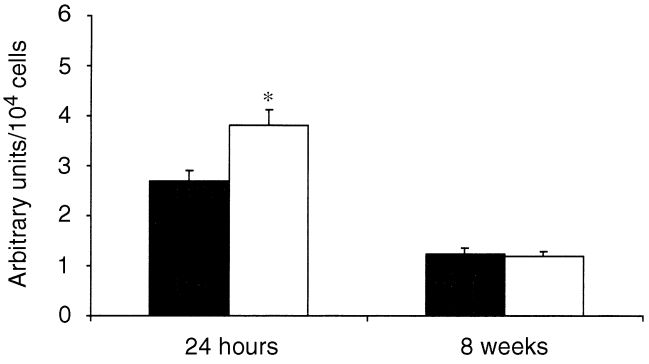

Fig. 3. Apoptosis in endothelial cell cultures treated with high glucose. Endothelial cells were cultured with high-glucose–containing media for 24 hours and for 8 weeks. Twenty microliters of cell lysate supernatant was assayed for oligonucleosomal DNA using a cell death-detection ELISA kit. Symbols are: (□) low-glucose– and (■) high-glucose–treated cells. The results are expressed as means ± SD. $*P < 0.05$ vs. short-term low glucose.

difference in the number of apoptotic cells after eight weeks of incubation with high and low glucose, with both control and high-glucose–treated cells showing lower apoptotic values than those at 24 hours (1.25 ± 0.08 vs. 1.18 ± 0.08 arbitrary units, high and low glucose, respectively; Fig. 3).

Nitric oxide production by endothelial cells

Oxidant stress is one of the mechanisms leading to the vascular dysfunction of diabetic patients, and several lines of evidence suggest that this may be due at least in part to altered NO [21, 22]. An increased NO production has been linked to the early diabetic hyperfiltration [23]. Furthermore, NO may be involved in the pathogenesis of diabetic vasculopathy indirectly through its toxic products such as peroxynitrite [24]. We questioned whether the exposure time could affect NO production by endothelial cells. The concentration of nitrate/nitrite in the supernatant of endothelial cell cultured in high glucose media was not different from that of endothelial cells cultured with low glucose either at 24 hours (3.85 ± 0.4 vs. 4.2 ± 0.7 μmol/L, low and high glucose, respectively) or at 8 weeks (4.3 ± 0.6 vs. 4.5 ± 0.58 μmol/L, low and high glucose, respectively).

Production of collagen IV

Collagen deposition is one of the hallmarks of the atherosclerotic process. Endothelial cells have been shown to be able to modulate the extracellular matrix turnover [25]. While at 24 hours of incubation there was no significant accumulation of collagen IV in the supernatant of endothelial cells treated with high glucose compared with low glucose cultured cells, after 8 weeks of incubation, high glucose induced a significant increase of collagen concentration in the cell supernatant (Fig. 4A). To determine whether the accumulation of collagen in the supernatant was caused by an increased synthesis and not by a decreased degradation, collagen type IV mRNA levels were measured by reverse transcription-polymerase chain reaction (RT-PCR; Fig. 4B). Densitometric analysis showed a twofold increase in the collagen IV/β-actin mRNA ratio in endothelial cells treated for eight weeks with high glucose (Fig. 4C). Since the 24-hour incubation may be not a sufficient time to demonstrate a stimulated change in collagen protein, the collagen type IV mRNA level was also measured. There was no difference in the collagen IV/β-actin ratio in endothelial cells treated for 24 hours with high glucose compared with controls.

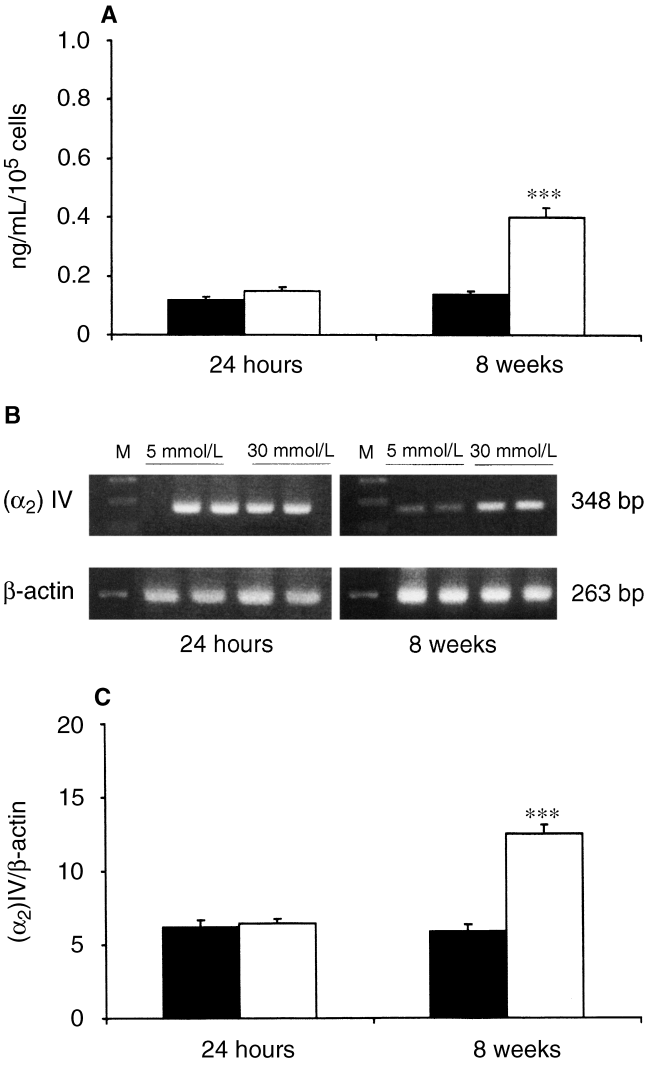

Fig. 4. Effect of short- and long-term high glucose exposure on (α2)IV collagen mRNA and protein in endothelial cell cultures. (*A*) Endothelial cells were cultured for 24 hours and for 8 weeks with high-glucose media. Media were then changed with media containing 0.1% fetal calf serum. After 24 hours, media were collected. Cells were counted, and ELISA for collagen type IV was performed. (*B*) (α2)IV and β-actin mRNA levels were analyzed by reverse transcription-polymerase chain reaction. (*C*) Densitometric analysis. Symbols are: (■) low-glucose– and (□) high-glucose–treated cells. Results are expressed as means ± SD. $***P < 0.001$ vs. long-term low glucose.

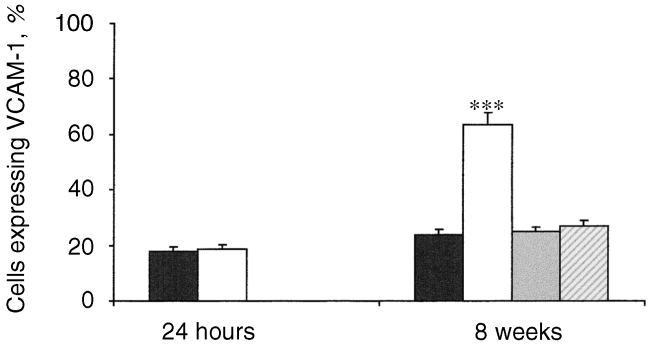

Fig. 5. Effect of short- and long-term high glucose exposure on the expression of VCAM-1 on endothelial cells. Endothelial cells were cultured for 24 hours and 8 weeks with high glucose. VCAM-1 expression on cell surface was then evaluated by flow cytometry using anti–VCAM-1 antibody. In some experiments, cells were incubated with staurosporin and mannitol. Symbols are: (■) low-glucose– and (□) high-glucose–treated cells; (▩) cells pretreated with staurosporine; (▨) cells treated with mannitol. Results are expressed as means ± SD. ***$P < 0.001$ vs. long-term low glucose vs. staurosporine vs. mannitol.

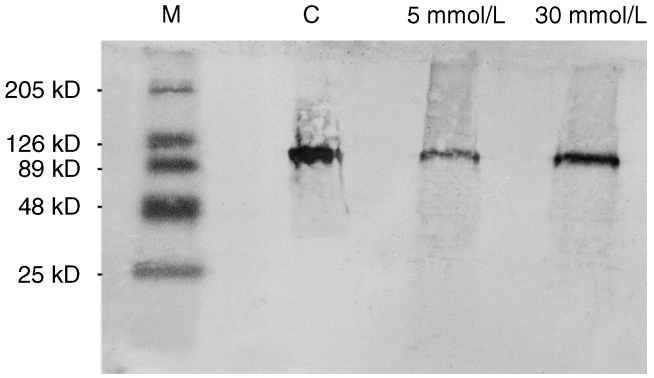

Fig. 6. Immunoblotting of solubilized cell membrane from long-term high-glucose– and low-glucose–treated endothelial cells. Cells were treated for eight weeks with low and high glucose. Cell membranes were solubilized. Protein samples were separated by gel electrophoresis, and immunoblot was performed as described in the **Methods** section. Abbreviations are: M, markers; C, VCAM-1; 5 mmol/L, cell membrane protein sample from low-glucose–treated endothelial cells; 30 mmol/L, cell membrane protein sample from high-glucose–treated endothelial cells.

Expression of adhesion molecule by endothelial cells

Adhesion molecules modulate the adherence of monocytes to the endothelium and their migration into the vessel wall where they start the atherosclerotic process. Since atherosclerosis is accelerated in diabetic patients, we questioned whether exposure to high glucose could change the pattern of adhesion molecules expression and whether this change could be dependent on the length of the exposure. We investigated the expression of VCAM-1, ICAM-1, and E-selectin by flow cytometry. Endothelial cells grown in high glucose for eight weeks but not for 24 hours demonstrated an increased expression of VCAM (63.6 ± 28.0% vs. 18.8 ± 5.2%, $P < 0.001$). No differences were found in the expression of ICAM-1 and E-selectin either at 24 hours or 8 weeks of incubation with high-glucose–containing media. A 24-hour incubation with the protein kinase C (PKC) inhibitor staurosporin (5 nmol/L) reduced the up-regulation of VCAM-1 expression by long-term exposure to high glucose. The incubation of endothelial cells with mannitol had no effect on VCAM-1 expression (Fig. 5). These results were confirmed by immunoblotting analysis (Fig. 6).

Peripheral blood mononuclear cell adhesion to endothelial monolayer

An early and critical phase of the atherosclerotic process is the adhesion of monocyte to the endothelium. This phase is preceded by the up-regulation of adhesion molecules such as VCAM-1. We asked whether the up-regulation of VCAM-1 by long-term exposure to high glucose in endothelial cell cultures was followed by an increased adherence of PBMCs. To test whether the adhesion was specifically led by VCAM-1, we preincubated endothelial cells with anti–VCAM-1 blocking antibodies. The adhesion of PBMCs to endothelial cell monolayer was significantly increased by long-term high-glucose exposure (Fig. 7). The adhesion was completely abrogated by anti-VCAM-1 antibodies (Fig. 7), whereas nonimmune Ig had no effect (data not shown). Pretreatment with cycloheximide completely prevented the adhesion of PBMCs to high-glucose–treated cells (Fig. 7). Even though the adhesion of PBMCs to the endothelial monolayer treated with high glucose for 24 hours was also increased, it did not reach the same extent as the long-term incubation and was not reduced by anti–VCAM-1 antibodies, suggesting that the adhesion was driven by a different mechanism.

Advanced glycation end products that result from a nonenzymatic reaction between glucose and amino groups are thought to mediate the effects of hyperglycemia, especially those responsible for the diabetic vasculopathy. We hypothesized that AGEs formed on endothelial cells that are exposed long-term to glucose could mediate the adhesion of leukocytes to the monolayer. To confirm this hypothesis, endothelial cells exposed to high glucose for 24 hours and for 8 weeks were preincubated with anti-RAGE antibodies, whereas endothelial cells grown in low glucose were incubated with AGE-albumin or were incubated with AGE-albumin after a two-hour incubation with anti-RAGE antibodies. Anti-RAGE antibodies completely prevented leukocyte adhesion to endothelial cells grown for eight weeks in high-glucose–containing media, but they did not reduce the adhesion at 24 hours. AGE-BSA-induced adhesion of leukocyte mimicked the effect of high glucose and was reduced by anti-RAGE antibodies (Fig. 7).

DISCUSSION

This study confirms the multiple effects of high glucose on endothelial cells but differentiates, to our knowledge for the first time, the effects caused by a short-term exposure from those observed after a long-term treatment.

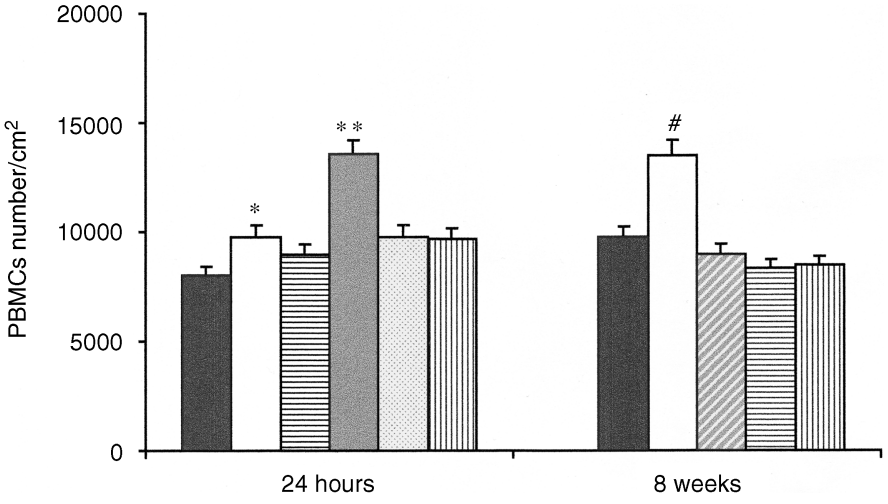

Fig. 7. PBMC adhesion to the endothelial cell monolayer. Endothelial cells were cultured for 24 hours and 8 weeks with high glucose. PBMCs were added to culture media. After incubation for two hours at 37°C, nonadherent cells were removed, and adherent cells were counted. Symbols are: (■) low-glucose– and (□) high-glucose–treated cells; (▤) high-glucose + anti–VCAM-1 antibody-treated cells; (▩) low-glucose + AGE-albumin–treated cells; (▦) low-glucose + AGE-albumin + anti-RAGE antibody-treated cells; (▥) high-glucose + anti-RAGE antibody-treated cells; (▨) high glucose + cycloheximide-treated cells. Results are expressed as means ± SD. $*P < 0.05$ short-term high glucose vs. short-term low glucose; $**P < 0.01$ short-term low glucose + AGE-albumin vs. short-term low glucose + AGE-albumin + anti-RAGE antibody; $\#P < 0.01$ long-term high glucose vs. long-term low glucose vs. long-term high glucose + anti-VCAM-1 antibody vs. long-term high glucose + anti-RAGE antibody.

Persistent hyperglycemia and postprandial glucose spikes are common in diabetic patients. We reasoned that the effects of high glucose on endothelial cells could depend on the exposure time. The acute effects could be directly induced by glucose itself, while the long-term changes could be mediated by products that accumulate in high-glucose conditions. These considerations led us to perform our study on endothelial cells harvested from bovine aortas and human skin cultured in low and high glucose for 24 hours and 8 weeks.

An increased proliferation has been shown in cells incubated with high glucose [26] and in the early phase of diabetic nephropathy in experimental animals [27]. Our results are in agreement with these studies, suggesting that high glucose indeed increases the proliferation of endothelial cells; however, endothelial cell proliferation seems to be an early time-limited effect since it is not observed after 48 hours of incubation. Furthermore, there was no difference in the cell-growth curve when endothelial cell monolayers were grown for eight weeks in high-glucose compared with low-glucose medium. The acceleration in cell growth at 24 hours was paralleled by a slightly increase of the apoptotic cells, which was not observed at eight weeks. Although proliferation and apoptosis are contrasting processes, leading, respectively, to an increase and a decrease in cell number, the presence of both is not surprising since the same stimulus may activate both processes or alternatively the increased proliferation could function as a stimulus for apoptosis, which constitutes the most predominant form of cell growth control [28].

Several lines of evidence suggest a role for oxidative stress in the pathogenesis of diabetic vascular complications [29, 30], that is, diabetic nephropathy [22, 31, 32], with the changes in NO production or stability leading to diabetic hyperfiltration. Furthermore, the inhibition of NO synthesis could be one of the central mechanisms leading to the extracellular matrix deposition in the long-term exposure to high glucose [33]. However, an increased synthesis of NO is not always sought as beneficial since NO may be converted to damaging reactive nitrogen species such as peroxynitrite, which has been proved very harmful for endothelial cells [24, 34]. No changes in nitrate concentration were found in the supernatant of endothelial cells exposed to high glucose either for 24 hours or for 8 weeks. Although these results suggest that NO is not involved in the changes induced in endothelial cells by high glucose, we cannot exclude this possibility since NO may be converted to more reactive products that were not measured.

Diabetes mellitus is one of the major causes of endothelial dysfunction leading to atherosclerosis. Leukocyte adhesion to vascular endothelium is an essential step of the atherosclerotic process. Adherence of leukocytes is accomplished by the induction of adhesion molecules such as ICAM-1 and VCAM-1 on the surface of endothelial cells. In this contest, VCAM-1 is of particular interest because its expression has been linked to the early phase of atherosclerosis in animal models [35, 36]. In our study, high glucose induced a significant increase in PBMCs adherence, an effect already present after 24 hours but more pronounced after an 8-week exposure of endothelial cells to high glucose, as already shown by Kim et al [37]. Endothelial cells exposed to high glucose for eight weeks exhibited an increased expression of VCAM-1, and the adhesion of leukocytes was completely abrogated by anti–VCAM-1 antibodies and by staurosporine, suggesting that the up-regulation of VCAM-1 via a PKC

pathway was essential for the adherence of leukocytes to endothelial cell monolayer.

Although high glucose induced an increased PBMC adherence to endothelial monolayer either after 24 hours or after 8 weeks of incubation, our study shows that different mechanisms are turned on in the acute and chronic exposure. After an 8-week exposure, the adherence of PBMCs was completely prevented by anti-RAGE antibodies, suggesting that AGE binding to its receptor plays a central role in VCAM-1–mediated adherence. At 24 hours, neither anti-RAGE nor anti–VCAM-1 antibodies prevented the adherence of PBMCs to endothelial cells treated with high glucose. These results demonstrate that AGEs are important mediators of high-glucose–induced endothelial dysfunction after long-term exposure, whereas the same changes in acute exposure occur with the intervention of different mediators. Our study confirms previous findings by Schmidt et al showing the central role of AGEs in the up-regulation of VCAM-1 in cultured human endothelial cells and increased adhesivity of the monolayer for Molt-4 cells [38]. In their study, binding of Molt-4 cells was inhibited by preincubation of endothelial cells with cycloheximide and anti-RAGE antibodies, demonstrating the importance of the de novo endothelial protein synthesis and the role of the AGEs–RAGE interaction. Data from Morigi et al also indicate that high glucose promotes leukocyte adhesion to endothelial cell through a VCAM-1 up-regulation [14]. Although these data were obtained after a short-term exposure (24 h) to high glucose, their work does not contradict our results. Their findings were obtained with endothelial cells harvested from umbilical veins, and the leukocyte adherence assay was performed under flow conditions. This may explain in part the difference found after 24 hours of exposure. However, they also found that AGE-albumin added to the endothelial cell monolayer markedly increased leukocyte adherence, which our findings confirmed. AGE products begin to form only after four weeks of incubation with glucose, which means that the effects of short- and long-term exposure to high glucose is mediated through different pathways. That high glucose is able to induce a direct effect on cultured cells after short-term exposure has been demonstrated in several cell types, including vascular smooth muscle, endothelial, and mesangial cells [39–41]. These effects are mediated either through the activation of PKC, as demonstrated by Williams et al, or other intermediate molecules [42]. The long-term effects may be mediated through the interaction AGE–RAGE as shown by our study, particularly because AGEs accumulate during hyperglycemia and high-glucose exposure [8].

Diabetes also favors extracellular matrix deposition in vessel wall accelerating the atherosclerotic process. There are several reports on the effects of high glucose on extracellular matrix synthesis [43], and it has been shown that hyperglycemia stimulates the production of transforming growth factor-β [44] and of connective tissue growth factor [45], two molecules known to play a central role during fibrogenic processes. Our study confirms that endothelial cells may be important in the vessel wall remodeling and that they react to environmental stimuli modulating their functions. After long-term exposure to high glucose, endothelial cells demonstrated an increased expression of collagen type IV, which was paralleled by an increase of collagen type IV in the cell supernatant. The increase of collagen type IV production was not observed after a 24-hour exposure.

Endothelial cells exposed to high glucose for eight weeks exhibited a different morphology when compared with control cells. Instead of the common cobblestone appearance, they were elongated. Although we have only demonstrated the effect of high glucose on the synthesis of collagen type IV, the different appearance shown by endothelial cells exposed to high glucose may be due to the changes in the composition of subendothelial matrix as shown for other cell types [46–48].

In conclusion, changes induced by high glucose exposure in cultured endothelial cells is time dependent. Different mediators and pathways are responsible for the derangement of endothelial functions observed after short- and long-term exposure to high glucose.

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