High Glucose–Induced Alterations in Subendothelial Matrix Perlecan Leads to Increased Monocyte Binding

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Objective—Hyperglycemia is an independent risk factor for cardiovascular disease in diabetic patients, although the link between the two is unknown. These studies were designed to model effects of high glucose on an early event in atherogenesis: the binding of monocytes to subendothelial matrix (SEM).

Methods and Results—SEM was prepared from human bovine aortic endothelial cells (HAECs) and bovine aortic endothelial cells (BAECs) cultured in the presence of low (5 mmol/L) or high (30 mmol/L) glucose for 3 to 5 days. Monocyte binding was significantly higher (P<0.05) to the SEM of both HAEC and BAEC exposed to high glucose. This increase was a result of changes in SEM heparan sulfate proteoglycans (HSPGs). Metabolic radiolabeling of BAEC demonstrated a 24% decrease in [35S]sulfate incorporation into SEM HSPG produced by cells incubated in 30 mmol/L versus 5 mmol/L glucose, whereas no glucose-associated differences were measured in [35S]methionine incorporation into proteoglycans (PGs) or non-PG proteins. Autoradiography revealed 2 high-molecular weight SEM HSPGs. One was a hybrid PG that contained both heparan sulfate and chondroitin sulfate/dermatan sulfate chains. Both PGs were identified by Western blotting as perlecan.

Conclusions—These results illustrate that hyperglycemia-induced structural changes in perlecan may result in a SEM that is more favorable to retention of monocytes. (Arterioscler Thromb Vasc Biol. 2004;24:858-863.)

Key Words: perlecan ■ endothelial cell ■ monocyte binding ■ diabetes ■ hyperglycemia
2 mmol/L 1-glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and MEM vitamins.

Subendothelial Matrix
Confluent EC monolayers were washed with balanced salt solution (BSS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.45 mmol/L KH₂PO₄, 20.3 mmol/L Na₂HPO₄) and incubated with 20 mmol/L NH₄OH + 0.1% TritonX-100 for 5 minutes at room temperature, followed by 3 washes with BSS and 3 with DMEM, 3% BSA, to remove detached cells. Microscopic examination revealed a clearly detectable matrix but no remaining cells. Cell monolayers of replicate cultures were digested with 0.5 mol/L NaOH and used to measure cell protein, or removed by trypsin to determine cell number.

Metabolic Radiolabeling
Cells were radiolabeled for 48 to 72 hours with media containing indicated glucose concentrations and 50 μCi/mL [³⁵S]sulfate or 25 μCi/mL [³⁵S]methionine/cysteine (NEN, Boston, Mass).

THP-1 Cells
Cells were incubated with 10 μCi/mL [³H]thymidine (NEN) for 24 hours. Radiolabeled cells were isolated by centrifugation at 300g for 5 minutes, washed 4 times, and resuspended in DMEM, 3% BSA. Specific activity was calculated as dpm/cell based on scintillation counting and cell number determination in an aliquot of radiolabeled THP-1 cells.

PG Isolation and CPC Precipitation
After removal of cells, the SEM was extracted by 4 mol/L GdnHCl with protease inhibitors overnight at 4°C and dialyzed with 0.03 mol/L Na₂SO₄ and 0.02 mol/L NaCl. PG were precipitated in 1% 1-hexadecylpyridinium chloride (CPC) for 24 hours at 25°C, separated by centrifugation, and isolated by ethanol precipitation.

GAG Identification by Enzymatic Digestion
Aliquots of PGs were dialyzed against ABC buffer (0.1 mol/L Tris, 0.03 mol/L sodium acetate, 0.01 mol/L EDTA, pH 8.0) and incubated for 16 hours at 37°C with 0.05 U of chondroitinase ABC (Seikagaku Inc. Ijamsville, Md) in buffer containing 1 mg/mL BSA and protease inhibitors. HSPGs were ethanol-precipitated and chondroitin sulfate/dermatan sulfate (CS/DSPG) calculated by subtracting HSPGs from total PGs. HS-GAGs were degraded by heparanase I and III (Sigma) in ABC buffer without EDTA for 16 hours at 37°C.

Monocyte Binding
THP-1 cells (3x10⁷ per well for all conditions) were added to either EC monolayers or SEM in 24-well plates for 1 hour at 37°C. Wells were washed 4 times with DMEM, 3% BSA, to remove unbound monocytes and solubilized with 0.1 N NaOH and 0.1% SDS overnight at room temperature. Samples were neutralized with 0.1 N HCl and analyzed by scintillation counting. Bound radioactivity was converted to number of cells bound. To identify involvement of specific GAGs in monocyte binding, EC monolayers or SEM were incubated with 1 unit/mL heparanase or chondroitinase enzymes for 1 hour at 37°C and washed with BSS before monocyte binding.

SDS-PAGE
Purified PGs were either left intact, digested with heparanase, chondroitinase ABC, or both enzymes as described and ethanol precipitated. Samples were reduced and resolved by 4% to 12% gradient SDS-PAGE for autoradiography or transfer to nitrocellulose.

Western Blotting
Nitrocellulose membranes were blocked overnight with PBS, 0.1% Tween, 5% nonfat dry milk, washed and incubated in PBS, 0.1% Tween, 2.5% nonfat dry milk, and an antiperlecan core protein antibody (final concentration 1 μg/mL) (Upstate Biotechnology, Lake Placid, NY). Membranes were washed and incubated with horseradish peroxidase-conjugated antirat IgG diluted 1:10,000, treated with chemiluminescence reagents, and exposed to film for visualization of bands. Detection of core proteins was consistently improved by enzymatic removal of GAG chains.

Immunoprecipitation
[³S] sodium sulfate-labeled SEM PGs (315±17 and 312±12 μg SEM protein for 5 mmol/L and 30 mmol/L glucose samples, respectively) were treated with chondroitinase ABC as described, adjusted to 2.5 mg/mL BSA, and incubated overnight at 4°C with 10 μg/mL antiperlecan antibody and 50 μL of (50% slurry) protein-A beads (Amersham Pharmacia Biotech, NJ). Beads were pelleted by centrifugation at 10,000g, washed extensively (150 μmol/L NaCl, 25 mmol/L Tris-HCl,1% Triton, 10 μg/mL peptatin, 10 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L PMSE), and boiled 2 minutes in SDS-PAGE sample buffer to release bound antigen. Bound and unbound radioactivity was measured by scintillation counting.

Immunofluorescence
BAECs were grown to confluence, washed twice with PBS, and fixed in 3.7% formaldehyde in PBS for 10 minutes at 25°C. After further washing and blocking (1% BSA in PBS, 10 minutes at 25°C), antiperlecan (10 μg/mL in PBS, 1% BSA), or PBS, 1% BSA alone (as control) were added for 30 minutes at 25°C. Cells were washed with PBS and incubated in rhodamine-conjugated goat antirat (Jackson ImmunoResearch, West Grove, Pa) (1:200 dilution) for 20 minutes at 25°C, then postfixed with 3.7% formaldehyde in PBS at 25°C for 10 minutes and mounted in glycerol.

Statistical Analysis
Experiments were performed in triplicate and repeated with consistent results. Data were analyzed by a Student t test and ANOVA and differences were considered statistically significant at P<0.05.

Results
High Glucose Increases Monocyte Binding to SEM
The effect of high glucose on monocyte binding to SEM was examined by incubating actively growing HAECs and BAECs in media containing normal (5 mmol/L) or high (30 mmol/L) glucose for 5 days, by which time they were confluent. Cell number was similar between low- and high-glucose–treated cultures (data not shown). The SEM was exposed by removal of the cells and monocyte binding to SEM was measured. As shown in Figure 1A, monocyte binding was significantly higher to BAEC SEM produced in the presence of 30 mmol/L versus 5 mmol/L glucose. Similarly, SEM of high-glucose–treated HAEC (Figure 1B) exhibited a 3-fold increase in monocyte binding.

Increased Monocyte Binding to SEM Results From Changes in HSPG
Previous studies have shown that HSPG may impede attachment of monocytes to the SEM and that enzymatic removal of HS-GAG results in increased binding of monocytes to SEM. We investigated high-glucose-mediated effects on this process. Monocyte binding to the cell layer was unchanged in high-glucose–treated HAEC (Figure 2). Moreover, digestion with GAG-degrading enzymes did not affect monocyte binding to the cell layer, indicating that PGs are not involved in monocyte binding to the apical surface of endothelial cells. Degradation of HS-GAG (heparanase treatment) in the SEM of low-glucose–treated cells increased monocyte binding >2-fold and to a level similar to binding to intact SEM of high-glucose–treated cells. Monocyte binding
to SEM produced by high-glucose–treated cells was not increased beyond this level by HS-GAG degradation. Removal of CS/DS-GAG (chondroitinase ABC treatment) did not change monocyte binding to SEM. These results confirm an inhibitory role for HS-GAG in monocyte binding to SEM and suggest that in high-glucose conditions, alterations in HS-GAG result in increased monocyte retention in SEM.

**High Glucose Decreases [35S] Sulfate Incorporation into SEM HSPG**

Our previous studies indicated that HSPG secreted into the media by HAEC and BAEC is structurally modified in high-glucose conditions. We questioned whether similar effects were produced in SEM HSPG. BAECs were incubated in 5 mmol/L or 30 mmol/L glucose for 3 days in the presence of [35S]sodium sulfate. The SEM was exposed as described in Figure 1 and extracted by 4 mol/L Gdn HCl with protease inhibitors. Samples were treated with chondroitinase ABC enzyme to isolate HSPG. Values are means ± SEM. *P<0.05

**High Glucose Does Not Alter SEM HSPG Core Protein or Other Matrix Proteins**

To determine whether the glucose-induced decrease in [35S]sulfate incorporation into SEM HSPG resulted from a specific effect on the GAG component of HSPG and not an effect on SEM proteins, BAECs were incubated in media containing 5 mmol/L or 30 mmol/L glucose for 3 days in the presence of [35S]methionine/cysteine. SEM was prepared and extracted as described and SEM PGs were isolated by CPC precipitation. Neither total cell protein nor total SEM protein was decreased by high-glucose conditions and no glucose-associated changes in radiolabel incorporation were measured in either PG or non-PG proteins isolated from the SEM (Figure 4). Autoradiography revealed no changes in total protein banding or intensity with high

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Increased monocyte binding to SEM of high-glucose–treated cells. A, BAEC SEM. B, HAEC SEM. Cells were cultured in media containing 5 mmol/L or 30 mmol/L glucose for 5 days. SEM was exposed by incubation of cell layer with 20 mmol/L NH₄OH plus 0.1% triton X-100 for 5 minutes at room temperature. THP-1 monocytes (labeled with 10 µCi/mL [3H]thymidine 24 hours before binding) were added (3 × 10⁵ per well) for 1 hour at 37°C. Dishes were washed to remove unbound monocytes and binding was analyzed by scintillation counting.* P<0.05

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** High glucose increases monocyte binding to SEM but not to the cell surface and results from changes in SEM HSPG. HAECs were cultured in media containing 5 mmol/L or 30 mmol/L glucose for 5 days. Either the cell layer was left intact or SEM was exposed as described in Figure 1. In some samples, GAG-degrading enzymes, heparinase (Hep) and chondroitinase ABC (ABC) were incubated with the cell layer or SEM before monocyte binding. Binding of THP-1 monocytes was measured as described in Figure 1. *P<0.05

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** SEM HSPG, but not CS/DSPG, is reduced in high-glucose conditions. BAECs were incubated for 3 days in media containing 5 mmol/L or 30 mmol/L glucose and 50 µCi/mL [35S]sodium sulfate. The SEM was exposed as described in Figure 1 and extracted by 4 mol/L Gdn HCl with protease inhibitors. Samples were treated with chondroitinase ABC enzyme to isolate HSPG. Values are means ± SEM. *P<0.05

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** High glucose does not alter SEM HSPG core protein or other matrix proteins. To determine whether the glucose-induced decrease in [35S]sulfate incorporation into SEM HSPG resulted from a specific effect on the GAG component of HSPG and not an effect on SEM proteins, BAECs were incubated in media containing 5 mmol/L or 30 mmol/L glucose for 3 days in the presence of 25 µCi/mL [35S]methionine/cysteine. SEM was prepared and extracted as described and SEM PGs were isolated by CPC precipitation. Neither total cell protein nor total SEM protein was decreased by high-glucose conditions and no glucose-associated changes in radiolabel incorporation were measured in either PG or non-PG proteins isolated from the SEM (Figure 4). Autoradiography revealed no changes in total protein banding or intensity with high
Based on [35S]methionine/cysteine incorporation, PG core proteins comprised 51.6% and 56.9% of SEM proteins in low- and high-glucose cultures, respectively. Therefore, high glucose did not affect levels of either PG cores or other SEM proteins. Furthermore, the decrease in [35S]sulfate incorporation into SEM HSPG was not a result of a decrease in production of the intact proteoglycans, but rather was an effect specifically on the HS-GAG.

Identification of Perlecan as the SEM HSPG of BAEC
Perlecan is the primary HSPG secreted into culture media by BAECs and HAECs, but the identities of SEM PG are unknown. Autoradiography of intact [35S]sulfate-labeled SEM PGs (Figure 5A) revealed a major band above 250 kDa (lane 1). Chondroitinase ABC treatment caused the appearance of a lower-molecular-weight band (lane 2, arrow), indicating a hybrid PG that contained CS/DS chains sensitive to chondroitinase ABC, while maintaining HS chains that allowed detection at the lower molecular weight. The remaining higher-molecular-weight band (lane 2) indicated a PG not sensitive to chondroitinase ABC. Heparinase treatment alone resulted in a lighter band above 250 kDa (lane 3), presumably resulting from degradation of HS on a PG that maintained detectable CS/DS-GAG. The failure of heparinase to cause a detectable shift in the migration of this core suggests that it may contain more units of CS than HS-GAG. A combination of both enzymes resulted in disappearance of all bands (lane 4). Western analysis of GAG-free core proteins showed recognition of both bands by a core protein-specific antiperlecan antibody (Figure 5B). Therefore, the SEM of BAEC contains 2 forms of perlecan with core proteins of different sizes: 1 substituted with only HS chains, the other with both HS and CS/DS. The antiperlecan antibody was used to immunoprecipitate SEM perlecan and demonstrates that the decreased [35S] sulfate incorporation in SEM produced under high-glucose conditions was indeed the result of alterations in perlecan (Figure 5C). This antibody was also used (Figure 5D) to demonstrate, by immunofluorescence, the distribution of perlecan throughout the SEM.

Discussion
These studies have shown that exposure of HAECS and BAECs to high glucose results in the production of a modified SEM with increased capacity for monocyte binding. Moreover, it was shown that this increased binding was mediated by alterations in SEM HSPG, specifically involving a decrease in HS-GAG components. Monocyte binding to the SEM

![Figure 4](image-url) SEM protein is not decreased with high glucose treatment. BAECs were incubated for 3 days in media containing 5 mmol/L or 30 mmol/L glucose and 5 μCi/mL [35S]methionine/cysteine. The SEM was exposed as described in Figure 1 and extracted by 4 mol/L Gdn HCl with protease inhibitors. PG were isolated by precipitation with 1% CPC, followed by ethanol precipitation, and analyzed by scintillation counting. Values are means±SEM.

![Figure 5](image-url) Identification of SEM PG. A, SDS-PAGE of BAEC SEM PG. BAECs were radiolabeled with 50 μCi/mL [35S]sodium sulfate for 72 hours. The SEM was exposed as described in Figure 1, extracted by incubation in 4 mol/L GdnHCl with protease inhibitors, dialyzed, and subjected to GAG-degrading enzymes before loading onto a 4% to 12% gradient gel. B, Western Blot of SEM perlecan. After SDS-PAGE, PG described in (A, lane 4) were transferred to nitrocellulose and probed with antiperlecan antibody (1:1000). C, Immunoprecipitation of perlecan. [35S] sodium sulfate-labeled SEM PG, described in Figure 3, were treated with chondroitinase ABC and immunoprecipitated with antiperlecan antibody. D, Immunofluorescence of SEM perlecan. BAECs were grown to confluence and stained with antiperlecan primary antibody and rhodamine-conjugated secondary antibody.
apical surface of the endothelial cells did not involve HSPG and was not changed by high-glucose conditions.

Adhesion of monocytes to the endothelium involves “rolling” along the endothelial cell layer before binding and migrating through the endothelium into the SEM. Retention of monocytes in the SEM is poorly understood, and, in fact, monocytes can cross the endothelium and later egress from the basal to apical side of the endothelial cell layer. However, in atherosclerosis, monocytes are retained in the SEM and differentiate into macrophages; therefore, the retention of monocytes in the SEM, and not their migration across the endothelium, is thought to be pivotal in atherosclerosis development. Studies suggest that monocytes are retained in the SEM through binding to matrix proteins such as fibronectin and heparinase that in turn decreased SEM HS. Our studies show that the elevated monocyte binding to SEM produced in high-glucose conditions is also the result of a reduction in HS. It is unclear whether the high-glucose–associated lowering of HS is caused by increased degradation or decreased HS synthesis.

In our studies, high glucose did not alter levels of PG core proteins or other SEM proteins. Others have shown that collagen IV and fibronectin levels were increased in glomerular kidney of experimentally diabetic rats and in mesangial cells cultured in glucose for several passages. However, shorter exposure to glucose did not change collagen IV, fibronectin, or laminin levels in HAEC or fibroblasts. In our studies, 3 days of high-glucose treatment was sufficient to detect changes in HS-GAG in the absence of detectable changes in SEM proteins. These data and the observation that HS-GAG degradation in SEM of low-glucose cells increased monocyte binding to a level similar to that of intact SEM of high-glucose cells strongly implicate changes specific for HS in the SEM of high-glucose cultures.

Our studies did not show a glucose-associated difference in monocyte binding to the apical surface of endothelial cells. This is consistent with previous evidence that monocyte binding to endothelium is dependent on cell surface adhesion molecules rather than PG. Endothelium, activated by TNF-α, exhibited increased monocyte adhesion via endothelial–leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 expressed on endothelial cells. Studies addressing effects of diabetes-associated factors on monocyte–endothelial cell interactions are limited. Alloxan-induction of diabetes in rabbits was shown to increase leukocyte accumulation on the endothelial surface. In diabetic rats, increased monocyte binding may have resulted from elevated monocyte chemotactic protein-1. Furthermore, high glucose was shown to induce expression of intercellular adhesion molecule-1 in human umbilical vein endothelial cells. Increased concentrations of circulating adhesion molecules have been found in diabetes mellitus and atherosclerosis. A previous study showed increased monocyte binding to HAECS cultured for 7 to 10 days in high glucose. In that study, no detectable changes were observed in endothelial cell adhesion molecules and factors responsible for the increased binding were not identified.

Changes in arterial PG are seen in both atherosclerosis and diabetes. In both conditions, HS-GAG was decreased, with the greatest decrease in diabetic patients with atherosclerosis. The reasons for this and the significance of the decreased HS are poorly understood. The association between hyperglycemia, HS, and impaired organ function has been well studied in kidney, whereas HSPGs are important regulators of glomerular filtration. Heparinase degradation of kidney HS resulted in impaired glomerular filtration of ferritin and albumin. In addition, a decrease in the glomerular basement membrane content of HSPG is associated with diabetic nephropathy. Further studies revealed that the decrease occurs specifically in the HS-GAG component. Diabetic kidneys exhibited decreased immunofluorescence with HS-GAG–specific but not HS-core protein–specific antibodies. In addition, cultured mesangial and kidney visceral epithelial cells have shown a decrease in HSPG when cultured in high (30 mmol/L) versus control (5 mmol/L glucose) media. Again, data indicated effects specifically on HS-GAG.

Perlecan is a major HSPG in basement membranes of many tissues including glomerulus. Perlecan is conventionally described as a 470-kDa core protein with 3 to 5 HS-GAG chains. Our studies have shown 2 forms of perlecan in BAEC SEM, 1 with HS chains only and 1 with both HS and CS/DS chains. Previous studies using the Engelbreth-Holm-Swarm (EHS) tumor have described perlecan with different sizes of core proteins and both HS and CS/DS GAG chains. GAG-attachment sites on perlecan are grouped at the N-terminus in domain I of the perlecan core protein, and although HS are the primary GAG attached at these sites, they can be substituted with CS or DS GAG chains. Recently, domains IV and V of this 5-domain core protein were shown to be capable of bearing GAG chains and may provide greater potential for diversity in GAG attachment.

This heterogeneity of perlecan may lead to different disease risk or outcome. Changes in GAG content or composition may affect the structure and organization of other matrix components. Moreover, integrin-linked signaling events may be perturbed, leading to altered cell protein expression. A recently identified perlecan polymorphism in the GAG-attachment region of domain I is associated with diabetic nephropathy. The same polymorphism in a nondiabetic population was associated with a reduced atherogenic lipid risk profile but no decrease in cardiovascular disease. Perhaps the favorable effect on lipids was countered by adverse perlecan-mediated interactions in the arterial wall. Our studies suggest that in diabetes, hyperglycemia alone may modify perlecan to produce an SEM more conducive to monocyte retention. This provides a mechanism for the increased atherosclerosis development associated with diabetes mellitus.

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