Communication

Altered Cellular Interactions between Endothelial Cells and Nonenzymatically Glucosylated Laminin/Type IV Collagen*

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Laminin and type IV collagen are two major basement membrane glycoproteins. In previous studies it has been shown that nonenzymatic glucosylation induces structural alterations of these macromolecules and also reduces their ability to self-associate. In the present study, endothelial cells were tested for their ability to adhere and spread on nonenzymatically glucosylated laminin and type IV collagen. Adhesion and spreading were reduced when glucosylated macromolecules were used as substrates. Glucosylation-induced changes in adhesion and spreading may be an important initial event signaling other phenotypic modifications of cells in the microvasculature and may be a crucial factor in order to understand the pathogenesis of diabetic microangiopathy at the molecular level.

It is by now well established that nonenzymatic glucosylation is one of the main mechanisms by which hyperglycemia affects structurally and functionally various macromolecules (1-10). The major targets in this process are macromolecules with relatively long half-lives; among them, basement membrane components are of major interest because their alterations may be a key factor contributing to diabetic microangiopathy.

We have examined in the past the effect of nonenzymatic glucosylation on two major basement membrane glycoproteins, laminin and type IV collagen (11, 12). We have observed that their structural alterations due to high glucose concentration can lead to defective interactions that may have a profound influence on the molecular architecture of the basement membrane (11, 12). In the vasculature, however, basement membrane components interact not only with each other but also with the endothelial cells that line every vessel, and these interactions are crucial for various endothelial cell properties and ultimately for the structural and functional integrity of the vascular wall.

Recently, using bovine aortic endothelial cells as a model system, we have started to explore the effect of nonenzymatic glucosylation of basement membrane components on various endothelial cell properties. In the present report we have focused on two of the earliest types of interactions between endothelial cells and laminin/type IV collagen: cell adhesion and cell spreading.

EXPERIMENTAL PROCEDURES

Laminin and type IV collagen were isolated from the Engelbreth-Holm-Swarm tumor matrix according to previously described protocols (11, 13, 14).

Nonenzymatic Glucosylation of Laminin and Type IV Collagen—Laminin was dialyzed against phosphate-buffered saline (PBS) pH 7.4, containing 10 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml N-ethylmaleimide (Sigma), and 0.02% NaN3. Type IV collagen was dialyzed against the same buffer which also contained 0.5 M NaCl. Both proteins were centrifuged at 20,000 rpm for 30 min to remove large aggregates. The nonenzymatic glucosylation of both proteins started by the addition of glucose in the same buffers, so that the final concentration of laminin and type IV collagen was 250 μg/ml and those of glucose were 0 (control), 50, and 500 mM. The nonenzymatic glucosylation was performed at 29 °C for 60 min in the dark with occasional shaking. At the end of the incubation samples were dialyzed against PBS at 4 °C. The protein concentration was measured, and after appropriate adjustments, the samples were immediately used for coating plates. Nonenzymically glucosylated laminin and type IV collagen were subjected to SDS-polyacrylamide gel electrophoresis in 6% gels under reducing conditions (in the presence of 2 mM β-mercaptoethanol) using well established techniques. Similar gels were run using proteins labeled with 125I and then incubated in the absence or presence of glucose. The gels were then dried and subjected to autoradiography.

Glucose Incorporation in Nonenzymatically Glucosylated Type IV Collagen and Laminin—In order to determine the number of glucose molecules incorporated under our experimental conditions in type IV collagen and laminin, we used [6-3H]glucose (from ICN Radiochemicals, CA) to glucosylate these proteins. The commercially available solution of [6-3H]glucose was first dried under a stream of nitrogen. The dried radiolabeled glucose was then diluted in PBS containing 10 mg/ml bovine serum albumin (BSA from ICN ImmunoBiologicals, Costa Mesa, CA), 10 mM EDTA, pH 7.4 and incubated for 3 days at 37 °C. In order to separate the radiolabeled glucose from unknown contaminants that usually coexist in the commercial batches of radioactive glucose (15), the above mixture was loaded on a 25-ml Sephadex G-25 column previously equilibrated with PBS, 10 mM EDTA, pH 7.4, and eluted in a flow rate of 1 ml/min with the same buffer; 0.5-ml fractions were collected and counted in a scintillation counter, and the fractions containing free radioactive glucose were pooled and lyophilized. Unlabeled, lyophilized glucose was then added to a final concentration of 50 or 500 mM, and the pooled peak was combined with type IV collagen and/or laminin as above described. The nonenzymatic glucosylation was stopped with dialysis at 4 °C against 0.1 M phosphate buffer, pH 7.0, and the glycolated proteins were incubated with 200 molar excess of NaBH4 (sodium borohydride diluted in 0.2 M NaOH, from Sigma) for 10 min at room temperature and, then, for another 50 min at 4 °C. All the samples were dialyzed against 0.1 M phosphate buffer, pH 7.0, then against 1 M urea in 0.1 M phosphate buffer, pH 7.0, to remove nonspecifically bound radioactivity and, finally, against PBS containing 10 mM EDTA, pH 7.4. The protein content of each sample was measured, and aliquots from

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1 The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium.

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Each sample was precipitated with acetone and counted in the scintillation counter (Beckman LS 5000/TD). In order to assess the extent of cross-link formation, aliquots were examined in a Perkin Elmer LS3B fluorescence spectrometer at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

**Cell Cultures**—Bovine aortic endothelial cells were isolated from calf aortas after the protocol described previously (16) with a few modifications. Briefly, aortas were obtained from 6-month-old calves at a local slaughterhouse and processed within 2 h from the time of death. The vessels were washed with a sterile solution of Hanks' balanced salts (HBSS with Ca²⁺ and Mg²⁺), 50 mM HEPES (Sigma), 1,000 units/ml penicillin, 1,000 μg/ml streptomycin, and 500 μg/ml gentamicin sulfate, pH 7.4, and digested with a sterile solution of 2 mg/ml of collagenase C (from Worthington) in HBSS, 0.35 g/liter NaHCO₃, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 μg/ml gentamicin sulfate, pH 7.4. The cell suspension was centrifuged at 1,500 rpm for 10 min, and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM from Sigma) containing 10% fetal calf serum and the above antibiotics. The endothelial cells were identified by their characteristic cobblestone morphology, their contact inhibition upon reaching confluency and the positive staining for factor VIII antibody, as reported elsewhere (16). The endothelial cells were routinely grown in 75-cm² flasks in DMEM containing 10% calf serum and 10% fetal calf serum and were used continuously after the protocol described previously (16) with a few modifications. Briefly, aortas were obtained from 6-month-old calves at a local slaughterhouse and processed within 2 h from the time of death. The vessels were washed with a sterile solution of Hanks' balanced salts (HBSS with Ca²⁺ and Mg²⁺), 50 mM HEPES (Sigma), 1,000 units/ml penicillin, 1,000 μg/ml streptomycin, and 500 μg/ml gentamicin sulfate, pH 7.4, and digested with a sterile solution of 2 mg/ml of collagenase C (from Worthington) in HBSS, 0.35 g/liter NaHCO₃, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 μg/ml gentamicin sulfate, pH 7.4. The cell suspension was centrifuged at 1,500 rpm for 10 min, and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM from Sigma) containing 10% fetal calf serum and the above antibiotics. The endothelial cells were identified by their characteristic cobblestone morphology, their contact inhibition upon reaching confluency and the positive staining for factor VIII antibody, as reported elsewhere (16).

**Cell Adhesion Assays**—96-well plastic plates were routinely coated with control and nonenzymatically glucosylated type IV collagen and laminin by the addition of 50 μl of solution/well. Each macromolecule was used for coating at five different concentrations: 100, 50, 25, 12.5, and 1 μg/ml in PBS in quadruplicates. The plates were left overnight at 29 °C to dry and were kept at 4 °C, until used during the following 7-8 days.

Subconfluent (60-70% confluent) bovine aortic endothelial cells were labeled with 500 μCi of [35S]methionine (from ICN Radiochemicals) for 18 h. Cells were washed with an HBSS solution containing Ca²⁺ and Mg²⁺, then with HBSS containing 1 mM EDTA, and were detached by the addition of a 0.05% trypsin, 0.75 mM EDTA solution. The cells were then washed three times with 10 ml of DMEM by centrifugation at 1200 rpm for 5 min and finally diluted in 4 ml of a DMEM solution containing 25 mM HEPES, 2 mg/ml BSA, pH 7.4 (binding buffer). The number of cells was calculated using a hemocytometer. Plates coated with nonenzymatically glucosylated proteins were incubated with PBS containing 2 mg/ml BSA, pH 7.4, for 60 min at 37 °C in a humidified incubator. Subsequently, five thousand cells were added to each well in a volume of 200 μl containing 500 mM glucose increased the coating of laminin and type IV collagen by 1% and 8%, respectively; in 500 mM glucose these increases were 11% and 30%.

Bovine aortic endothelial cells were isolated, cultured, and characterized as described (16). Subconfluent cells were metabolically labeled with [35S]methionine, detached, washed, and allowed to adhere to coated substrates, as described above. Adhesion was examined after 15 min for type IV collagen and 20 min for laminin. The differences in time intervals used in this and subsequent assays were dictated by the fact that bovine aortic endothelial cells exhibit different affinities for the two substrates; they adhere more avidly to type IV collagen compared with laminin. The results are shown in Fig. 1A for type IV collagen and Fig. 1B for laminin. In the case of type IV collagen, a reduction of cell adhesion in the range of 30-40% was observed between control and glucosylated samples, in all coating concentrations used (100 to 1 μg/ml); differences in the samples glucosylated at low or high glucose concentrations were smaller and became statistically significant only at the highest coating concentration used (Fig. 1A).

In the case of laminin, a reduction of cell adhesion in the range of 20-30% was observed in all but the lowest coating concentration. However, samples glucosylated under different glucose concentrations did not exhibit any statistically significant difference when grown on laminin (Fig. 1B). We cannot satisfactorily explain this somewhat puzzling finding. It could be due to several factors: severe steric changes induced even at low glucose concentrations, differential adherence to plastic of heavily modified macromolecules, or lack of sensitivity of the technique used. These experiments were performed at least three times in quadruplicates and the data were analyzed for statistical significance.
Adhesion Spreading on Diabetically Modified Macromolecules

FIG. 1. Control and glucosylated type IV collagen (A) or laminin (B) were coated on plastic at different coating concentrations. The ability of metabolically labeled bovine aortic endothelial cells to adhere was measured and normalized for the amount of protein retained in each well. Five different concentrations were used in the coating solution: 100, 50, 25, 12.5, and 1 μg/ml. Data are expressed as percentage of cells adhering for each permutation. Circles, incubated in 0 mM glucose; squares, incubated in 50 mM glucose; triangles, incubated in 500 mM glucose. Bars indicate standard deviation.

The effect of nonenzymatic glucosylation on the ability of laminin and type IV collagen to promote cell spreading was examined next. Two methods were used to assess the extent of cell spreading. In the first method, the percent of cells exhibiting “long processes” was determined. A long process was operationally defined as a protrusion from the cell body longer than the maximal diameter of the cell under examination. The number of cells with long processes decreased by 43% between control and type IV collagen incubated in the presence of 50 mM glucose, and by 62% between control and type IV collagen incubated in the presence of 500 mM glucose. In the case of laminin, no reduction in the number of cells with long processes was observed when control was compared with glucosylated laminin at 50 mM glucose, but a 45% decrease in cell processes was seen when cells were allowed to spread on glucosylated laminin at 500 mM glucose. These data were obtained at coating concentrations of 50 μg/ml for each macromolecule. Lower coating concentrations tended to minimize these differences (data not shown).

As a complementary and more objective approach, the perimeter of cells was measured. Bovine aortic endothelial cells allowed to spread on control and glucosylated type IV collagen for 15 min or on control and glucosylated laminin for 30 min were examined using a Nikon Diaphot microscope and their perimeter was traced by using an Optomax camera connected to an Apple IIe computer. Again, the difference in time interval in which spreading was stopped by fixation was dictated by differences in affinity for these macromolecules observed with bovine aortic endothelial cells. In these experiments, three different coating concentrations were used (50, 25, and 12.5 μg/ml). At least six randomly chosen fields were analyzed per well, and at least 10 wells were used for every permutation. The data shown in Fig. 2A (for type IV collagen) and Fig. 2B (for laminin) demonstrate that nonenzymatic glucosylation reduced the ability of cells to spread. In this case, coating with heavily glucosylated macromolecules resulted in a pronounced reduction in cell perimeter compared with cells examined on lightly glucosylated type IV collagen and laminin. Because totally round cells exhibit a perimeter in the range of 70–80 μm, the magnitude of the observed reductions constitutes a major phenotypic change. Similar results were obtained when cell spreading was assessed for longer time intervals (up to 60 min). Fig. 3 shows representative fields of cells allowed to adhere and spread on type IV collagen for 15 min. It can be appreciated that compared with control (Fig. 3A), cells on glucosylated macromolecules (Fig. 3, B and C) exhibit less extensive processes. Also, more extensive glucosylation results in reduction in the number of existing processes (Fig. 3, compare panels B and C).

DISCUSSION

In this study, evidence is provided that endothelial cells undergo changes in their interactions with nonenzymatically
Adhesion Spreading on Diabetically Modified Macromolecules

Glycosylated laminin and type IV collagen, both important components of all basement membranes. These altered interactions resulted in a impaired adhesion and spreading to each of these basement membrane glycoproteins.

Baseline membrane macromolecules and matrix components in general, are important elements in the regulation of cellular phenotype (18). Matrix mediated changes of cell shape interfere with growth and differentiation. Extracellular matrix components are crucial in determining cell shape and induction of specific gene expression in several cells, including cultured adipocytes (19), mammary epithelial cells (20), pheochromocytoma cells (21), and hepatocytes (22). Adhesion and spreading should be considered as very early events in the interaction between cells and extracellular matrices; therefore, these early binding events may be very critical in determining the extent and the nature of such interactions.

Alterations in the ability to adhere and spread have been very well documented in the case of transformed cells and associated with the expressed malignant phenotype (23). The data presented in this study provide support to the hypothesis that in diabetes, hyperglycemia-induced matrix modifications may to some extent influence the cellular properties of endothelial and possibly other cell types. Differential adhesion and spreading under diabetic conditions may lead to differences in growth, proliferation, and secretory activity of specific cell types. It is well documented that in diabetic retinopathy retinal microvascular endothelial cells may have an increased proliferation whereas microvascular pericytes are reduced in number (24). In diabetic nephropathy a sizable increase in the mesangial matrix is observed (25). In vitro studies have suggested that nonenzymatically glycosylated mesangial matrix resulted in reduced ability of mesangial cells to proliferate (26). Thus, diverse effects could be caused on various aspects of cell behavior as a result of matrix nonenzymatic glycosylation.

Many factors are likely to be involved in diabetic microangiopathy. It has been suggested that endothelial cell behavior may be altered by exposure to a high glucose concentration, even for a short time interval (27, 28) and/or by excessive sorbitol formation via the polyol pathway (29). This report provides evidence for yet another important mechanism which may contribute to altered endothelial cell properties in diabetic conditions, suggesting that nonenzymatic glucosylation of laminin and type IV collagen may eventually influence cellular phenotype.

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