The glomerular epithelial cells and the glomerular basement membrane are important constituents of the permselective barrier in the kidney. These are affected in diabetic nephropathy, one of the long-term complications in diabetic patients. Nonenzymatic glycosylation resulting in the accumulation of advanced glycosylation end products correlates with the development of long-term complications in diabetes. The interaction of cells with extracellular matrix proteins plays a critical role in a variety of biological processes. Recent studies show that cell-matrix interactions mediated by integrins can transduce biochemical signals to the cell interior and regulate cell behavior. In this paper we demonstrate that interactions of human glomerular epithelial cells with a nonenzymatically glycated matrix are altered with defective cell spreading, reduced phosphorylation of focal adhesion kinase and reduced activity of mitogen-activated protein kinase. These data suggest that matrix glycation interferes with normal cell-matrix interactions and intracellular signaling that can potentially result in differential gene expression contributing to the changes seen in diabetic nephropathy.

The glomerular epithelial cells together with the glomerular basement membrane participate in the formation of the permselective barrier in the kidney. Thickening of the glomerular basement membrane and expansion of the mesangium are among the characteristic changes seen in diabetic nephropathy, and they correlate with declining renal function in diabetic patients (1).

In diabetes, hyperglycemia affects many metabolic pathways and each of these changes has important consequences in the development of various pathologic manifestations. There is much documented evidence, including that from The Diabetes Control and Complications Trial (2), that hyperglycemia and subsequent biochemical events correlate with the development of diabetic complications. One of the mechanisms by which elevated sugar levels compromise normal structure and function is by the phenomenon of nonenzymatic glycosylation with the formation of primary Amadori, intermediate and advanced glycosylation end products (3) that alter the structure and function of basement membrane macromolecules in vitro and in vivo (4–6). The accumulation of these products correlates with the development of long-term complications in diabetes (7).

It is well accepted that extracellular matrices, especially basement membranes, are involved in a dynamic and reciprocal relationship with cells and to a large extent determine cellular phenotype, function, and gene expression (8). These interactions of cells with matrix molecules are mediated largely by cell receptors of the integrin family (9–11). The integrins form clusters at sites of close cell-substrate contact termed focal contacts, and these serve to link the integrins to the cytoskeleton. However, focal contacts play more than a structural role; they serve as centers for the activation of several signal transduction pathways (12–14). Tyrosine phosphorylation is a common and ubiquitous response to integrin activation in many cell types including leukocytes, fibroblasts, and carcinoma cells. Tyrosine phosphorylation of important components of the focal adhesion complex such as focal adhesion kinase (FAK) and paxillin, occurs following integrin activation (15, 16). Integrin engagement has also been linked to activation of MAPK (17, 18). AP-1 is a sequence-specific transcriptional activator, and its activity is regulated by mitogen-activated protein kinases (19). Thus, one can see how integrin-mediated cell interactions with the extracellular matrix influence cell behavior and gene expression.

In this work, we examine the possibility that interactions of cells with a diabetically modified matrix interfere with normal cell-matrix interactions and consequently result in changes in cell behavior. We have investigated the alterations seen when human glomerular epithelial cells interact with glycated matrix. This provides an insight into the role played by the human glomerular epithelial cells in the pathogenesis of diabetic nephropathy.

MATERIALS AND METHODS

Cell Line and Culture Conditions—The T-SV40 immortalized HGEC were cultured as described (20). Earlier these cells were compared with primary HGEC for their integrin-mediated interactions with type IV collagen (21). The cells were synchronized to G0 phase of the cell cycle as follows. First, they were arrested in S phase using thymidine excess (1 mg/ml in Dulbecco’s modified Eagle’s medium/F-12 with 0.1% serum) for 24–28 h. Following this, the medium containing thymidine was replaced by Dulbecco’s modified Eagle’s medium/F-12 containing 0.1% fetal calf serum for 18–20 h. 75% of cells were in G0 phase (tested by flow cytometry) by this method. All experiments were performed using synchronized cells.

In Vitro Glycation of Type IV Collagen—We have used type IV col-
aden for these studies since it accounts for >50% of the dry weight of the glomerular basement membrane. Type IV collagen (tIV), isolated from the Engelbreth-Holm-Swarm tumor system (22) was glycated using previously described techniques (23). Briefly, tIV was incubated for 5 days in solutions containing 0.2 M phosphate buffer (pH 7.4), 0.5M NaCl, 0.5M NaH2PO4, 0.5M Na2HPO4, 0.1M Na2EDTA, 1mM phenylmethanesulfonfonyl fluoride, 1mM N-ethylmaleimide, 0.02% sodium azide, and one of the following: buffer only, 0.5 or 1m ribose. Ribose has been reported to be 16.6 times more reactive than glucose in the formation of Schiff bases with proteins and 129 times more reactive in the generation of advanced glycosylation end products (24, 29). The fluorescence of each sample was measured at the excitation/emission wavelength of 370/440 nm using a luminescence spectrometer (model LS 50 B; PerkinElmer). Fluorometric analysis revealed a 500 and 900% increase in fluorescence at 370/440 nm for tIV-0.5m ribose and tIV-1m ribose, respectively, compared with control-incubated tIV-C. Levels of increase in advanced glycosylation end product adducts were in the range of that seen in diabetes with long-term complications (7). Protein concentrations were estimated spectrophotometrically (25).

Cell Adhesion and Spreading on Type IV Collagen—The assays were done on 96-well microtiter plates. The coating efficiency of control and glycated tIV had been previously determined by the use of radiolabeled proteins (125I-tIV) in solid phase adhesion assays. Glycation increased the coating of type IV collagen by 30%. The coating efficiency of each control or differently glycated sample was adjusted accordingly. Type IV was coated in serial dilutions and dried overnight at 29°C. The maximum amount coated was 15 pmol/cm². For cell spreading, cells were used without radioisotopic labeling and allowed to spread on glass slides coated with control or glycated tIV. After 1 h, adherent cells were stained with Diff-Quik Stain Set (Baxter, Miami, FL). Random pictures were taken using TEC-470 Optronics CCD camera/Image 1, and MetaMorph software (Westchester, PA) was used to define regions of 75 cells from each condition and derive the cell area.

Flow Cytometry—Expression of integrin subunits was evaluated by flow cytometry. 10-cm culture dishes were coated with 10 ml of control tIV or glycated tIV to give 2.5 pmol/cm². These were dried overnight at 29°C and sterilized by UV irradiation. The plates were blocked with 100 ml of 5 mg/ml BSA for 2 h. The plates were washed with phosphate-buffered saline two times and trypanized HGEC were added. Cells were cultured and synchronized, and the integrin expression was studied using anti-integrin antibodies. The monoclonal antibody to α5(TS2/7) was a generous gift from Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). The monoclonal antibodies to α1(F1H5), α6(P3D11), α2(P4G9), α1(F1D6), and β1(P5D2) subunits of integrins were generously provided by Dr. Elizabeth Wayner and are previously described (26).

Immunofluorescence—Glass coverslips were coated with control and glycated tIV, and the cells were allowed to interact for 60 min in serum free medium containing 0.02% BSA. Unbound cells were washed off; the remaining cells were fixed in 2% paraformaldehyde in phosphate-buffered saline for 30 min and then permeabilized for 2 min with 0.5% Triton X-100 and 30 min with 1% BSA. The coverslips were incubated with 10 μg/ml anti-FAK (UBI, Lake Placid, NY) for 60 min. After this, the coverslips were washed extensively and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Boehringer Mannheim). The immunostained samples were examined with a Bio-Rad MRC 1024 confocal microscope equipped with a krypton/argon laser. Counting of focal contacts was done in Adobe Photoshop 4.0 using the image processing tool kit. Twenty-five 100-μm² cell areas were counted in each experiment. Results are expressed as mean ± S.D. from three experiments (number of focal contacts per 100-μm² cell area).

Western Blotting for Tyrosine Phosphorylation—10-cm culture dishes were coated with 10 ml of control tIV or glycated tIV to give 2.5 pmol/cm². 5 x 10⁶ cells were plated on a culture dish in binding buffer (serum free Dulbecco’s modified Eagle’s medium containing 2mg/ml BSA). After 60 min of incubation at 37°C, nonadherent cells were washed, and adherent cells were lysed at 4°C with 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.2, that contained 150 mM NaCl, 1 mM phenylmethanesulfonfonyl fluoride, 1 mM N-ethylmaleimide, 10 μg/ml leupeptin, 1% Triton X-100, 2 μg/ml aprotinin, 50 mM sodium fluoride, and 2 mM sodium orthovanadate). Lysates from cells kept in suspension for 60 min served as negative control. The lysates were clarified by cesium chloride at 12,000 x g for 15 min. Protein estimation was performed by the bicinchonic acid method. 50 μg of cell lysate was run on a 7.5% polyacrylamide gel. After electrophoresis, gels were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 2.5 h at 0.5 A. The membranes were blocked overnight with Tris-buffered saline containing 2% BSA and then incubated with monoclonal peroxidase-conjugated antiphosphotyrosine antibody (UBI) at 1 μg/ml in blocking buffer for 1 h at room temperature. After washing with Tris-buffered saline containing 0.05% Tween 20, the blots were developed using enhanced chemiluminescence (Amersham Corp.) according to the manufacturer’s protocol.

Immunoprecipitation—Experiment was performed as for the Western blotting, and cell lysates were immunoprecipitated overnight using polyclonal anti-pp125 FAK (UBI). Immune complexes were precipitated with protein A-Sepharose beads at 4°C for 2 h. The beads were washed four times with lysis buffer, and the precipitated proteins were released by boiling the beads in SDS sample buffer (80 mM Tris-HCl, pH 6.8, 3% SDS, 15% glycerol, 0.01% bromphenol blue, 5% β-mercaptoethanol) for 10 min. This was run on a 7.5% SDS polyacrylamide gel and first immunoblotted with antiphosphotyrosine antibody as described above. The immunoblots were stripped in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 55°C for 30 min as recommended by the manufacturer and then immunoblotted with anti-FAK antibody (1 μg/ml in 5% skim milk in phosphate-buffered saline). This was followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (Sigma). Blots were developed using enhanced chemiluminescence.

In Vitro Kinase Assay—MAPK activity was assayed according to Boulton and Cobb (27). Briefly cell lysates (300 μg/sample) were immunoprecipitated with 2.5 μg of anti-MAPK antibody (UBI). Immune complexes were precipitated with protein A-Sepharose beads at 4°C for 2 h. Immunoprecipitates were washed two times with 0.25M Tris (pH 7.6), and once with 0.1M NaCl and 50 mM Hepes, pH 8.0. The immunoprecipitated MAP kinases were incubated in 100 μl of a mixture containing [γ-32P]ATP, 50 mM ATP, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM benzamidine, 0.3 mg/ml myelin basic protein, and 25 mM Hepes, pH 8.0 at 30°C for 30 min. The reactions were stopped by removing the supernatants from the pelletted immunocomplexes and boiling in SDS sample buffer. The samples were electrophoresed on a 15% polyacrylamide gel. The gel was dried and autoradiographed. 15% of the immunoprecipitated MAPK was run on a 7.5% polyacrylamide gel and immunoblotted with MAPK antibody as a loading control.

All films were scanned using a Bio-Rad Model GS-700 imaging densitometer, and densitometric analysis was performed using Molecular Analyst Version 2.1.

RESULTS

Adhesion of HGEC on Control or Glycated Type IV Collagen—At the end of 1 h about 30% of the cells adhered to tIV. There was no difference in the absolute amount of adhesion of HGEC on control or glycated type IV collagen (Fig. 1). At 1 h the adhesion of various cell types in the kidney to tIV is as follows: primary HGEC, ~40%; immortalized human proximal tubular epithelial cells, ~30%; human microvascular endothel-
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Matrix glycation interferes with the initial events of cell-matrix interactions. The number of focal adhesions when cells adhered to a glycated tIV would mean an overall lesser cell-matrix contact compared with control tIV. Cells being smaller on a glycated tIV would mean an overall lesser cell-matrix contact area and therefore fewer focal adhesions in total. Since FAK is an important molecule associated with focal adhesions (34), we subsequently explored the possibility of alterations in intracellular signaling following cell interaction with a glycated matrix.

Changes in Intracellular Signaling—In preliminary experiments we studied the phosphotyrosine content of cell lysates as a function of incubation time. We observed that after 60 min the amount of phosphotyrosine detected reached its peak (data not shown). Therefore we decided to use the 60-min time point for further studies especially since changes in cell spreading were also noted at this time point, and we wanted to examine alterations in early cell-matrix interactions.

Lysates from HGEC interacting with both control and glycated t-IV displayed an increase in the phosphorylation of a band at 125 kDa (pp125FAK) and a band at 43 kDa (MAPK) compared with cells in suspension (Fig. 3).

To test if there were differences in the tyrosine phosphorylation of FAK following adhesion to glycated type IV collagen we immunoprecipitated FAK following adhesion of cells to control and glycated type IV collagen. The immunoprecipitates were first immunoblotted with antiphosphotyrosine antibody (Fig. 4A) and then with anti-FAK antibody (Fig. 4B) to check for equal amounts of FAK immunoprecipitated. Fig. 4A demonstrates that there is reduced phosphorylation of FAK following adhesion to glycated type IV collagen. Densitometric analysis across three experiments revealed that, compared with cells on tIV-C, cells adhering to tIV-0.5 M ribose had about 28% less phosphorylated FAK, and cells adhering to type IV glycated with 1 M ribose had about 50% reduction in the amount of phosphorylated FAK (Fig. 4C; p < 0.05, one-way ANOVA).

MAPK activity has been linked to FAK phosphorylation through adaptor proteins like Grb2 (18). Therefore we then investigated if there were differences in MAPK activity following interaction of cells with glycated tIV. For this, MAPK from cell lysates was immunoprecipitated with anti-MAPK antibody. The MAPK immunoprecipitates were then compared for...
their ability to phosphorylate a substrate, myelin basic protein (Fig. 5A). 15% of the immunoprecipitates were immunoblotted with anti-MAPK antibody to confirm that equal amounts of MAPK had been immunoprecipitated (Fig. 5B). Densitometric analysis across three experiments revealed that, compared with tIV-C, cells adhering to tIV-0.5M ribose had about 35% less MAPK activity and cells adhering to Type IV glycated with 1M ribose had about 50% reduction in the extent of MAPK activity (Fig. 5C; \( p < 0.05 \), one-way ANOVA).

These results demonstrate that there is a definite alteration in cell behavior when human glomerular epithelial cells interact with an altered matrix macromolecule, as evidenced by changes in cell spreading, phosphorylation of FAK, and activation of MAPK.

DISCUSSION

The mechanisms by which hyperglycemia results in altered cell behavior in vitro or in situ are multiple and complex. One of the mechanisms is nonenzymatic glycosylation, which is known to alter the structural and functional integrity of extracellular matrix. In the present report we provide evidence that these matrix modifications additionally affect cell-matrix interactions and behavior of human glomerular epithelial cells.

As cells adhere and spread they form focal adhesions. Focal adhesions are sites of integrin clustering and activation of several signal transduction pathways (16). It has been proposed that they may integrate mechanical signals associated with changes in cell shape with chemical signals elicited directly by integrin binding and thereby modulate downstream signaling (35). With increased cell spreading the total area of cell-extracellular matrix contact increases, hence focal adhesion formation, integrin binding, and therefore subsequent signaling events may vary in parallel. In addition it has been recently demonstrated that cell shape by itself is an important determinant of cell functions like survival and growth (36). Therefore alterations in cell spreading could both directly and indirectly affect normal cell responses.

Defects in cell spreading have been noted on interaction of mesangial cells and endothelial cells with a glycated matrix (29, 30) and changes in intracellular signaling have been observed under diabetic conditions in mesangial cells and endothelial cells (30, 37). However, to the best of our knowledge there is no report of such studies in human glomerular epithelial cells in diabetic conditions, and changes in MAPK activation have not been investigated in these earlier reports.

In diabetic nephropathy some of the changes occurring in the glomerulus are a thickening of the glomerular basement membrane and expansion of the mesangial matrix. The thickening of the glomerular basement membrane and the expansion of the mesangial matrix are due to an increased deposition of extracellular matrix macromolecules such as collagen, fibronectin, laminin, and proteoglycans (38–42). It has been seen that this could be due to an imbalance between matrix deposition and degradation. In streptozotocin-induced diabetes...
activity could potentially cause alterations in the expression and secretion of matrix-degrading enzymes that we are currently investigating.

Altered cell-matrix interactions followed by alterations in intracellular signaling could translate downstream into differential expression of matrix components, matrix degrading enzymes, and/or their inhibitors, growth factors, and cytokines that could lead to the changes occurring in a long-term diabetic patient.

Acknowledgments—We acknowledge Dr. Elizabeth Wayner for the generous donation of antibodies, Dr. Aristidis Charonis for useful suggestions, and Howard Higsen for technical assistance.

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Fig. 5. MAPK activity in vitro kinase assay. Cells in suspension and cells attached for 60 min to control or glycated tIV were lysed and immunoprecipitated with anti-MAPK. The activity of the immunoprecipitated MAPK was measured using myelin basic protein as substrate and [γ-32P]ATP. The phosphorylated myelin basic protein was separated by a 15% SDS gel, dried, and autoradiographed (A). 15% of MAPK immunoprecipitates were immunoblotted with anti-MAPK to confirm equal amounts immunoprecipitated (B). Densitometric analysis was done on the autoradiograms and the MAPK immunoblots. MAPK activity in tIV-C has been expressed as 100%. The relative % of MAPK activity in each of the other lanes was calculated using the following equation:

\[
\text{Relative % of MAPK activity} = \frac{\text{Autoradiogram value tIV-C} \times \text{MAPK immunoblot value tIV-C}}{\text{Autoradiogram value test lane} \times 100} \quad \text{(Eq 2)}
\]

The bars in panel C represent the mean ± S.D. from three experiments of the relative % of MAPK activity in HGEC interacting with control and glycated tIV. Compared with cells on tIV-C, cells adhering to tIV-0.5 M ribose and tIV-1 M ribose had about 35 and 50% less MAPK activity, respectively (p < 0.05, one-way ANOVA).

in rats, there is an up-regulation of expression of collagenases, a down-regulation of the expression of collagens, and an up-regulation of the expression of tissue inhibitors of collagenases (43).

AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos families, and its activity is regulated by Mitogen activated protein kinases (19). In addition, it has been demonstrated that the AP-1 binding site regulates the transcription of the collagenase gene in response to integrin-mediated signals (44). Therefore reduced MAPK activity could potentially cause alterations in the expression and secretion of matrix-degrading enzymes that we are currently investigating.