Decreased Interaction of Fibronectin, Type IV Collagen, and Heparin due to Nonenzymatic Glycation. Implications for Diabetes Mellitus†

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ABSTRACT: The nonenzymatic glycation of basement membrane proteins, such as fibronectin and type IV collagen, occurs in diabetes mellitus. These proteins are nonenzymatically glycated in vivo and can also be nonenzymatically glycated in vitro. After 12 days of incubation at 37 °C with 500 mM glucose, purified samples of human plasma fibronectin and native type IV collagen showed a 13.0- and 4.2-fold increase, respectively, in glycated amino acid levels in comparison to control samples incubated in the absence of glucose. Gelatin (denatured calfskin collagen) was glycated 22.3-fold under the same conditions. Scatchard analyses were performed on the binding of radiolabeled fibronectin to gelatin or type IV collagen. It was found that there is a 3-fold reduction in the affinity of fibronectin to type IV collagen due tc the nonenzymatic glycation of fibronectin. The dissociation constant (K_D) for the binding of control fibronectin to type IV collagen was 9.6×10^{-7} M while the K_D for glycated fibronectin and type IV collagen was 2.9×10^{-6} M. This was similar to the 2.7-fold reduction in the affinity of fibronectin for gelatin found as a result of the nonenzymatic glycation of fibronectin (K_D of 4.5×10^{-7} M for the interaction of control fibronectin with gelatin vs. K_D of 1.2×10^{-6} M for the interaction of nonenzymatically glycated fibronectin with gelatin). The molecular association of control fibronectin or its glycated counterpart with [^{3}H]heparin was also determined. Scatchard analyses of this interaction showed no difference between control fibronectin and glycated fibronectin in [^{3}H]heparin binding. Both a low-affinity ($K_D = 2 \times 10^{-7}$ M) binding site and a high-affinity [$K_D = (2–3) \times 10^{-8}$ M] binding site were found for heparin on each of these fibronectin molecules. However, when fibronectin and [^{3}H]heparin were incubated together with type IV collagen at fixed concentrations, the binding of [^{3}H]heparin to fibronectin was significantly enhanced by the addition of type IV collagen. This positive cooperative binding of heparin with fibronectin and type IV collagen was reduced approximately 36% when fibronectin was nonenzymatically glycated, 64% when type IV collagen was nonenzymatically glycated, and 70% when both fibronectin and type IV collagen were nonenzymatically glycated (12 days of incubation with excess glucose). The results of the latter binding studies are almost identical with those conducted with fibronectin, heparin, and gelatin [Tarsio, J. F., Wigness, B., Rhode, T. D., Rupp, W. M., Buchwald, H., & Furcht, L. T. (1985) *Diabetes 34*, 477–484] even though type IV collagen was nonenzymatically glycated to a significantly lesser extent than gelatin. In addition, a slight but significant reduction in the binding of [^{3}H]heparin to fibronectin and type IV collagen could be observed even with fibronectin nonenzymatically glycated for 1 day in vitro (a level of glycation 3.2-fold that of nonglycated fibronectin). These observations with native type IV collagen are discussed relevant to changes in the molecular composition of basement membranes that occur in diabetes mellitus.

Diabetes mellitus is a chronic illness characterized by absolute or relative insulin deficiency, elevations in fasting plasma glucose, and glycosuria (Reynolds et al., 1978). Standard treatment of diabetes including dietary adjustment, insulin injection, and administration of oral hypoglycemic agents does not prevent or correct the multiple tissue defects that occur in this disease. These complications include a generalized macroangiopathy and microangiopathy with the development of tissue damage to the lens, peripheral nerves, retina, and kidney and with accelerated atherosclerosis (Alberti & Press, 1982; Brownlee & Cerami, 1981). In these tissues, basement membrane thickening is a prominent finding (Hansen & Lundbaek, 1970; Williamson & Kilo, 1976; Kefalides et al., 1979; Brownlee & Cerami, 1981; Alberti & Press, 1982; Martinez-Hernandez, 1983) in both type I (insulin-dependent) and type II (non-insulin-dependent) diabetics (National Diabetes Data Group, 1979). Nephropathy in particular seems to parallel the development of glomerular basement membrane thickening with a corresponding increase in mesangial volume (Østerby, 1975; Steffes et al., 1979, 1980). An early renal change in diabetics may be a decrease in the content of heparan sulfate proteoglycan in glomerular basement membranes (Cohen & Surma, 1981; Rohrbach et al., 1982; Parthasarathy & Spiro, 1982; Kanwar et al., 1983). The loss of heparan sulfate proteoglycan has been speculated to cause a disruption in the anionic-charged nature of the glomerular filtration barrier (Kanwar & Rosenzweig, 1982). Other investigations suggest that the loss of heparan sulfate proteoglycan may result in a compensatory synthesis of other basement membrane components such as fibronectin (Pettersson & Colvin, 1978; Scheinman et al., 1981) or type IV collagen (Grant et al., 1976), which may lead to basement membrane thickening. Mesangial expansion is found in diabetic patients, and an accumulation of fibronectin has been reported to occur in the mesangium of these patients (Scheinman et al., 1981).

Another key biochemical change in diabetes, associated with hyperglycemia, is an increase in levels of the chemical attachment of glucose to proteins brought about without the

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involvement of enzymes (nonenzymatic glycation) (Bunn, 1981; Perejda & Uitto, 1982; Brownlee et al., 1984; Kennedy & Baynes, 1984). The mechanism of this reaction involves the formation of a Schiff base between glucose and the N-terminal amino group of a polypeptide or the ϵ-amino group of lysine in the polypeptide with subsequent formation of a stable ketoamine linkage via an Amadori rearrangement (Bunn et al., 1977). Many proteins such as hemoglobin, albumin, low-density lipoproteins, basement membrane type IV collagen, lens crystallin, and peripheral nerve proteins have been found to be nonenzymatically glycated to a greater extent in diabetes when compared to normals. It is also known that various tissue extracts and purified proteins can be nonenzymatically glycated in vitro by incubation with excess glucose (Bunn, 1981; Perejda & Uitto, 1982; Brownlee at al., 1984; Kennedy & Baynes, 1984).

Recently it was shown that fibronectin, a major component in plasma and in the glomerular basement membrane, is glycated 2–3-fold greater in the plasma of alloxan-induced diabetic beagle dogs above that of age-matched controls. High-performance liquid chromatographic analysis revealed that the site of enhanced glycation of plasma fibronectin in diabetes was the result of the addition of glucose to the ϵ-amino groups of lysine residues within the polypeptide chains of the dimeric molecule (Tarsio et al., 1985). Because of this finding and because of the various alterations that have been suggested in the turnover and/or molecular association of basement membrane or connective tissue components in diabetes, we have nonenzymatically glycated fibronectin, type IV collagen, and gelatin (purified denatured calfskin collagen) in vitro and have conducted ligand binding studies using these glycated molecules or their normal counterparts. This study is the first report of abnormalities in the molecular association of basement membrane proteins due to nonenzymatic glycation presented from an actual analysis of affinity constants with Scatchard plots (Scatchard, 1949; Rodbard, 1981). This study shows that the nonenzymatic glycation of fibronectin decreases its affinity for gelatin or type IV collagen. This consequently has a deleterious effect on the subsequent incorporation of [^{3}H]heparin into complexes of fibronectin–collagen (viewed as a model for heparan sulfate proteoglycan incorporation into extracellular-like basement membrane material).

Materials and Methods

In Vitro Nonenzymatic Glycation. Human plasma fibronectin purified as previously described (Smith & Furcht, 1982), type IV collagen purified from EHS tumors grown in mice (Kleinman et al., 1982), and gelatin (Eastman Kodak, Rochester, NY) were individually glycated in vitro essentially as previously described (Tarsio et al., 1985) by incubation of 25 mg of each protein (at 1 mg/mL) at 37 °C with 500 mM D-glucose in phosphate-buffered saline (PBS, pH 7.4) containing 1 mM sodium azide as a preservative. In this study, protease inhibitors were also added to the incubation buffer to give a final concentration of 372.2 mg/L disodium ethylenediaminetetraacetate (Na_2EDTA), 34.8 mg/L phenylmethanesulfonyl fluoride (PMSF), 0.7 mg/L pepstatin A, 0.5 mg/L leupeptin, and 4.5 trypsin inhibitor units of aprotinin/mL (all from Sigma, St. Louis, MO). After 12 days of incubation, unreactive sugar was removed by extensive dialysis against PBS buffer. Control samples of each protein were also each incubated in PBS and protease inhibitors as above but without the addition of glucose. The extent of nonenzymatic glycation of the in vitro glycated proteins and their corresponding controls was determined by labeling the glucose moieties on the proteins by reductive tritiation with NaB^3H_4 (Bookchin & Gallop, 1968). The protein was then hydrolyzed, and the labeled glycated amino acids were separated on boronate columns (1.5 × 8 cm, Affi-Gel 601, Bio-Rad Laboratories, Richmond, CA) (Brownlee et al., 1980). The level of nonenzymatic glycation of each in vitro glycated protein and its corresponding nonglycated control is then expressed as cpm of glycated amino acids per milligram of protein (Brownlee et al., 1980; Tarsio et al., 1985).

Radioiodination of Fibronectin. Normal (control) human plasma fibronectin and its nonenzymatically glycated counterpart (after 12 days of in vitro glycation) were radiolabeled with ^{125}I and with 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen) as the oxidizing agent (Salacinski et al., 1981). Specifically, 50 μg of Iodo-Gen (Pierce, Rockford, IL) in 0.1 mL of chloroform was applied to coat the bottom and sides of each 1-mL capacity conical glass React-Vial (Pierce) by evaporation with N_2 and allowed to incubate overnight at 4 °C in a vacuum desiccator as previously described (Salacinski et al., 1981). Each vial was then rinsed 2 times with PBS buffer (pH 7.4) to remove any loose flakes, and the rinse was discarded. Fibronectin (0.5 mg) was then added in 0.5 mL of PBS and the vial capped with a resealable Teflon/silicone disc closure (Pierce). Next, 2.5 mCi (25 μL) of $Na^{125}I$ solution (carrier free, 100 mCi/mL in NaOH solution, pH 7–11, Amersham, Arlington Heights, IL) was added. The protein was allowed to react with $Na^{125}I$ for 15 min at 4 °C. The reaction mixture was then removed from the vial with a syringe and chromatographed on a column of Bio-Gel P6DG (1.5 × 7.5 cm, Bio-Rad) equilibrated in PBS buffer (pH 7.4), and 1-mL fractions of eluant were collected in glass test tubes. A total of 5 μL of each fraction was then counted in a γ counter. Tubes collected from the void volume of the column showing appreciable radioactivity were then pooled, and the fibronectin concentration of the pooled fractions was determined in a spectrophotometer (using an extinction coefficient of 1.28 at A_{280} for 0.1% w/v solution of fibronectin). Four 5-μL aliquots of the pooled radiolabeled fibronectin were also counted in a γ counter. The specific radioactivity of the radioiodinated fibronectin was then expressed as the total average cpm per picomole of protein (taking 440 000 as the molecular weight of fibronectin) and was in the range of (0.9–3.0) × 10^6 cpm/pmol per iodination experiment. Radioiodinated control fibronectin or its nonenzymatically glycated counterpart was then used at this specific radioactivity as free ligand in the concentration range of 10^{-9}–10^{-7} M in binding experiments to gelatin and type IV collagen immobilized on nitrocellulose filters. In order to use ligand concentrations of 10^{-7}–10^{-6} M in the binding assay, this high specific activity radiolabeled control fibronectin or its nonenzymatically glycated counterpart was diluted approximately 100-fold with cold control fibronectin or nonenzymically glycated fibronectin to give a higher protein concentration. At these latter concentrations of protein, the specific radioactivity of fibronectin was approximately 20 000 cpm/pmol.

Iodination of normal or nonenzymatically glycated fibronectin did not alter its electrophoretic mobility as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis performed in the presence of 2-mercaptoethanol (Laemmli, 1970) with a 5% stacking gel and a 7.5% separating gel. Aliquots of each of these types of fibronectin before and after radioiodination ran only as a M_r 220 000 doublet judged to be at least 96% pure by gel electrophoresis and autoradiography of the dried gel.

Fibronectin Binding to Gelatin or Type IV Collagen. Gelatin, glycated gelatin, type IV collagen, and glycated type IV

collagen were obtained as described above, dialyzed extensively against 0.5 M acetic acid, and lyophilized. The lyophilized proteins were then weighed on an analytical balance and solubilized in 0.5 M acetic acid to yield stock solutions of 1 mg/mL. Nitrocellulose filter spots (6-mm filtration diameter, 0.45-μm porosity) held in place at the bottom of a 96-well microtiter plate (Millititer HA filtration plate, Millipore, Bedford, MA) were each coated at room temperature with 5 μg of gelatin or type IV collagen diluted into PBS buffer (pH 7.4). These molecules, labeled by reductive methylation as described in the text, were used previously to determine that it takes the addition of 10 μg of gelatin or 22 μg of type IV collagen in 50 μL of PBS buffer incubated for 30 min to produce a coating of approximately 5 μg of either molecule. Following coating with gelatin or type IV collagen, the nitrocellulose filters were washed 4 times with 0.2 mL of PBS buffer. Any remaining adsorptive sites were blocked by incubation of each well with 0.2 mL of 5 mg/mL bovine serum albumin (BSA, fatty acid free, Miles Scientific, Elkhart, IN) for 30 min as above to prevent nonspecific binding of labeled ligand to uncoated areas of the filter. The filters were then washed 4 times with PBS buffer as above. Various concentrations (1×10^{-9} to 1×10^{-6} M) of ^{125}I-labeled control fibronectin (Figures 1A and 2A) or ^{125}I-labeled nonenzymatically glycated fibronectin (Figures 1B and 2B) were then incubated in a total volume of 0.2 mL/well for 3 h at room temperature with moderate agitation of the filter plate. The unbound fibronectin was then removed by immediate aspiration and washing of the wells by the gentle addition and aspiration of ten 0.2-mL washes of PBS. The filters were then air-dried with a hair dryer and punched out from the microtiter plate, by a Millititer filter punch (Millipore), and counted in a γ counter. Background binding for each fibronectin concentration was determined as above but with BSA-coated nitrocellulose filters without prior coating with gelatin or type IV collagen. These values were subtracted from the total binding of fibronectin per well to give the specific binding of fibronectin. The specific binding expressed as cpm was converted to moles of fibronectin bound per 0.2 mL of buffer in the wells (and subsequently to molar concentrations of fibronectin bound) by comparison of the specific cpm bound per well obtained with the cpm of standard known amounts of ^{125}I-labeled fibronectin.

Heparin Binding. Heparin binding to fibronectin and/or other basement membrane components was determined in solution in a total volume of 0.2 mL of PBS buffer (pH 7.4) in the wells of a 96-sample polyvinyl chloride microtiter plate (Dynatech, South Windham, ME). Control fibronectin or nonenzymatically glycated fibronectin was added at 50 μg/well (final concentration of 5.7×10^{-7} M) along with various final concentrations of [^{3}H]heparin (1×10^{-8} to 1×10^{-6} M, based on an average M_r of 12000; sp act. 0.14 mCi/mg, New England Nuclear, Boston, MA). Quadruplicate wells were used for each sample condition, and the microtiter plate was incubated for 3 h at room temperature with moderate agitation on a platform shaker. After 3 h, the contents of each well were collected by filtration through a 0.45 μm porosity nitrocellulose sheet held in the bottom of a replicate Millititer HA filtration plate (with the flow valve open and the vacuum operating). Residual samples of associated matrix compounds were transferred from the wells of the polyvinyl chloride microtiter plate to the wells of the nitrocellulose filtration plate with two 0.1-mL PBS washes of the microtiter plate. Each filter spot in the filtration plate was then washed 10 times each with 0.2 mL of PBS (with the flow valve open and the vacuum operating) to remove unbound heparin, since unbound heparin is freely permeable through the filter. After equilibration at room temperature for 10 min, the filter spots were dried and counted, and the specific binding of [^{3}H]heparin was determined for each well as described above.

Table I: Levels of Nonenzymatic Glycation of in Vitro Glycated Proteins

sample[a]	cpm of glycated amino acids/ mg of protein	glycation relative to controls
FN	13874	1.0
g-FN	179734	13.0
type IV collagen	24119	1.0
g-type IV collagen	102437	4.2
gelatin	19443	1.0
g-gelatin	434382	22.3

[a] Samples of each protein that have undergone incubation in vitro at 37 °C with 500 mM D-glucose in PBS buffer for 12 days are designated by the prefix g. The extent of nonenzymatic glycation of control samples that were incubated in PBS buffer as described above but without the addition of D-glucose (no prefix) is also given above.

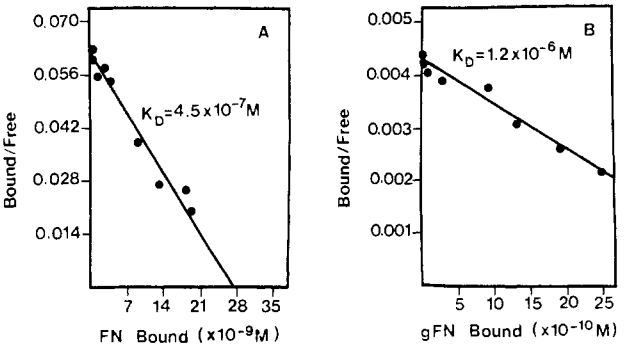

FIGURE 1: Scatchard plots of binding of ^{125}I-labeled control fibronectin (A) and ^{125}I-labeled nonenzymatically glycated fibronectin (B) to gelatin-coated nitrocellulose filters. Each filter disk attached in the bottom of the wells of a 96-well microtiter plate was coated with 5 μg of gelatin. Unbound material was removed by extensive washing, and uncoated areas of the disk were then blocked with 5% (w/v) BSA. The wells were then incubated for 3 h at 25 °C with various concentrations of each ^{125}I-labeled fibronectin sample (1×10^{-9} to 1×10^{-6} M). After incubation, the filters were washed extensively, and the amount of ^{125}I-labeled fibronectin bound to each filter was determined as described under Materials and Methods.

RESULTS

In Vitro Nonenzymatic Glycation. The levels of nonenzymatic glycation of each in vitro glycated protein and its corresponding control are given in Table I. Native type IV collagen glycated in vitro (gIV) was only glycated 4.2-fold greater with respect to its control (IV), while gelatin incubated with excess glucose (g-gelatin) showed a 22.3-fold greater increase in nonenzymatic glycation than its corresponding control (gelatin). Human plasma fibronectin incubated in vitro with excess glucose (gFN) showed a 13.0-fold greater level of nonenzymatic glycation than its control (FN).

Fibronectin Binding to Type IV Collagen or Gelatin. Scatchard analysis of the binding of ^{125}I-labeled control fibronectin (Figure 1A) and ^{125}I-labeled 12-day nonenzymatically glycated fibronectin (Figure 1B) to gelatin-coated filters shows an approximate 2.7-fold reduction in the affinity of binding in comparison to that of controls. The dissociation constant (K_D) for control fibronectin binding to gelatin was 4.5×10^{-7} M, similar to that which has been previously reported (Bing et al., 1982) for the binding of gelatin to human plasma fibronectin immobilized on plastic tubes (K_D = 1.31

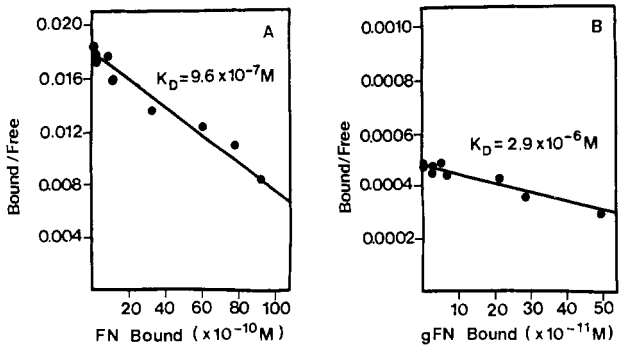

FIGURE 2: Scatchard plots of binding of ^{125}I-labeled control fibronectin (A) and ^{125}I-labeled nonenzymatically glycated fibronectin (B) to type IV collagen coated nitrocellulose filters. Experimental conditions and concentrations were the same as in Figure 1 except that each filter disk was coated with 5 μg of type IV collagen.

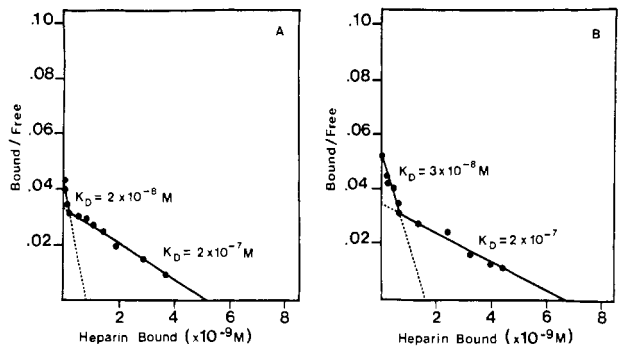

FIGURE 3: Scatchard plots of binding of [^{3}H]heparin in solution to control fibronectin (A) or nonenzymatically glycated fibronectin (B). [^{3}H]heparin was incubated at various concentrations (1 × 10^{-8} to 1 × 10^{-6} M) in sample wells of a microtiter plate containing either 50 μg/well control fibronectin or 50 μg/well nonenzymatically glycated fibronectin in PBS buffer (pH 7.4). After incubation for 1 h at 25 °C, the contents of the sample wells were collected by filtration, and the amount of [^{3}H]heparin bound to fibronectin in each sample well was determined as described under Materials and Methods.

× 10^{-7} M). On the other hand, the K_D for the binding of nonenzymatically glycated fibronectin to gelatin was 1.2 × 10^{-6} M.

Scatchard plots of ^{125}I-labeled fibronectin–type IV collagen interactions show that nonenzymatic glycation of fibronectin (Figure 2B) results in a 3.0-fold reduction in its affinity for type IV collagen in comparison to the binding of control fibronectin to type IV collagen (Figure 2A). The K_D for binding of control fibronectin to type IV collagen was 9.6 × 10^{-7} M while the K_D for the binding of nonenzymatically glycated fibronectin to type IV collagen was 2.9 × 10^{-6} M. An approximate 3-fold reduction in the affinity of fibronectin to gelatin or to type IV collagen due to the nonenzymatic glycation of fibronectin was also shown with ^{3}H-labeled fibronectin molecules (data not shown).

Heparin Binding. Scatchard analysis of the binding of [^{3}H]heparin to control fibronectin (Figure 3A) and to nonenzymatically glycated fibronectin (Figure 3B) produced almost identical plots. In each situation, two binding sites for heparin were found per molecule of fibronectin. One site had K_D of (2–3) × 10^{-8} M while the other site had K_D of 2 × 10^{-7} M with little variance in the affinity of either site for heparin observed between control and nonenzymatically glycated fibronectin (Figure 3, parts A and B). Similar high- and low-affinity sites have been previously reported on fibronectin for heparin (Yamada et al., 1980). There was also very little difference in the number of total extrapolated binding sites for heparin between control and nonenzymatically glycated

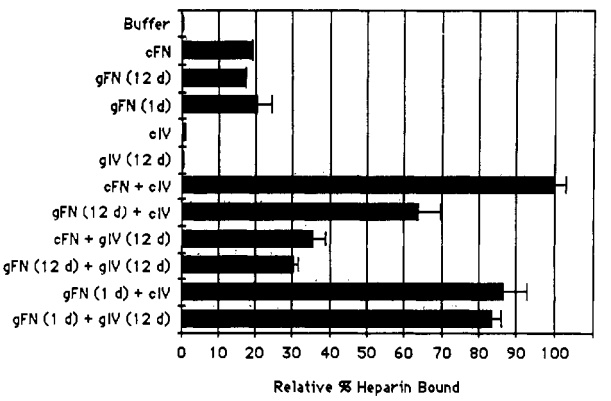

FIGURE 4: Effects of nonenzymatic glycation of fibronectin and type IV collagen on binding of [^{3}H]heparin in solution. Binding assays were done according to Figure 3 but with a constant concentration of [^{3}H]heparin (6 × 10^{-7} M) in the presence of 50 μg/well control fibronectin (cFN) or fibronectin that had been nonenzymatically glycated (gFN) for 1 (1 d) or 12 days (12 d) in vitro, 20 μg/well control type IV collagen (cIV) or 12-day nonenzymatically glycated type IV collagen (gIV), or combinations of these molecules as described to the left of the bars. Values represent the mean ± SD of four separate incubations for each sample and are expressed relative to the maximal binding of [^{3}H]heparin found for sample wells containing both control fibronectin and type IV collagen (100%).

fibronectin (Figure 3, parts A and B).

In another experiment, the effects of nonenzymatic glycation of fibronectin and type IV collagen on [^{3}H]heparin binding were determined. This experiment was conducted as above but with a constant concentration of [^{3}H]heparin (6 × 10^{-7} M) and constant amounts, as indicated in Figure 4, of unlabeled control fibronectin or nonenzymatically glycated fibronectin (at a final concentration of 50 μg/well or 5.7 × 10^{-7} M) or control type IV collagen or nonenzymatically glycated type IV collagen (at a final concentration of 20 μg/well); glycation levels were as indicated in Table I. In addition, in some of the wells, fibronectin that was nonezymatically glycated for 1 day to a level 3.2-fold that of control fibronectin [approximating the level of nonenzymatic glycation of fibronectin found in vivo by Tarsio et al. (1985)] was used in place of 12-day nonenzymatically glycated fibronectin.

Figure 4 shows that the binding of [^{3}H]heparin to the mixture of control (normal) fibronectin and control type IV collagen was the greatest of all the various combinations of molecules (3.1 × 10^{-8} mol of heparin bound/L, or approximately 5.1% of the total heparin input), and this was arbitrarily set at 100% (with an SD of ±2.8). However, [^{3}H]heparin binding to normal fibronectin alone was 19.2 ± 0.6% of the maximal binding of [^{3}H]heparin achieved above when both normal fibronectin and normal type IV collagen were incubated together with [^{3}H]heparin (the maximal level). The level of binding of [^{3}H]heparin to nonenzymatically glycated fibronectin alone (17.2 ± 0.2% of the maximal level) was similar to that found for heparin binding to normal fibronectin alone. In contrast, [^{3}H]heparin bound very poorly to either control or nonenzymatically glycated type IV collagen when each of these molecules was incubated alone with [^{3}H]heparin. These values were between 1 and 2% of the maximal binding. However, when fibronectin and type IV collagen were added concomitantly to the wells, the nonenzymatic glycation of fibronectin resulted in an approximate 36% reduction in the maximal binding of [^{3}H]heparin to 64.2 ± 6.4% of the maximal level. When fibronectin and nonenzymatically glycated type IV collagen were added concomitantly to the wells, there was approximately a 64% decrease in [^{3}H]heparin binding to 35.9 ± 3.5% of the maximal binding. A more substantial

reduction to 30.5 ± 1.6% of the maximal [^{3}H]heparin binding was observed when [^{3}H]heparin was incubated with both fibronectin and type IV collagen that were both nonenzymatically glycated. These results with 12-day nonenzymatically glycated fibronectin and type IV collagen are similar to the findings we have previously reported for 12-day nonenzymatically glycated fibronectin and gelatin (Tarsio et al., 1985). Figure 4 also shows that the binding of [^{3}H]heparin to 1-day nonenzymatically glycated fibronectin (21.0 ± 3.9) is approximately the same as control or 12-day nonenzymatically glycated fibronectin. However, when both 1-day nonenzymatically glycated fibronectin and control type IV collagen are incubated together with [^{3}H]heparin, there is a small but significant reduction in binding to 87.0 ± 6.4% of the maximal binding. A somewhat greater reduction in the binding of [^{3}H]heparin is observed when both 1-day glycated fibronectin and 12-day glycated type IV collagen are incubated together with [^{3}H]heparin (84.1 ± 2.6% of that found for the nonglycated molecules). These latter conditions, where both matrix components are nonenzymatically glycated, more closely approximate what may occur in vivo.

Discussion

In this study, we have nonenzymatically glycated fibronectin, native type IV collagen, and gelatin (denatured calfskin collagen) in vitro by incubation with excess glucose. Although incubated under identical conditions, significantly different increases in levels of nonenzymatic glycation were observed for each of these molecules (Table I). For example, after nonenzymatic glycation in vitro, the fibronectin, type IV collagen, and gelatin samples showed a 13.0-, 4.2-, and 22.3-fold increase, respectively, in final glycated amino acid levels in comparison to those of control samples (incubated without excess glucose). The differences observed for fibronectin, type IV collagen, and gelatin in the final levels of nonenzymatic glycation achieved in vitro by incubation with excess glucose may be due to differences among these proteins in the content of key amino acids, such as lysine and hydroxylysine, available for condensation with glucose. It is also possible that there are differences among these proteins in the accessibility of key amino acids to glucose imposed by various structural constraints of the molecules (Rogozinski et al., 1983; Garlick et al., 1984). Native type IV collagen, for example, is a relatively rigid coil composed of three chains with extensive helical structure except in its noncollagenous domains (portions of the collagen chains not having the amino acid sequence Gly-X-Y) (Timpl et al., 1981). On the other hand, gelatin being denatured would be composed of a mixture of unfolded chains that would be more readily accessible for interaction with glucose (Dessau et al., 1978).

Ligand binding experiments with normal or nonenzymatically glycated proteins were also conducted in this study. Figure 3 shows that there is no difference in the binding of [^{3}H]heparin to fibronectin or to nonenzymatically glycated fibronectin when [^{3}H]heparin is incubated alone with each of these molecules. However, effects of nonenzymatic glycation on [^{3}H]heparin binding become apparent when collagen molecules are concomitantly added with fibronectin (Figure 4). When type IV collagen is added concomitantly with normal fibronectin, there is approximately a 5-fold increase in heparin binding above the levels found for [^{3}H]heparin binding to normal fibronectin alone. This positive cooperative effect is similar to the findings of other investigations made for fibronectin and gelatin (Ruoslahti & Engvall, 1980; Tarsio et al., 1985). The positive cooperative binding of heparin by normal fibronectin and normal type IV collagen is decreased approximately 36% when fibronectin is nonenzymatically glycated, 64% when type IV collagen is nonenzymatically glycated, and 70% when both fibronectin and type IV collagen are nonenzymatically glycated for 12 days in vitro. More importantly, the cooperative binding of heparin by fibronectin and type IV collagen was significantly reduced when either fibronectin or type IV collagen was nonenzymatically glycated to levels approximating those found for these molecules in vivo in diabetic animals (Cohen et al., 1980; Tarsio et al., 1985). Under these conditions, the most significant decrease in binding of [^{3}H]heparin was observed when both protein constituents were nonenzymatically glycated, which is what would be occurring in vivo.

In this study, we use varying concentrations of radiolabeled normal or nonenzymatically glycated fibronectin to assess binding of these fibronectin molecules to nitrocellulose filters coated with collagen molecules. By Scatchard analyses of the binding data we show that nonenzymatic glycation of fibronectin results in a 2.7-fold reduction in affinity between fibronectin and gelatin (Figure 1, parts A and B) and a 3-fold reduction in affinity between fibronectin and type IV collagen (Figure 2, parts A and B). These studies more precisely define the nature of the defects in the ligand binding properties of fibronectin due to nonenzymatic glycation. Previous studies had only utilized fixed concentrations of radiolabeled fibronectin or its nonenzymatically glycated counterpart and showed a reduction in the total amount of fibronectin bound to gelatin immobilized on Sepharose (Cohen & Ku, 1984) or on nitrocellulose filters (Tarsio et al., 1985) due to the nonenzymatic glycation of fibronectin.

These studies also suggest that the first step in the decreased cooperative binding of heparin by fibronectin and gelatin or by fibronectin and type IV collagen due to nonenzymatic glycation of fibronectin is a decrease in the affinity between fibronectin and collagen molecules. In a similar approach, it has been found that polygalactosamine glycosylation of a 44-kDa gelatin binding fragment from human placental fibronectin (an enzymatic glycation in this case) resulted in a 3-fold reduction in its affinity for gelatin (Zhu & Laine, 1985). It was speculated in these studies that the weakened binding affinity of polygalactosamine fibronectin may be due to interference of the gelatin binding site on fibronectin by the proximity of polar saccharide groups on the glycosylated molecule or that the folding of the protein is somehow affected by these adducts. Since either of the collagen molecules used in the above studies profoundly influenced [^{3}H]heparin binding (Figure 4), it is not surprising that a change in the level of interaction of fibronectin with gelatin or in the interaction of fibronectin with type IV collagen due to nonenzymatic glycation will have a disproportionately greater effect on the net amount of heparin bound.

A previous study (Rohrbach et al., 1982) has shown decreased levels of heparan sulfate proteoglycan in basement membrane produced by EHS tumors grown in diabetic mice. The loss of heparan sulfate proteoglycan from the glomerular basement membrane of diabetic animals has also been well documented (Cohen & Surma, 1981; Parthasarathy & Spiro, 1982; Kanwar et al., 1983). Therefore, it is tempting to postulate that the alterations that we have observed in vitro in the molecular association of fibronectin, collagen, and glycosaminoglycans, due to nonenzymatic glycation, are related to these phenomena.

An approximate 2–3-fold increase in the level of nonenzymatic glycation of glomerular basement membrane type IV collagen in streptozotocin-diabetic rats 3–4 weeks after the

induction of diabetes has been reported (Cohen et al., 1980). This level of nonenzymatic glycation in relatively short-term diabetic animals is only slightly lower than the in vitro level of nonenzymatic glycation of type IV collagen that was used in studies reported here. At present, the levels of nonenzymatic glycation of fibronectin associated with the glomerular basement membranes of diabetic animals or humans are not known. However, we have found that fibronectin in the plasma of short-term alloxan-diabetic dogs is nonenzymatically glycated 2–3-fold above that of control animals (Tarsio et al., 1985). Although Scatchard analyses were not done, this level of nonenzymatic glycation of fibronectin was sufficient to perturb the association of fibronectin with gelatin at fixed concentrations (Tarsio et al., 1985) or the association of fibronectin and type IV collagen with heparin (Figure 4). An accumulation of fibronectin in the mesangial matrix and glomerular capillary walls in the kidneys of human diabetics has been reported (Scheinman et al., 1981). A slower turnover of glomerular fibronectin in diabetics may therefore result in significantly higher levels of nonenzymatic glycation of this fibronectin than that of plasma fibronectin, but this remains to be proven. If alterations in the molecular association of glomerular fibronectin with type IV collagen and heparan sulfate proteoglycan do occur in vivo due to nonenzymatic glycation, this may substantially affect the integrity of the glomerular basement membrane filtration barrier. Specifically, a loss of heparan sulfate proteoglycan, due at least in part to reduced affinity with nonenzymatically glycated molecules, may decrease the anionic charge of the glomerular basement membrane and affect its charge selectivity. The decrease in glomerular basement membrane heparan sulfate proteoglycan may also be a precursor to basement membrane thickening. This could be due to compensatory glomerular basement membrane synthesis or perhaps trapping of plasma proteins by the altered glomerular basement membrane. The change in the molecular association of basement membrane or extracellular matrix component constituents reported here, though perhaps subtle, may have profound functional effects. Further work therefore will be necessary to discern the relationship between these biochemical abnormalities and functional alterations that may occur in poorly controlled diabetes mellitus.

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Altered Expression of Glycosaminoglycans in Metastatic 13762NF Rat Mammary Adenocarcinoma Cells†

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ABSTRACT: A difference in the expression and metabolism of sulfated glycosaminoglycans between rat mammary tumor cells derived from a primary tumor and those from its metastatic lesions has been observed. Cells from the primary tumor possessed about equal quantities of chondroitin sulfate and heparan sulfate on their cell surfaces but released fourfold more chondroitin sulfate than heparan sulfate into their medium. In contrast, cells from distal metastatic lesions expressed approximately 5 times more heparan sulfate than chondroitin sulfate in both medium and cell surface fractions. This was observed to be the result of differential synthesis of the glycosaminoglycans and not of major structural alterations of the individual glycosaminoglycans. The degree of sulfation and size of heparan sulfate were similar for all cells examined. However, chondroitin sulfate, observed to be only chondroitin 4-sulfate, from the metastases-derived cells had a smaller average molecular weight on gel filtration chromatography and showed a decreased quantity of sulfated disaccharides upon degradation with chondroitin ABC lyase compared to the primary tumor derived cells. Major qualitative or quantitative alterations were not observed for hyaluronic acid among the various 13762NF cells. The metabolism of newly synthesized sulfated glycosaminoglycans was also different between cells from primary tumor and metastases. Cells from the primary tumor continued to accumulate glycosaminoglycans in their medium over a 72-h period, while the accumulation of sulfated glycosaminoglycans in the medium of metastases-derived cells showed a plateau after 18–24 h. A pulse–chase kinetics study demonstrated that both heparan sulfate and chondroitin sulfate were degraded by the metastases-derived cells, whereas the primary tumor derived cells degraded only heparan sulfate and degraded it at a slower rate. These results suggested that altered glycosaminoglycan expression and metabolism may be associated with the metastatic process in 13762NF rat mammary tumor cells.

The dissemination of tumor cells to distant sites involves a multitude of complex interactions between the tumor cells and the host environment (Nicolson & Poste, 1983; Nicolson, 1984). An essential aspect of the metastatic process is the tumor cell's ability to migrate and invade the surrounding host tissue. Although the exact mechanisms of cellular locomotion are unknown, the process is thought to consist of a coordinate sequence of cell adhesions and dissociations (Heaysman, 1978; Weiss & Ward, 1983). Proteoglycans and glycosaminoglycans (GAGs)[1] have been proposed to play key functional roles in the adhesive and migratory properties of cells (Lark & Culp, 1982, 1983, 1984; Hook et al., 1984). In addition to modulating its own matrix, malignant cells have been shown to degrade the host's surrounding basal laminae (Jones, 1979; Sloane et al., 1981; Kramer et al., 1982; Nakajima et al., 1984).

A number of investigations have focused on qualitative and quantitative modulation of GAGs between transformed or tumor cells and their normal counterparts [reviewed by Kraemer (1979) and Hook et al. (1984)]. Differential expression of GAGs has been observed in virally or chemically transformed cells (Underhill & Toole, 1982; Angello et al., 1982a; Shanley et al., 1983). In addition, structural heterogeneity of GAGs has also been demonstrated in several transformed cells and hepatomas, in which heparan sulfate (HS) was shown to have a lower degree of sulfation than normal cells (Underhill & Keller, 1975; Keller et al., 1980; Winterbourne & Mora, 1981; Nakamura & Kajima, 1981; Robinson et al., 1984). The decreased sulfated HS was shown to be synthesized by mouse embryo clones with greater tu-

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[1] Abbreviations: GAG, glycosaminoglycans; HS, heparan sulfate; CS, chondroitin sulfate; HA, hyaluronic acid; AMEM, α-modified minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; TRU, turbidity-reducing units.

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