Inhibition of Fibronectin Binding to Matrix Components by Nonenzymatic Glycosylation

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SUMMARY

We examined the effect of nonenzymatic glycosylation on the ability of fibronectin, an extracellular glycoprotein that interacts with cell surfaces and matrix components, to bind to collagen and heparin. Nonenzymatic glycosylation was accomplished by incubation of the protein with glucose, both cold and [^{14}C]-labeled, and documented by measurement of ketoamine-bound carbohydrate with the thiobarbituric acid assay. Effect on binding was assessed with affinity chromatography on heparin-Sepharose and gelatin-Sepharose, and with an in vitro assay that detects complexation of fibronectin with [^{3}H]-heparin. Glycosylated fibronectin did not bind to these immobilized matrix components, and in vitro binding of the glycosylated protein was reduced compared with that of nonglycosylated fibronectin. Inhibition of heparin binding in the in vitro assay was observed even with levels of glycosylation about threefold those of control, which is comparable to the degree of glycosylation determined in fibronectin isolated from plasma of two patients with uncontrolled diabetes. The findings indicate that nonenzymatic glycosylation of fibronectin inhibits its binding to connective tissue components, and suggest that this process contributes to faulty integrity of extracellular matrices in diabetes. DIABETES 1984; 33:970–74.

Nonenzymatic glycosylation of proteins has received increasing attention in recent years in view of the recognition that this process is increased in patients with diabetes and attendant hyperglycemia, that it may alter physicochemical properties of involved proteins, and that it could consequently contribute to the pathogenesis of certain chronic complications of diabetes (for reviews, see refs. 1–3). Many of these complications involve extracellular matrices; in particular, the typical lesions of diabetic microangiopathy affect basement membranes, resulting in their accumulation and defective organization, and in functional alterations associated with increased permeability of the capillary filtration barriers. Increased nonenzymatic glycosylation of basement membranes in diabetes has been described,[4,5] and there is some evidence that excess glycosylation effects cross-linking of this extracellular matrix.[6,7] Information on the impact of nonenzymatic glycosylation on structure-function relationships of specific extracellular matrix components is scant, however.

In the present investigation, we examined the effect of nonenzymatic glycosylation on the ability of fibronectin, an extracellular glycoprotein that interacts with cell surfaces and with other matrix components (see ref. 8) to bind to denatured collagen (gelatin) and glycosaminoglycans (heparin). These studies were prompted by the above considerations, coupled with recognition of the essential role that fibronectin binding to extracellular matrix components plays in its attachment and biologic function.

MATERIALS AND METHODS

Fibronectin was purified from human plasma by described techniques,[9] or was purchased commercially (Collaborative Research, Waltham, Massachusetts). For some experiments, fibronectin was the generous gift of Dr. Deane Mosher. The protein was either used directly after solubilization in 0.1 M cyclohexylaminopropane sulfonic acid (CAPS) buffer, pH 11.0, or nonenzymatically glycosylated with unlabeled or [^{14}C]-glucose. Nonenzymatic glycosylation was performed by incubating fibronectin for varying times at room temperature in 10 ml of phosphate-buffered saline (PBS) containing 0.02% sodium azide and made 10–40 mM in glucose. For preparation of [^{14}C]-labeled glycosylated fibronectin, the incubation contained 25 μCi of U-[^{14}C]-glucose, 9mCi/mM (New England Nuclear, Boston, Massachusetts), in PBS made 40 mM in unlabeled glucose, giving a specific activity of 62.5 μCi/mmol in the reaction mixture. The material was

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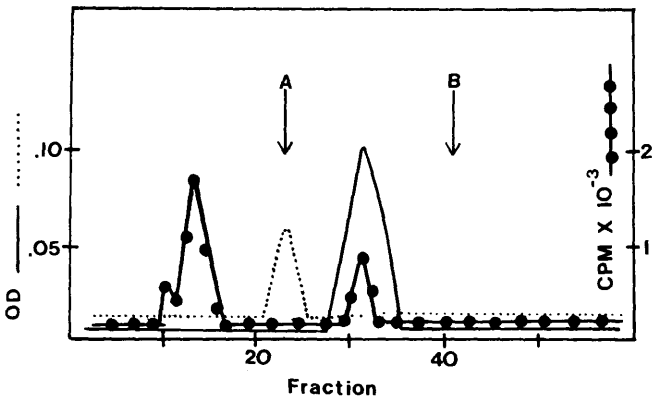

FIGURE 1. Gel filtration on Sepharose 4B of fibronectin monomer (——), [^{14}C]-glycosylated fibronectin (110 nmol HMF/mg protein) (●——●), and unlabeled glycosylated fibronectin (15 nmol HMF/mg protein) (·····). Elution positions of fibrinogen (A) and albumin (B) are marked with arrows. Fractions of 5 ml were collected and monitored for absorbances at 280 nm or for radioactivity in a liquid scintillation counter. Void volume was in fraction 11.

dialyzed against several changes of dilute PBS (for chromatography) or dilute CAPS buffer (for in vitro heparin binding) and, in the case of radiolabeled fibronectin, until the dialysates were free of radioactivity. The glycosylated preparations were clarified by centrifugation and the supernatants used for further analysis. Analysis included measurement of the level of nonenzymatic glycosylation by the thiobarbituric acid assay for ketoamine-bound carbohydrate,[10] gel filtration, and affinity chromatography. Glycosylated fibronectin prepared with unlabeled glucose was used to establish the relation between time and glucose concentration on nonenzymatic glycosylation, for chromatographic studies, and for the in vitro assay to detect complexation of fibronectin with [^{3}H]-heparin (vide infra). [^{14}C]-glycosylated fibronectin was used for chromatographic studies.

Gel filtration was performed on Sepharose 4B, equilibrated, and eluted with PBS, pH 7.2. Affinity chromatography on heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, New Jersey) essentially followed published procedures that describe the binding of fibronectin to these immobilized matrix components.[11,12] For heparin-Sepharose chromatography, a 2 × 10-cm column was equilibrated in and eluted with PBS, followed by stepwise elution with 0.3 M, 0.5 M, and 1 M NaCl. For gelatin-Sepharose chromatography, a 0.8 × 8-cm column was equilibrated in and eluted with PBS, followed by stepwise elution with 0.5 M, 1 M, 2 M, 4 M, and 8 M urea in 0.05 M Tris buffer, pH 7.5. Eluants were monitored for absorbance at 280 nm and, when radiolabeled, for [^{14}C]-radioactivity in a liquid scintillation counter.

In vitro binding of fibronectin to heparin was assayed essentially as described by Yamada et al.[13] Control or unlabeled glycosylated fibronectin at a concentration of 0.5–1.4 μM (based on fibronectin monomer of Mr ≈ 220,000) was incubated in 0.1 ml of PBS supplemented with an additional 10 mM sodium phosphate, pH 7.4, and varying amounts of [^{3}H]-heparin (0.332 mCi/mg, New England Nuclear). After 60 min at room temperature, the samples were briefly agitated, then quickly vacuum filtered through 0.45-μ nitrocellulose (Millipore) filters. The filters were washed 3 times with 3 ml of PBS, placed in vials containing Aquasol, and counted. Control incubations containing [^{3}H]-heparin without fibronectin were simultaneously conducted with each assay. Results were corrected for nonspecific adherence of [^{3}H]-heparin to the nitrocellulose filter by subtracting counts obtained with [^{3}H]-heparin alone from total counts obtained with fibronectin plus [^{3}H]-heparin at each concentration of radiolabeled heparin.

RESULTS

Incubation of fibronectin with 10–40 mM glucose for 1–4 days resulted in the formation of glycosylated fibronectin that was dependent on time and glucose concentration. The level of glycosylation, determined by the thiobarbituric acid assay, ranged from 2 to 110 nmol hydroxymethylfurfuraldehyde (HMF)/mg protein at 1 day in 10 mM glucose and 4 days in 40 mM glucose, respectively. Visible precipitates appeared in the latter preparations, suggesting the formation of insoluble aggregates at neutral pH. After dialysis, the glycosylated protein in the soluble material eluted on Sepharose 4B gel filtration correspondent with an estimated mol wt > 400,000, consistent with fibronectin multimers. [^{14}C]-

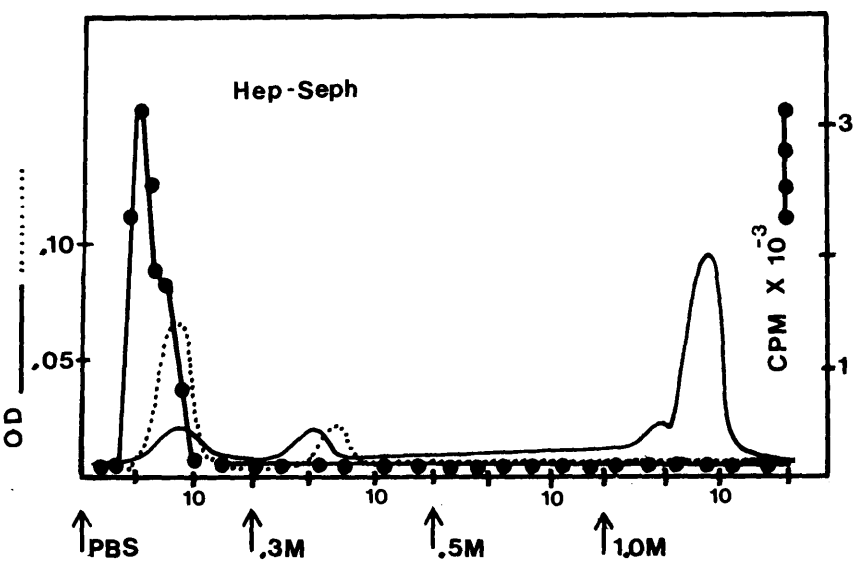

FIGURE 2. Affinity chromatography on heparin-Sepharose of purified fibronectin (——), unlabeled glycosylated fibronectin (·····), and [^{14}C]-glycosylated fibronectin (●——●). Fractions of 1 ml were collected and monitored for absorbance at 280 nm or for radioactivity in a liquid scintillation counter. Arrows indicate start of each stepwise change in eluting solution: PBS, 0.3 M NaCl, 0.5 M NaCl, and 1.0 M NaCl.

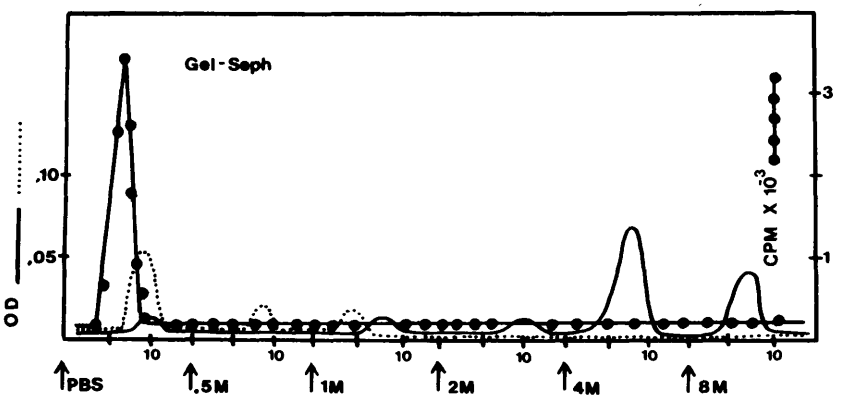

FIGURE 3. Affinity chromatography on gelatin-Sepharose of purified fibronectin (——), unlabeled glycosylated fibronectin (·····), and [^{14}C]-glycosylated fibronectin (●——●). Fractions of 1 ml were collected and monitored for absorbance at 280 nm or for radioactivity in a liquid scintillation counter. Arrows indicate start of each stepwise change in eluting solution: PBS, and 0.5 M, 1.0 M, 2.0 M, 4.0 M, and 8.0 M urea in 0.05 M Tris buffer, pH 7.5.

glycosylated fibronectin incubated for 4 days in 40 mM glucose had a specific activity of 700 cpm/μg, and most of the radioactivity eluted as multimers on Sepharose 4B gel filtration (Figure 1). That the radioactivity in the [^{14}C]-labeled material represented nonenzymatic glycosylation and not covalent binding of radiolabeled impurities in the radioactive glucose preparation[14] was corroborated by the demonstration of thiobarbituric acid reaction products; when compared with results with this assay, however, [^{14}C] incorporation overestimated the degree of nonenzymatic glycosylation by a factor of 5–10-fold, as has been previously reported.[14,15] With lesser degrees of nonenzymatic glycosylation (≈15 nmol HMF/mg protein), fibronectin eluted as dimer of mol wt ≈ 400,000 (Figure 1).

Affinity chromatography of purified fibronectin on heparin-Sepharose produced the expected elution profile, with adsorption of the protein to the glycosaminoglycan gel and elution of most of the bound material with 1 M NaCl[12,13] (Figure 2). Glycosylated fibronectin, in contrast, did not bind to heparin-Sepharose; all of the radioactivity in [^{14}C]-glycosylated fibronectin and the absorbance of unlabeled glycosylated fibronectin appeared in the initial PBS wash (Figure 2). The level of glycosylation in these preparations was 110 and 75 nmol HMF/mg protein, respectively.

Binding of fibronectin to gelatin-Sepharose was also inhibited by nonenzymatic glycosylation. As shown in Figure 3, purified fibronectin adsorbed to the gelatin and was largely eluted with 4 M urea, with residual adsorbed protein eluting with 8 M urea.[11,16] In contrast, glycosylated fibronectin did not bind to the gelatin, and all of the radioactivity ([^{14}C]-glycosylated) and most of the absorbance (nonradiolabeled glycosylated preparation) appeared in the initial PBS wash (Figure 3). The degree of glycosylation in these samples was the same as that in the material applied to heparin-Sepharose.

Inhibition of fibronectin binding to the glycosaminoglycan heparin as a result of nonenzymatic glycosylation was further confirmed with the in vitro binding assay, which takes advantage of the protein's relative insolubility at neutral versus alkaline pH. The complexation product formed between fibronectin and [^{3}H]-heparin incubated at neutral pH is retained on the nitrocellulose filters; since [^{3}H]-heparin alone is not retained, binding to heparin can be detected by measuring the radioactivity on the filters.[13] We found that a small but significant amount of radioactivity, proportional to the amount added, of free [^{3}H]-heparin was retained by the nitrocellulose filters and, thus, subtracted this from total counts retained with fibronectin plus [^{3}H]-heparin at each concen-

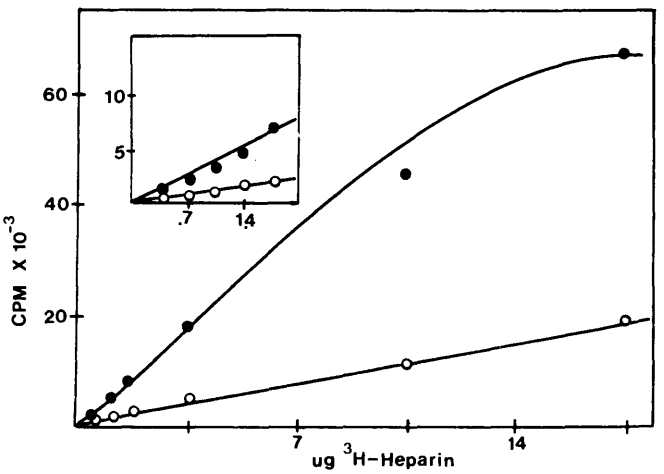

FIGURE 4. In vitro assay of [^{3}H]-heparin binding to purified human fibronectin (●——●) and glycosylated human fibronectin that had been incubated for 3 days in 20 mM glucose (○——○). The glycosylated fibronectin was dialyzed against 0.05 CAPS buffer, and contained 9 nmol HMF/mg protein. Results represent duplicate determinations at each concentration of [^{3}H]-heparin and are corrected for nonspecific adherence of the radiolabel to the nitrocellulose filters. Incubations were performed in 0.1 ml containing 15 μg (inset) or 30 μg of either control or glycosylated fibronectin and [^{3}H]-heparin in the amounts indicated.

TABLE 1
[^{3}H]-heparin binding to glycosylated fibronectin

Fibronectin	[^{3}H]-heparin μg Added	[^{3}H]-heparin cpm Bound	% Inhibition
Control	3.45	10,145 ± 1120	—
Glycosylated	3.45	8013 ± 630	21.0
Control	10.35	23,432 ± 2055	—
Glycosylated	10.35	17,055 ± 1445	27.2

In vitro assay of [^{3}H]-heparin binding to purified human fibronectin. Control experiments were performed with nonglycosylated fibronectin that had been incubated for 3 days in PBS without added glucose; the glycosylated fibronectin contained 3.1 nmol HMF/mg protein. Results represent mean ± SEM of four separate assays for each fibronectin preparation with each concentration of heparin noted. Results are given as cpm bound, and have been corrected for nonspecific adherence of radiolabel to the nitrocellulose filters.

tration of the radiolabeled glycosyaminoglycan. The specificity of [^{3}H]-heparin binding to fibronectin was corroborated by demonstrating its inhibition in the presence of cold heparin 1 mg/ml; cold hyaluronic acid (1 mg/ml) did not inhibit [^{3}H]-heparin binding. With fixed concentration of fibronectin, specific binding was proportional to the amount of [^{3}H]-heparin added (Figure 4). Binding of [^{3}H]-heparin to glycosylated fibronectin was about 25–50% of that with the nonglycosylated protein at every concentration of radiolabeled glycosaminoglycan that was examined. This effect was initially observed with fibronectin glycosylated at a level of 75–100 nmol HMF/mg protein; subsequent experiments confirmed that [^{3}H]-heparin binding was similarly inhibited at levels of glycosylation as low as 9 nmol HMF/mg protein (Figure 4). Control incubations with nonglycosylated fibronectin for these experiments were conducted with purified fibronectin that had been incubated for 4 days at room temperature in PBS to which glucose had not been added.

To probe whether glycosylation of fibronectin that occurs in vivo is sufficient to have similar physiologic effect, we isolated fibronectin from normal and diabetic human plasma, the latter obtained from 2 patients who had blood glucose concentrations in excess of 400 mg/dl for several days. The degree of glycosylation in the diabetic samples was 2–3-fold that of control (3.6 and 2.5 versus 1.1 and 1.2 nmol HMF/mg protein, respectively). We then assessed the ability of fibronectin glycosylated in vitro to a level of 3 nmol HMF/mg protein to bind to [^{3}H]-heparin, using the nitrocellulose assay described above. Binding was significantly reduced in these preparations compared with control (Table 1).

DISCUSSION

Fibronectin is one of several extracellular glycoproteins that interact with cell surfaces and with each other to influence cell growth, development, and adhesion, and to modulate biologic properties of extracellular matrices.[8] It is present in large amounts in plasma as a soluble protein, and in an insoluble form at cell surfaces. The two forms are similar but not identical, and contain subunits of approximately 220,000 daltons that are joined by disulfide bonds into dimers and multimers.[17–20] Fibronectin is synthesized by a variety of cells, including fibroblasts and endothelial cells,[21–24] and it has been demonstrated by immunoflourescent techniques in the connective tissue matrix[25,26] and in basement membranes.[26,27] Although it is not clear whether fibronectin that has been found in glomerular basement membrane derives from local production[24,28,29] or from entrapment of circulating fibronectin,[30] it is believed that the protein exerts ligand binding and biologic activities in this matrix that are similar to those that it intrinsically possesses and exhibits in other tissue sites.[29] This includes cell-substratum adhesion, binding to collagen and glycosaminoglycans, and attachment of cells to extracellular matrix (reviewed in ref. 8). Indeed, the results of a recent study directly suggest a role for fibronectin in glomerular cell adhesion.[31]

The findings reported herein indicate that nonenzymatic glycosylation of fibronectin inhibits its ability to bind to gelatin, which is essentially denatured collagen, and to the glycosaminoglycan heparin. This inhibition was demonstrated by using the affinity chromatography techniques that have been traditionally employed to examine interactions of extracellular molecules with substrates. An in vitro binding assay that measures fibronectin-heparin complexation confirmed that nonenzymatic glycosylation compromises the ability of fibronectin to bind to this glycosyaminoglycan. It is likely that glycosylation of lysine residues, which appear necessary for binding to gelatin,[8] and probably the negatively charged glycosaminoglycan as well, is responsible for these effects. Since basement membranes contain collagenous proteins[32] and glycosaminoglycans, of which heparan sulfate is the principal species,[33,34] these findings are of interest with respect to the impact of diabetes on organizational features of basement membranes. The interaction of fibronectin with basement membrane components, which may play a role in maintaining the integrity of the microvascular matrix, would be compromised as a result of increased nonenzymatic glycosylation of this protein in diabetes. Although the degree of excess glycosylation in vivo observed in samples isolated from plasma of diabetic patients was modest, this level was nevertheless associated with inhibition of binding to heparin when examined in vitro. It should be noted that circulating fibronectin has a relatively short half-life, whereas tissue fibronectin may be incorporated into the extracellular matrix and have a longer half-life.[35] Thus, measurement of the level of nonenzymatic glycosylation in the circulating form may underestimate the extent of this reaction in tissue sites, where cumulative glycosylation with exposure to a hyperglycemic milieu would further impact on the protein's matrix-binding properties.

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