Nonenzymatic Glycosylation Reduces the Susceptibility of Fibrin to Degradation by Plasmin

MICHAEL BROWNLEE, HELEN VLASSARA, AND ANTHONY CERAMI

SUMMARY

The effect of nonenzymatic glycosylation on the susceptibility of fibrin to degradation by the specific fibrinolytic enzyme plasmin was evaluated using both a fibrin plate assay and a fluorogenic synthetic plasmin substrate assay. Data from both types of experiments demonstrate that nonenzymatic glycosylation reduces the susceptibility of fibrin to plasmin degradation. Acetylation and carbamylation have qualitatively similar effects, indicating that chemical modification of lysine amino groups is the underlying phenomenon responsible for the observed degradative defect produced by glucose. Experimental conditions that increased the rate of nonenzymatic protein glycosylation (higher monosaccharide concentration, glucose-6-phosphate) were associated with correspondingly greater degrees of resistance to degradation by plasmin. Such reduced degradation of nonenzymatically glycosylated proteins in vivo may contribute to the accumulation of fibrin and several other proteins observed in those tissues most frequently affected by the complications of diabetes. DIABETES *32*:680–684, July 1983.

Accumulation of fibrin has been reported to occur in those diabetic tissues most affected by long-term complications, prompting speculation that abnormal fibrin turnover plays a significant role in the pathogenesis of vascular damage in kidney, retina, nerve, and artery.[1–4] Currently, the sequence of events leading from abnormal glucose homeostasis to fibrin accumulation and vascular damage is not known. A diabetes-associated hypercoagulable state has been suggested by the observation that disappearance of radioiodinated autologous fibrinogen is accelerated in hyperglycemic diabetic patients,[5] but interpretation of these data is complicated by associated changes in vascular permeability and by the existence of nonspecific fibrinogen removal pathways.[6,7] Clinical clotting assays such as prothrombin time, activated partial thromboplastin time, and thrombin time are normal in both hyper- and euglycemic diabetic patients.[5] Activity of the fibrinolytic system has also been examined in diabetic patients. Release of plasminogen activator from vascular endothelium and euglobulin lysis time have both been reported to be abnormal in some cases,[8,9] but decreased fibrinolytic activity has not been consistently observed by all investigators.[10]

In the studies described in this report, we have evaluated the effect of nonenzymatic glycosylation on the susceptibility of fibrin to degradation by the specific fibrinolytic enzyme plasmin. Excessive nonenzymatic glycosylation resulting from sustained hyperglycemia has been reported to occur in vivo in a variety of proteins, including fibrinogen.[11,12] Lysine residues, with their free ε-amino groups, are the major sites of this glucose attachment to proteins.[11] Lysine residues are also centrally involved in the fibrinolytic process, with plasmin hydrolysis of deposited fibrin occurring specifically at arginyl- and lysyl-peptide bonds.[13] Using both the fibrin plate assay and a fluorogenic synthetic plasmin substrate assay, we have found that glucose blocking of the ε-amino group of lysines in the fibrin molecule interferes with the specific fibrinolytic enzyme–substrate interaction. Defective fibrin degradation induced by excessive nonenzymatic glycosylation in vivo could lead to the fibrin accumulation observed in various diabetic tissues.

METHODS AND MATERIALS

Purification of fibrinogen. Fibrinogen (Sigma, St. Louis, Missouri) was purified to remove contaminating plasminogen and serum activators and inhibitors of fibrinolysis, using a modification of the method of Laki.[14] Fibrinogen, dialyzed against 0.6 M sodium chloride, was further purified by resuspension in sodium phosphate–lysine buffer and reprecipitated with 95% ethanol according to the method of Mosesson et al.[15] Fibrinogen was then dialyzed against a 0.3-M sodium chloride solution, filtered through a 0.2-μm Millipore,

From the Laboratory of Medical Biochemistry, Rockefeller University, 1230 York Avenue, New York, New York 10021.
Address reprint requests to Michael Brownlee, M.D., at the above address.
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and stored frozen for future use after protein determination by the Bradford method.[16] Aliquots of fibrinogen were radioiodinated using the ICl method of Helmkamp et al.[17] Ten millicuries of carrier-free ^{125}I was used to iodinate 20 mg fibrinogen.

In vitro glycosylation of fibrinogen. Iodinated fibrinogen (0.1 mg/ml, 2.0 × 10^6 cpm/mg) was incubated for 21 days at 23°C in phosphate-buffered saline, pH 7.4, 1 mM sodium azide with (1) no additives (negative control), (2) the nonreactive sugar alcohol sorbitol (positive control), (3) glucose (two concentrations), and (4) glucose-6-phosphate (two concentrations). At the end of the incubation period, each sample was dialyzed to remove the unreacted sugar, and then plated in a multi-well tissue culture plate (1.7 cm × 1.6 cm) in 500-μl aliquots for a standard fibrin plate assay.[18] 1.0 × 10^5 cpm per well were plated and all saline washes from the fibrin plate were counted to determine the total amount of fibrin remaining in each well at the beginning of the assay. The assays were carried out using 500 μl of 0.1 M Tris buffer, pH 8.1, containing 0.5 mU urokinase and 2.4 μg plasminogen. At each time point in the assay, 50-μl aliquots were taken, and the percentage of fibrin cpm solubilized by the action of plasmin was determined. Additional control fibrin plate assays were performed using nonglycosylated substrate and urokinase/plasminogen solutions made 500 mM glucose, glucose-6-phosphate, or sorbitol, immediately before pipetting into the wells.

In vitro glycosylation of activated fibrin plates. Activated nonglycosylated fibrin plates were also incubated with buffer containing 500 mM glucose, 500 mM glucose-6-phosphate, and no added sugars in order to allow detection of potentially significant glycosylation differences resulting from conformational changes in the fibrinogen-to-fibrin conversion process. Fibrin plate assays were performed on these samples after incubation at 37°C for 3 wk and 6 wk.

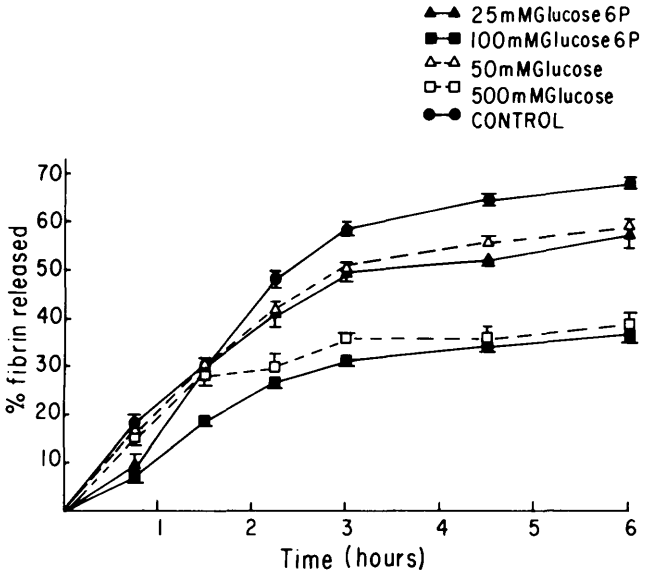

FIGURE 1. Comparisons of the rates of degradation by plasmin of control fibrin and glycosylated fibrin. Fibrinogen was incubated under the indicated conditions for 3 wk as described in METHODS AND MATERIALS. Control fibrinogen was incubated in buffer alone. Each point represents the mean ± SEM of 24 determinations.

Plasminogen preparation. Plasminogen was prepared from fresh human plasma according to the method of Strickland et al.[19] Fibrinogen was removed using a 20% $(NH_4)_2SO_4$ precipitation followed by chromatography on a lysine-sepharose column equilibrated with phosphate-buffered saline, pH 7.3, at 4°C. This column was washed with 0.3 M potassium phosphate buffer, pH 7.3, to remove unwanted protein, and plasminogen was then eluted with 0.2 M ε-amino caproic acid in 0.1 M KPO_4 buffer, pH 7.3. Purified plasminogen was obtained by a final precipitation with 40% $(NH_4)_2SO_4$.

Glycosylation of fluorogenic plasmin substrate. The synthetic fluorogenic peptide substrate for plasmin, t-Boc-Glu-Lys-Lys-methylcoumarin amide (Vega Biochemicals, Albuquerque, New Mexico) was dissolved in dimethylsulfoxide to a final concentration of 10.8 mM. TES buffer (0.05 M, pH 7.5) with 1 mM NaN_3 was added to give a final concentration of 5% DMSO. Aliquots were made 500 mM with glucose or glucose-6-phosphate, and incubated at 37°C for 3 wk and 6 wk.

Acetylation and carbamylation of fluorogenic plasmin substrate. Five milligrams of fluorogenic plasmin substrate were dissolved in 0.5 ml DMSO, and 1.5 mg of 0.05 M TES buffer, pH 7.5, was added. Acetic anhydride (1.5:1 molar ratio) was added slowly with stirring on ice. Samples were stirred for an additional 30 min and diluted for assay. The pH was adjusted to 7.5 with sodium hydroxide. Carbamylation was accomplished by adding NaNCO to the standard substrate incubation at a final concentration of 200 mM. Unreacted cyanate was removed by lowering the pH to 1.5 with HCl with stirring on ice. After standing 1 h, the solution was retitrated to a pH of 7.4 with sodium hydroxide and assayed.

Spectrofluorometric assay of fibrinolytic activity. One hundred microliters of each incubation of the synthetic substrate was diluted to 3.0 ml with 0.05 M TES, 5% DMSO, pH 7.5. Plasminogen (25 μl, 1 mg/ml) was added and fluorescence was monitored continuously by using a Perkin-Elmer 204A spectrofluorometer (Perkin-Elmer, Norwalk, Connecticut) equipped with a chart recorder. Excitation and emission wavelengths (383 nm and 440 nm, respectively) were chosen such that free amino methylcoumarin gave a relative fluorescence 500-fold greater than that of an equivalent amount of unhydrolyzed fluorogenic substrate.[20] The instrument was standardized daily using subdilutions of a 10-mM amino methylcoumarin solution and a calibration curve was constructed. Chart speed was 5 cm/h, sensitivity = 5, selector = 1/10 with 0.01 V full scale. Once a stable baseline was achieved, 100 μl of 100 mU/ml urokinase (10 mU) was added and the rate of substrate hydrolysis was determined for 1 h. Results were calculated and expressed as nanomoles methylcoumarin amide released per minute.

RESULTS

In vitro glycosylation of fibrinogen. Fibrin plates prepared from fibrinogen that had been previously incubated with sugars consistently showed a significant decrease in susceptibility to degradation by the specific fibrinolytic enzyme plasmin (Figure 1). Higher incubation concentrations of monosaccharide were associated with greater resistance to degradation by plasmin. Incubation with glucose-6-phosphate inhibited the subsequent release of soluble fibrin degradation products more efficiently than did incubation with

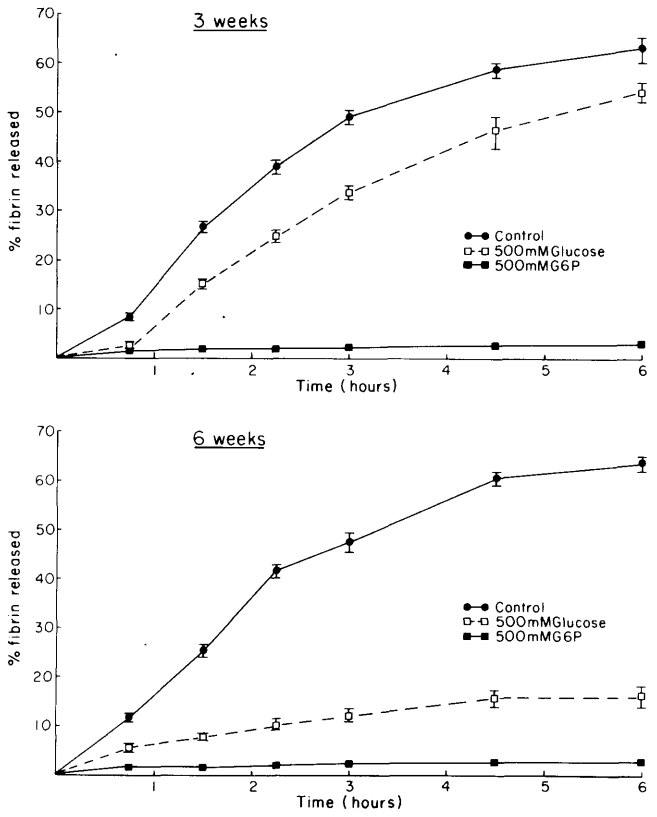

FIGURE 2. Comparison of the rates of degradation by plasmin of control fibrin and glycosylated fibrin. Activated fibrin plates were incubated under the indicated conditions as described in METHODS AND MATERIALS. Control fibrin plates were incubated in buffer alone. Each point represents the mean ± SEM of 24 determinations. Assays were performed after 3 wk of incubation (upper panel) and after 6 wk of incubation (lower panel).

glucose. The rate of nonenzymatic protein glycosylation has previously been shown to increase significantly when glucose-6-phosphate is substituted for glucose in equimolar concentrations.[21,22] Incubations of fibrinogen with identical concentrations of the nonreactive sugar-alcohol sorbitol had no effect on subsequent plasmin degradation in the fibrin plate assay. The percent of fibrin released at each timepoint was identical to buffer controls. Addition of sugars to nonglycosylated fibrin plates immediately before assay likewise had no effect (data not shown).

In vitro glycosylation of activated fibrin plates. Assays performed using fibrin plates prepared from nonglycosylated fibrinogen and subsequently incubated with 500 mM glucose or 500 mM glucose-6-phosphate showed resistance to plasmin degradation similar to that observed when fibrinogen was glycosylated in solution (Figure 2). High levels of nonenzymatic glycosylation achieved by glucose-6-phosphate incubation resulted in essentially complete resistance of fibrin to enzymatic degradation by 3 wk. The less highly glycosylated fibrin plates resulting from incubation with equimolar concentrations of glucose showed a reduction in susceptibility to fibrinolysis that was correspondingly less (Figure 2, upper panel). Longer incubation time was associated with greater resistance to degradation by plasmin (Figure 2, lower panel). The quantitative differences between changes in susceptibility to plasmin degradation observed when sugars are incubated with activated fibrin plates rather than with fibrinogen in solution may reflect significant glycosylation differences resulting from conformational changes in the fibrinogen-to-fibrin conversion process.

TABLE 1
Fluorometric assay for plasmin activity

Substrate	AMC released (nmol/min)	Percent of control
Control	0.360	100%
500 mM Glucose	0.150	42%
100 mM Glucose 6-P	0.110	31%
500 mM Glucose 6-P	0.042	12%
Acetylated	0.013	3.6%
Carbamylated	0.026	7.2%

Glycosylation of fluorogenic plasmin substrate. A fluorogenic synthetic peptide substrate for plasmin, t-Boc-Glu-Lys-Lys-methylcoumarin amide, was used in further studies to confirm the specific inhibitory effect of nonenzymatic glycosylation on fibrinolysis. Such fluorometric assays for plasmin activity correlate well with the standard ^{125}I-labeled fibrin plate assay.[20] Substrate incubated with glucose or glucose-6-phosphate showed significant resistance to degradation by plasmin (Table 1). The similarity of these results to those obtained with the fibrin plate assay suggests that the effects observed in that system resulted specifically from nonenzymatic glycosylation of lysine ε-amino groups in the protein.

Acetylation and carbamylation of fluorogenic plasmin substrate. Blocking of lysine ε-amino groups in the synthetic plasmin substrate by either acetylation or carbamylation resulted in essentially complete resistance to degradation by plasmin (Table 1). These data suggest that the observed effects of nonenzymatic glycosylation on fibrin degradation reflect steric hindrance at the normal plasmin cleavage site in the fibrinogen molecule.

DISCUSSION

The studies described in this article demonstrate that nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by the specific fibrinolytic enzyme plasmin. Acetylation and carbamylation have qualitatively similar effects, indicating that chemical modification of lysine amino

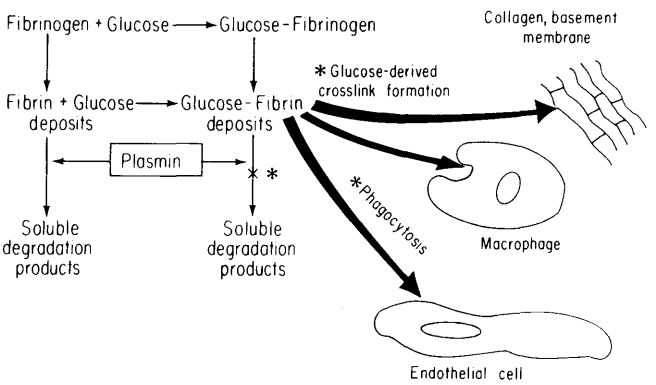

FIGURE 3. Schematic representation of the mechanisms (*) by which excessive nonenzymatic glycosylation may lead to fibrin accumulation in various diabetic tissues.

groups is the underlying phenomenon responsible for the observed degradative defect produced by glucose. Analogous reductions may also occur in susceptibility of other excessively glycosylated proteins to degradation by proteases cleaving preferentially at substrate lysine residues. Such reduced degradation of nonenzymatically glycosylated proteins in vivo may contribute to the accumulation of fibrin and several other proteins observed in those tissues most frequently affected by the complications of diabetes.

In addition to interfering with the specific fibrinolytic enzyme–substrate interaction, glucose attachment to lysine residues in the fibrin molecule would result in fewer sites being available for factor XIII, the fibrin stabilizing factor, to form ε-(gamma glutamyl)-lysine intramolecular crosslinks.[23] Instead, glycosylated fibrin monomers might form glucose-derived intermolecular crosslinks with vascular collagen or basement membrane,[24] contributing by this mechanism to the thickening of those structures, which is associated with long-term diabetes. Nonenzymatic glycosylation of fibrin might also act as a signal for preferential uptake of this protein by vascular endothelial cells. Such cells have been shown to avidly take up glycosylated albumin, while unmodified albumin is excluded from ingestion.[25] An analogous process involving degradation-resistant glycosylated fibrin could lead to significant intracellular deposition of this material. These mechanisms are summarized schematically in Figure 3.

In the diabetic kidney, immunohistochemical studies have documented the presence of fibrin in glomerular capillary basement membrane. Persistence of this fibrin or its degradation products, deposited in the glomerulus as a result of increased permeability,[26] may contribute to the capillary occlusion and progressive glomerular dropout in long-term diabetes. Local responses to mesangial and endothelial trapping of fibrinogen may result in the mesangial enlargement characteristic of diabetes, and could represent the initial phase of Kimmelstiel-Wilson nodule development.[27] Similar morphologic features have been described both in diabetic retinal capillaries,[2] and in small epineurial arterioles from patients with long-standing diabetic neuropathy.[3] Significant fibrin deposition is also a constant feature of atherosclerotic plaques.[4] Recent evidence suggests that fibrin in the arterial wall enhances the proliferation of arterial smooth muscle cells, while fibrin degradation products (fragments D and E) inhibit the proliferation of smooth muscle cells.[28] Defective degradation of glycosylated fibrin in the arterial wall could thus play an important role in the development of diabetic macrovascular disease as well.

It should be noted that the rate of nondegradable glycosylated fibrin accumulation in diabetic tissues would be directly proportional to the number of fibrin lysine residues blocked by nonenzymatic glycosylation. This, in turn, is a function of both glucose concentration and time.[11] Quantitative estimates of in vivo accumulation rates cannot be extrapolated from the data reported here, however, since higher than physiologic concentrations of both glucose and fibrinolytic enzymes were used in these experiments. A rather slow rate of in vivo accumulation would be most consistent with the long period of time known to be required for clinical complications to develop.

The observations described in this report provide a biochemical link between excessive protein glycosylation induced by hyperglycemia and abnormal tissue accumulation of protein at sites where diabetic complications occur. Therapeutic inferences from such data must be regarded as highly speculative at present, but more physiologic patterns of blood glucose response in diabetic patients would seem to be an increasingly desirable therapeutic goal.

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