COVALENT ATTACHMENT OF SOLUBLE PROTEINS BY NONENZYMATICALLY GLYCOSYLATED COLLAGEN

Role in the In Situ Formation of Immune Complexes*

By MICHAEL BROWNLEE,[‡] SANDOR PONGOR, and ANTHONY CERAMI

*From the Laboratory of Medical Biochemistry, The Rockefeller University,
New York, New York 10021*

Hyperglycemia appears to be the major risk factor for the development of diabetic microvascular disease (1–3). However, the sequence of events leading from abnormal glucose homeostasis to irreversible tissue damage remains to be elucidated.

Morphologically, diabetic microvascular disease is characterized by thickening of capillary basement membranes that characteristically exhibit intense linear immunofluorescent staining for albumin and IgG (4). The protein attached to diabetic kidney extracellular matrix remains bound even after prolonged washing with phosphate-buffered saline (PBS), hypotonic or hypertonic sodium chloride buffers, and potassium thiocyanate (5). Such persistent renal accumulation of albumin, IgG, or other circulating proteins may be the first step in a process of in situ formation of immune complexes (6) in diabetic tissues. Experimentally, a diabetes-like glomerulonephritis has been induced by this mechanism (7, 8), and in diabetic patients, extensive renal deposition of complement membrane-attack complexes has recently been described (9).

The diabetes-associated excessive attachment of glucose to extravascular matrix proteins such as basement membrane (10) and collagen (11) could be the biochemical link between persistent hyperglycemia, excessive high affinity binding of plasma constituents, and immunologically mediated tissue damage initiated by the in situ formation of immune complexes. The initial, rapidly formed ketoamine products of nonenzymatic protein glycosylation, which have been well-characterized in a variety of diabetic tissue components (12–14), undergo, over time, a variety of degradations, dehydrations, and rearrangements to form nonenzymatic browning pigments that have highly reactive carbonyl groups. Through these carbonyls, late glycosylation products become capable of reacting with additional amino groups on other proteins to form intermolecular cross-links (15, 16). Trapping of plasma constituents by such reactive products of nonenzymatic glycosylation could result in the extravascular diabetic protein accumulation described above. Subsequently, some of these tightly bound proteins could function as planted antibodies or antigens in the in situ formation of

---

immune complexes (6), leading ultimately to diabetic tissue damage.

In this report, we have tested this hypothesis, using nonenzymatically glycosylated collagen. Binding of both albumin and IgG averaged four times the amount bound to unmodified collagen. Both albumin and IgG bound to nonenzymatically glycosylated collagen retained their ability to form immune complexes in situ with free antibody and antigen, respectively.

Materials and Methods

Collagen Immobilization.   Soluble collagen from calf skin (Elastin Products, Elastin Company, Pacific, MO) was immobilized on agarose by a previously described technique (17), using Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA), rather than CNBr-activated resin. The amount of collagen bound to the resin (determined by quantitating total amino acids after acid hydrolysis of an aliquot) was 17.2 mg/ml of gel (18).

Nonenzymatic Glycosylation of Immobilized Collagen.   Immobilized collagen (86 mg) was washed with 50 ml of 0.5 M phosphate buffer, pH 7.5, and then resuspended in 5 ml of buffer to which D-glucose was added to a final concentration of 500 mM. The immobilized collagen samples were incubated with glucose at 37°C. After 10 d, the gels were washed with 200 ml of PBS and the samples were then stored at 4°C. Unmodified immobilized collagen was prepared by incubating and washing as described without added glucose. All solutions contained 3 mM NaN_3.

Radioiodination of Proteins.   Bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO) and rabbit anti-BSA IgG (Miles Laboratories, Elkhart, IN) purified on diethylaminoethyl (DEAE)-Affi-Gel Blue (Bio-Rad Laboratories) were iodinated with ^{125}I-NaI using the IODOGEN procedure (19). Specific activities of BSA and IgG were 507,000 cpm/nmol and 492,000 cpm/nmol, respectively. Samples were counted in a Packard Tricarb Scintillation Counter (Model 3002; Packard Instrument Co. Inc., United Technologies, Downers Grove, IL) and protein was determined by the Bradford method (20).

Binding of Lysine and Proteins to Immobilized Collagen.   [14C]L-lysine (New England Nuclear, Boston, MA) S.A. 75 cpm/nmol, ^{125}I-BSA, and ^{125}I-IgG were incubated with both unmodified immobilized collagen and nonenzymatically glycosylated collagen (8.6 mg of each) at 37°C for 10 d. 20 nmol [14C]lysine and 1 nmol ^{125}I-labeled protein, respectively, were added per milligram of collagen.

The amount of ligand bound to immobilized collagen was determined by filtering aliquots corresponding to 1 mg of collagen on Durapore hydrophilic membranes (Millipore, Bedford, MA). Filters were washed six times with 0.5 ml of 0.2 M glycine buffer, pH 8.0, followed by six washes of the same buffer containing 2 M NaCl. Samples containing ^{14}C were hydrolyzed with 6 N HCl at 110°C for 12 h and then neutralized before addition of hydrofluor scintillation fluid. Samples containing ^{125}I were counted directly.

Binding of lysine and proteins to agarose alone was assessed under similar conditions. No binding of [14C]lysine was detected, and binding of ^{125}I proteins gave blank values of 2–9 pmol/aliquot.

In Situ Formation of Immune Complexes.   Binding of unlabeled proteins to nonenzymatically glycosylated collagen was accomplished by incubating immobilized collagen (8.6 mg) with BSA or IgG (25 nmol/mg collagen) as described above. After filtering and washing, 0.5 ml of PBS containing 500 µg of ^{125}I-BSA or ^{125}I-IgG was added to the filter and incubated at room temperature for 10 min. This procedure was repeated four times and the samples were then washed six times with 1 ml of buffer. The amount of radioactive antigen or antibody fixed to the corresponding planted antibody or antigen bound to immobilized collagen was determined by counting material retained on the filter.

Data Analysis.   Statistical significance of differences observed between paired samples were evaluated using Student's *t*-test.

Results

Binding of Lysine and Proteins to Nonenzymatically Glycosylated Collagen.   Incubation of [14C]lysine with nonenzymatically glycosylated collagen immobilized on

agarose 5 M resulted in a nearly fivefold increase in the amount of amino acid bound over that observed after incubation with unmodified collagen (Table I). The number of nanomoles of radiolabeled amino acid bound to reactive glycosylated lysine residues in these experiments corresponds to 1.86% of the total collagen lysine residues present. This is equivalent to one glucose-derived amino group binding site for each 200-nm length of individual tropocollagen molecules which, when linked end-to-end and side-to-side, make up the collagen fibril (21). The increase in binding of ^{125}I-serum albumin to nonenzymatically glycosylated collagen was very similar in degree to that observed with lysine. Incubation of ^{125}I-IgG with nonenzymatically glycosylated collagen resulted in a nearly identical number of picomoles being bound. The number of glucose-derived reactive binding sites cannot be extrapolated from this data, however, since an unknown number of lysine residues within the protein may not be accessible. Several lysine residues within a single protein molecule may simultaneously bind at separate reactive sites, and steric hinderance by bound protein molecules may act to prevent further protein binding.

In Situ Formation of Immune Complexes.   The ability of antigen (BSA) or antibody (IgG) bound by nonenzymatically glycosylated collagen to form immune complexes in situ was established by incubating each with ^{125}I-BSA and with ^{125}I-IgG (Table II). Nonspecific binding of free ligand (antigen or antibody) was evaluated by incubating each with its glycosylated-collagen-bound form. Low levels of nonspecific binding were observed in both cases. In contrast, the amount of ^{125}I-antigen (BSA) or ^{125}I-antibody (IgG) fixed to the corresponding planted antibody or antigen bound to glycosylated collagen was strikingly similar to that observed in the simple binding experiments described above.

TABLE I

*Binding of Lysine and Proteins to Unmodified and Nonenzymatically Glycosylated Collagen**

Ligand	Unmodified collagen (C)	Nonenzymatically glycosylated collagen (NEG-C)	$\frac{NEG\text{-}C}{C}$	P
L-Lysine	0.75 ± 0.23 nmol[‡]	3.49 ± 0.61 nmol	4.65 ± 0.17	<0.005
Serum albumin	45.3 ± 8.1 pmol	172.1 ± 13.2 pmol	3.79 ± 0.07	<0.001
IgG	60.2 ± 13.2 pmol	145 ± 13.3 pmol	2.41 ± 0.09	<0.005

* Immobilized calf skin collagen (8.6 mg) was incubated with either 20 nmol of [14C]-L-lysine or with 1 nmol ^{125}I-labeled protein in phosphate buffered saline (3 mM NaN3) at 37°C for 10 d.
‡ Values indicate the amount of ligand bound to 1 mg of immobilized calf skin collagen (mean ± SEM), $n = 4$.

TABLE II

In Situ Formation of Immune Complexes by Planted Antigen (BSA) and Planted Antibody (IgG) Bound to Nonenzymatically Glycosylated Collagen

Free antigen or antibody	IgG (anti-BSA) bound to glycosylated collagen*	BSA bound to glycosylated collagen
^{125}I-BSA	269.7 ± 51.8 pmol[‡]	16.7 ± 9.4 pmol
[125]I-IgG (anti-BSA)	4.4 ± 13.1 pmol	216.5 ± 29.1 pmol

* Binding of unlabeled proteins to nonenzymatically glycosylated collagen was accomplished by incubating immobilized calf skin collagen (8.6 mg) with 2.5 mg protein as described in Table I.
‡ Amount of ^{125}I-antigen (BSA) or ^{125}I-antibody (IgG) fixed to the corresponding planted antibody or antigen bound to glycosylated collagen (mean ± SEM), $n = 4$.

Discussion

The reuslts reported in this communication demonstrate that amino acids and proteins are bound specifically by nonenzymatically glycosylated, long-lived structural proteins such as collagen. Proteins bound in this manner to nonenzymatically glycosylated collagen are able to function as planted antigens or planted antibodies in the subsequent in situ formation of immune complexes.

It should be noted that the collagen utilized in these experiments, though obtained from skin of young animals, undoubtedly had undergone some degree of nonenzymatic glycosylation in vivo before isolation. These nonenzymatic glycosylation products could form subsequently the brown pigments that cross-link proteins, and could account for the low levels of binding consistently observed with "unmodified" collagen. The nonphysiological glucose concentration used in these experiments was selected to accelerate the rate of reactive-site formation. In vivo, this process would occur over a much longer period of time, consistent with the slow evolution of clinically demonstrable diabetic vascular disease. The concentrations of serum protein used in these experiments were considerably less than those in plasma, however, a factor that would tend to reduce the amount of protein bound.

Previously published immunopathological studies of diabetic microvascular disease in the kidney have demonstrated IgG in a linear pattern resembling that seen in anti-basement membrane nephropathy (4, 5). However, the failure to show fixation of IgG from eluates of diabetic kidneys to normal extracellular basement membranes in vitro was interpreted as evidence against the participation of this type of immunological mechanism in the pathogenesis of diabetic microvascular disease. More recently, discontinuous granular ("lumpy-bumpy") deposits of immunoglobulins identified by electron microscopy as immune complexes have been reported in diabetic kidneys (22), and activated-complement, (poly C9) membrane-attack complex deposition has also been described (9). Both patterns of immunoglobulin deposition are consistent with current concepts of in situ immune complex formation (6).

Both the binding of plasma protein constituents to nonenzymatically glycosylated perivascular structural proteins and their subsequent participation in the in situ formation of immune complexes may be favored in vivo by several pathophysiologic and biochemical features of the diabetic state. The well-described increase in vascular permeability associated with diabetes would result in a higher concentration of extravasated plasma proteins available for binding either primarily, as planted antigen or antibody, or secondarily, in immune complex formation. Removal of proteins or immune complexes bound to nonenzymatically glycosylated matrix may be reduced due to diabetes-associated defects in mesangial clearance mechanisms (23) and perhaps also because of a decreased susceptibility to proteolytic degradation associated with nonenzymatic protein glycosylation (24).

The data presented in this report suggest that the chronic tissue damage associated with long-term diabetes mellitus may arise in part from continuous accumulation of serum proteins and in situ immune complex formation by accumulated immunoglobulins and/or antigens bound to long-lived structural proteins that have undergone excessive nonenzymatic glycosylation. The essen-

tial chemical irreversibility of late nonenzymatic glycosylation products once formed, together with the extremely long survival times of many structural tissue proteins, suggest that therapeutic attempts to normalize blood glucose in diabetic patients late in the course of the disease may have little effect on the progression of these diabetic complications once they have become clinically manifest.

Summary

The chronic tissue damage associated with long-term diabetes mellitus may arise in part from in situ immune complex formation by accumulated immuno-globulins and/or antigens bound to long-lived structural proteins that have undergone excessive nonenzymatic glycosylation. In this report, we have tested this hypothesis using nonenzymatically glycosylated collagen. Binding of both albumin and IgG averaged four times the amount bound to unmodified collagen. Both albumin and IgG (anti-BSA) bound to nonenzymatically glycosylated collagen retained their ability to form immune complexes in situ with free antibody and antigen.

References

1. Cudworth, A. G., H. J. Bodansky, and K. M. West. 1982. Genetic and metabolic factors in relationship to the prevalence and severity of diabetic complications. *In* Complications of Diabetes, second edition. H. Keen and J. Jarrett, editors. Edward Arnold (Publishers) Ltd., London. pp. 1–18.

2. Pirart, J. 1978. Diabetes mellitus and its degenerative complications: prospective study of 4,400 patients observed between 1947 and 1973. *Diabetes Care.* 1:168–188; 252–263.

3. Brownlee, M., and A. Cerami. 1981. The biochemistry of the complications of diabetes mellitus. *Annu. Rev. Biochem.* 50:385.

4. Miller, K., and A. F. Michael. 1976. Immunopathology of renal extracellular membranes in diabetes: specificity of tubular basement-membrane immunofluorescence. *Diabetes.* 25:701.

5. Michael, A. F., and D. M. Brown. 1981. Increased concentration of albumin in kidney basement membranes in diabetes mellitus. *Diabetes.* 30:843.

6. Couser, W. G., and D. J. Salant. 1980. In situ immune complex formation and glomerular injury. *Kidney Int.* 17:1.

7. Golbus, S. M., and C. B. Wilson. 1979. Experimental glomerulonephritis induced by in situ formation of immune complexes in glomerular capillary wall. *Kidney Int.* 16:148.

8. Fleuren, G., J. Grond, and P. J. Hoedemaeker. 1980. In situ formation of subepithelial glomerular immune complexes in passive serum sickness. *Kidney Int.* 17:631.

9. Falk, R. J., J. I. Scheinman, S. M. Mauer, and A. F. Michael. 1983. Polyantigenic expansion of basement membrane constituents in diabetic nephropathy. *Diabetes.* 32(suppl 2):34.

10. Cohen, M. P., E. Urdanivia, M. Surma, and V. Y. Wu. 1980. Increased glycosylation of glomerular basement membrane collagen in diabetes. *Biochem. Biophys. Res. Commun.* 95:765.

11. Rosenberg, H., J. B. Modrak, J. M. Hassing, W. A. Al-Turk, and S. J. Stohs. 1979. Glycosylated collagen. *Biochem. Biophys. Res. Commun.* 91:498.

12. Stevens, V. J., C. A. Rouzer, V. M. Monnier, and A. Cerami. 1978. Diabetic cataract formation: potential role of glycosylation of lens crystallins. *Proc. Natl. Acad. Sci. USA.* 75:2918.

13. Vlassara, H., M. Brownlee, and A. Cerami. 1981. Non-enzymatic glycosylation of peripheral nerve protein in diabetes mellitus. *Proc. Natl. Acad. Sci. USA.* 78:5190.

14. Yue, D. K., S. McLennan, and J. R. Turtle. 1983. Non-enzymatic glycosylation of tissue protein in diabetes in the rat. *Diabetologia.* 24:377.

15. Reynolds, T. M. 1963. Chemistry of non-enzymatic browning I. *Adv. Food Res.* 12:1.

16. Reynolds, T. M. 1965. Chemistry of non-enzymatic browning II. *Adv. Food Res.* 14:167.

17. Moscatelli, D., E. Jaffe, and D. B. Rifkin. 1980. Tetradecanoyl phorbol acetate stimulates latent collagenase production by cultured human endothelial cells. *Cell.* 20:343.

18. Moore, S., and W. H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* 211:907.

19. Pierce Chemical Co. 1981. IODO-GEN, Rockford, IL pp. 1–4.

20. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248.

21. Fietzek, P. P., and K. Kuhn. 1976. The primary structure of collagen. *Int. Rev. Connect. Tissue Res.* 7:1.

22. Cavallo, T., J. A. Pinto, L. C. Abbott, and S. Rajaraman. 1983. Immune complex disease complicating diabetic glomerulosclerosis. *Lab. Invest.* 48:13A.

23. Mauer, S. M., M. W. Steffes, A. F. Michael, and D. M. Brown. 1976. Studies of diabetic nephropathy in animals and man. *Diabetes.* 25(suppl. 2):850.

24. Brownlee, M., H. Vlassara, and A. Cerami. 1983. Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. *Diabetes.* 32:680.