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Glycated Albumin Stimulates Fibronectin and Collagen IV Production by Glomerular Endothelial Cells under Normoglycemic Conditions

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Albumin modified by Amadori glucose adducts, formed in increased amounts in diabetes, stimulates the synthesis of matrix by renal glomerular mesangial cells and has been causally linked to the pathogenesis of diabetic nephropathy. However, the effect of glycated albumin on the biology of glomerular endothelial cells, which elaborate a basement membrane that undergoes thickening in diabetes, has not been investigated. We used well-characterized rat glomerular endothelial cells to examine the influence of glycated albumin on the synthesis of extracellular matrix proteins by these cells in culture. Concentrations of glycated albumin that are present in clinical specimens stimulate fibronectin and collagen IV production by glomerular endothelial cells, and this effect is operative under normoglycemic conditions. These results support the hypothesis that increased glycated albumin contributes to glomerular basement membrane thickening in diabetes.

Thickening of the renal glomerular basement membrane, an extracellular matrix situated between and produced by the juxtaposed glomerular endothelial and epithelial cells, is a characteristic morphologic feature of diabetic nephropathy (1-3). Identification of factors in the diabetic milieu which influence the synthesis of basement membrane components by the relevant glomerular cells is therefore important. A cell culture approach is mandatory for such studies in which effects of single variables with potential growth or other modulating activities are to be determined. This approach has proved successful in the case of glomerular mesangial cells, and has led to an appreciation of the roles of glucose, glycated proteins, and the cytokine TGF-β_1 in the increased mesangial matrix production that is another hallmark of diabetic nephropathy (4-11). Relatively few studies, however, have been conducted with glomerular endothelial cells, which have been difficult to isolate and propagate (12-16). Although high glucose concentration has been shown to affect the biology of endothelial cells cultured from large blood vessels and umbilical veins (17-21), there is evidence that the endothelium from capillary beds such as the glomerular tuft behaves differently from endothelium in larger vessels (13,22-26). Thus, biosynthetic responses of cultured glomerular endothelial cells to potential pathophysiologic factors warrants investigation.

We have been interested in the role of glycated serum proteins in the development of diabetic nephropathy, and have shown that albumin modified by Amadori glucose adducts stimulates the production and gene expression of Type IV collagen by mesangial cells in culture (9-11). The possibility that glycated albumin affects the biology of other glomerular cell types has not been examined, although several considerations indicate that it might do so. For example, injection of nondiabetic mice with glycated plasma proteins has been reported to induce thickening of the glomerular basement membrane (27). Glomerular hyperfiltration can be induced in normal rats by transfusion of glycated plasma proteins in concentrations similar to those found in streptozotozin-diabetic rats (28). Such findings suggest that hemodynamic and biosynthetic properties of the glomerular endothelium are altered when it is exposed to serum containing increased concentrations of glycated proteins, as is the case in diabetes.

In the present experiments, we used a recently available, well-characterized cell line to probe the influence of glycated albumin on glomerular endothelial cell biology (29). We demonstrate, for the first time, that glycated albumin in concentrations that are present in clinical specimens stimulates the elaboration of fibronectin and Type IV collagen by these cells in culture. We further show that this effect is operative under normoglycemic conditions and derives from Amadori-glucose adducts in the glycated protein.

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MATERIALS AND METHODS

Glomerular endothelial cells in culture (gift of Dr. F.N. Ziyadeh) were derived from glomeruli isolated from Sprague-Dawley rats as described (29). This homogeneous cell line fulfills criteria for endothelial cells and has been characterized by positive staining for factor VIII, CD31 and endothelial leukocyte adhesion molecule-1, expression of angiotensin converting enzyme, and release and synthesis of endothelin-1 in response to angiotensin II (29). Cells were carried in DMEM containing 10 mM glucose and 10% fetal calf serum (FCS). To initiate experiments, 1×10^4 cells were seeded onto 96 well microtiter plates, allowed to attach, rested for 24 hours in serum-free media containing 10 mM glucose, and then grown for 48 hours in fresh media under the experimental conditions described below. Preliminary experiments established that these conditions were satisfactory for cell adherence, maintenance and uniform growth in all wells. Cells were not exposed to glucose concentrations above 10 mM in order to avoid effects that might accrue from an independent influence of elevated glucose on growth or fibronectin production.

Experimental culture conditions were introduced after the rest period upon the addition of fresh DMEM containing 5.5 mM glucose, 50 μg each of ascorbic acid and β-aminopropionitrile, without or with the described supplements in the indicated concentrations. Media supplements consisted of purified glycated or nonglycated albumin (200-600 μg/ml) and Fab fragments of the A717 monoclonal antibody in matching concentrations. The albumin concentrations represent those of glycated albumin that are found in clinical specimens and encompass the concentrations that previously have been found to stimulate mesangial cell α_1(IV) collagen production, mRNA expression and transcriptional activation (9-11). In nondiabetic individuals, approximately 1% of serum albumin is in the glycated form, which is equivalent to concentrations between 300-400 μg/ml of glycated albumin; the concentration of glycated albumin is in this range or is increased one and a half to three fold in diabetic subjects, according to recent glycemic status (30,31). A717 concentrations were selected to approximate molar equivalency (Fab fragment $\approx$50 kDa; albumin $\approx$66 kDa).

Albumin was purified from human plasma by chromatography on Affi gel blue and DEAE-Sepharose, obtaining a homogeneous band of approximately 66,000 molecular weight on SDS gel electrophoresis. The glycated protein was separated from the nonglycated protein by affinity chromatography on phenylboronate agarose (PBA), which binds Amadori adducts. This protocol has been shown to yield glycated albumin containing approximately 1 mol glucose/mol albumin and reactive with the A717 monoclonal antibody that specifically recognizes albumin modified by Amadori-glucose adducts (32). The epitope defined by the A717 monoclonal antibody encompasses an albumin domain containing a deoxyfructosylline residue. The PBA pass-through was used as source for nonglycated albumin. This material contained $<$0.05 mol glucose/mol albumin, migrated as a single 66 kDa band on gel electrophoresis, and did not react with the A717 monoclonal antibody.

A717 was harvested from ascitic fluid of mice injected with the A717 cell line, purified by affinity chromatography on Protein G, and digested with papain to produce Fab fragments. Specificity and reactivity of the Fab fragments with the glycated albumin epitope was confirmed as described (33,34).

Media were collected at the end of the experimental period and the cells were harvested after light trypsinization for counting in a cell chamber. Fibronectin in the media was measured by an ELISA (Exocell, Phila., PA) in which fibronectin in standard or sample was bound to gelatin that had been immobilized onto microtiter wells, and was detected with HRP-conjugated monoclonal antibody against fibronectin (35). The assay is sensitive to 10 ng/well. Type IV collagen was measured by competitive ELISA (9,10) using Type IV collagen from EHS tumor as standard, rabbit anti-mouse Type IV collagen as primary antibody (both from collaborative Research), and HRP-conjugated goat anti-rabbit IgG for development (BioRad). The assay is sensitive to 5 ng/well.

TABLE 1

Effect of Glycated Albumin on Fibronectin Production by Glomerular Endothelial Cells

Supplement	Concentration μg/ml	Fibronectin (ng/ml)
None	—	79.6 ± 15.4
Nonglycated albumin	300	73.0 ± 10.9
	600	102.6 ± 12.5
Glycated albumin	300	206.0 ± 22.1*
	600	262.0 ± 19.6*

Note. Results represent mean ± SEM of six independent cultures in each of which media were collected after 48 hours of incubation without or with nonglycated versus glycated albumin in the indicated concentrations. *$p < 0.05$ compared with nonglycated albumin at same concentration.

RESULTS AND DISCUSSION

The purpose of these experiments was to determine whether glycated albumin influences growth or biosynthetic properties of glomerular endothelial cells. Our approach to these studies adhered to conditions in which the glycated protein was represented by the Amadori construct, which is the principal form in which glycated albumin exits *in vivo* and to which endothelial cells are exposed in the diabetic milieu (30,31,36,37). To this end, we compared the effect of the purified glycated protein with that of its carbohydrate-free counterpart, and corroborated that the observed effects were induced by Amadori-glucose adducts by examining responses in the presence of site specific antibodies that have been shown to bind to and neutralize the effects of these biologically active epitopes (32-34).

Supplementation of cultures with either glycated or nonglycated albumin did not affect cell growth, determined as cell number. The number of cells per well at the end of the incubation period was consistent between wells, ranging from 0.9-1.2 $\times$ 10^5 and without significant differences under the different incubation conditions. Fibronectin concentrations in cultures supplemented with nonglycated albumin did not differ significantly from those in cells cultured in unsupplemented media. However, fibronectin concentrations in media from cells cultured with glycated albumin were significantly increased compared with both unsupplemented and nonglycated albumin-supplemented incubations (Table 1). These increases pertained when results were normalized to cell numbers (Figure 1).

Blocking experiments with the A717 monoclonal antibody confirmed that changes in fibronectin production induced by glycated albumin were functionally related to the Amadori-glucose modification. Cell numbers in cultures supplemented with A717 plus glycated albumin did not differ from those in cultures supplemented

with glycated albumin alone, indicating that the antibody did not affect cell growth. However, inclusion of A717 in culture media prevented the glycated albumin-induced increase in fibronectin (Figure 1).

Glycation albumin also stimulated production of Type IV collagen by glomerular endothelial cells. Collagen IV concentrations in media from cells incubated with nonglycated albumin were higher than in those in unsupplemented media, consistent with a general supportive effect and underscoring the need to examine the influence of the glycated protein relative to that of the nonglycated counterpart. However, collagen IV concentrations in media from cells incubated with glycated albumin were significantly greater than those in media from nonglycated albumin-supplemented cultures (Table 2). The glycated albumin-induced increase was prevented in the presence of A717 monoclonal antibody (Table 2).

Several investigators have examined responses to high media glucose concentration of various cell types involved in complications of diabetes (4-7,16-20). We have demonstrated that proteins modified by Amadori glucose adducts also can induce abnormalities (9-11,21), whereas other workers have reported on effects of advanced glycation end products (AGE), a heterogeneous group of substances that arise from rearrangement, oxidation and polymerization reactions in nonenzymatically glycated proteins (31,38-41). The results of these studies clearly support the postulate that factors in the diabetic milieu can trigger maladaptive biosynthetic programs that likely contribute to glomerular and vascular pathobiology in diabetes. The present findings, which demonstrate for the first time that glycated albumin disturbs glomerular endothelial cell production of extracellular matrix proteins, are in keeping with this hypothesis.

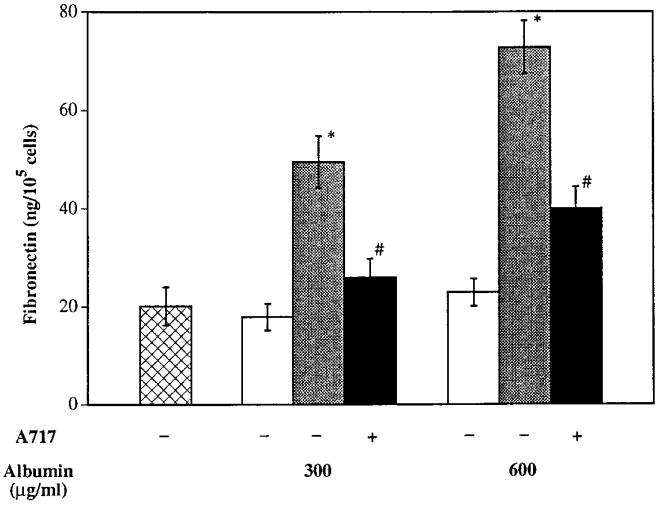

FIG. 1. Effect of A717 monoclonal antibody on glycated albumin-induced stimulation of fibronectin production. Cells cultured for 48 hours with nonglycated (open bars) or glycated (shaded bars) albumin, without or with (solid bars) equal concentrations of A717 Fab fragments. Results represent mean ± SEM of six observations. *$p<0.05$ compared with nonglycated albumin at same concentration. #$p<0.05$ compared with no A717.

TABLE 2

Effect of Glycated Albumin on Collagen IV Production by Glomerular Endothelial Cells

Supplement	A717	Collagen IV (ng/ml)	Collagen IV ng/10^5 cells
None	–	233.7 ± 29.4	59.0 ± 7.4
Nonglycated albumin	–	392.0 ± 68.8#	87.5 ± 15.3#
Glycated albumin	–	980.0 ± 120.7*#	272.2 ± 50.2*#
Glycated albumin	+	382.5 ± 69.2#	85.4 ± 15.4#

Note. Results expressed as mean ± SEM of six independent cultures in which cells were incubated with 400 μg/ml of nonglycated or glycated albumin. A717 was added at 400 μg/ml. *$p < 0.05$ compared with nonglycated albumin; #$p < 0.05$ compared with no supplement.

It is noteworthy that glycated albumin stimulates elaboration of matrix proteins by glomerular endothelial cells in physiologic (5.5 mM) glucose concentration. Hyperglycemia is the driving force for increased albumin glycation, but increased production of fibronectin and collagen IV in response to glycated albumin does not require elevated glucose to be operative. Extrapolated to the *in vivo* situation, this means that the influence of increased glycated albumin, which has a circulating half-life of about two weeks, on endothelial cell biology can continue after normoglycemia is restored. It is also interesting to note the discordant effects of glycated albumin on matrix production in glomerular versus aortic endothelial cells, in which it inhibits synthesis of Type IV collagen (21). It is not unusual for cell biology modulators to have dissimilar or opposite effects on a single biologic process in different cell types (42).

In summary, albumin modified by Amadori-glucose adducts stimulates the production of fibronectin and Type IV collagen by glomerular endothelial cells without affecting the growth of these cells relative to growth in nonglycated albumin-supplemented cultures. This effect, which may be mediated by cell-associated receptors for the glycated albumin ligand (43,44), is operative under normoglycemic conditions, and likely contributes to the thickening of the glomerular basement membrane that occurs in diabetes.

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