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Glycated Albumin Promotes a Generalized Vasculopathy in the *db/db* Mouse

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Increased protein glycation has been mechanistically linked to accelerated vascular pathobiology in diabetes. Because glycated albumin induces biosynthetic abnormalities in cultured aortic endothelial cells that resemble those associated with macrovascular disease, we sought evidence that increased glycated albumin is operative in the genesis of diabetic vasculopathy *in vivo*. Plasma concentrations of fibronectin, a sensitive marker of endothelial cell damage, were increased two-fold in diabetic *db/db* mice compared with their nondiabetic *db/m* littermates. Treatment with monoclonal antibodies specifically reactive with albumin modified by Amadori glucose adducts normalized fibronectin in diabetic animals despite persistent hyperglycemia. These findings suggest that increased glycated albumin causally contributes to diabetic vasculopathy, and that blocking this influence ameliorates vascular damage.

The Diabetes Control and Complications Trial (DCCT) established that intensive treatment of hyperglycemia in patients with insulin dependent diabetes lowers the risk for development of diabetic retinopathy, nephropathy and neuropathy and decreases the incidence of cardiovascular events (1,2). Among the challenges remaining in the aftermath of the DCCT are elucidation of the mechanisms by which hyperglycemia promotes vascular damage and determining if the results can be appropriately extrapolated to patients with noninsulin dependent (Type II) diabetes, in whom cardiovascular disease is a major cause of morbidity and mortality. Given that cardiovascular events represent advanced disease, the identification of animal models of Type II diabetes in which early biochemical markers of vascular disease can be ascertained would enable study of its pathogenesis and the ability of potential therapeutic interventions to arrest its progress.

In the present experiments, we used the *db/db* mouse, a genetically diabetic rodent model of Type II diabetes, to address these issues. The *db/db* mouse develops hyperglycemia in association with insulin resistance and obesity, and has emerged as a suitable small animal model for investigation of diabetic nephropathy (3). Notably, it develops abnormalities in renal glomerular morphology and function that parallel in nature and chronology those that characterize human diabetic nephropathy (4–7). We chose to measure plasma fibronectin, the high molecular weight noncollagenous protein that is considered to be a sensitive marker of endothelial damage, as an index of vasculopathy in this model (8–14). Produced by endothelial and various other cells, fibronectin serves cell adhesion and motility, tissue repair, and coagulation and opsonic functions, is released during platelet aggregation, and promotes platelet adhesion and spreading on matrix substrates (14–18). Because the increased concentration of nonenzymatically glycated albumin in diabetes has been shown to be an important causative factor in the development of diabetic nephropathy (4–6,19,20), we examined the ability of treatment with a monoclonal antibody that neutralizes the effects of excess glycated albumin to modulate changes in plasma fibronectin concentrations.

MATERIALS AND METHODS

Diabetic *db/db* mice and their lean nondiabetic *db/m* littermates were obtained from the Jackson Laboratory (Bar Harbor, ME) at age 6–8 weeks. The animals were individually housed and given free access to food and water. Beginning at age

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15 weeks, one half of the *db/db* and *db/m* mice were treated for nine consecutive weeks with either monoclonal antibody A717 (specifically reactive with Amadori-modified glycated albumin) (21), and the other half received weekly injections of vehicle. The monoclonal antibody, harvested from ascitic fluid and purified by affinity chromatography on protein-G sepharose, was administered as Fab fragments, prepared by papain digestion and purified on protein-A sepharose. Antibody preparations were sterile-filtered and administered in buffered saline as intraperitoneal injections of 150 μg/mouse/week. This treatment regimen was selected on the basis of earlier experiments which showed that the elevated plasma glycated albumin concentrations in *db/db* mice were significantly reduced when measured 48 hours after injection of 100 μg A717 (Fab fragments), and the previously described rationale that takes into account stoichiometric relationships and the circulating half-life of albumin (4,5).

Blood was obtained from the retro-orbital sinus. Glucose was determined by the glucose oxidase method, and creatinine by the picric acid colorimetric procedure (Sigma Chem. Co., St.Louis, MO). These assays were adapted for use with 2-20 μL of plasma, and were sensitive to 10 and 30 mg/dL, respectively. Plasma fibronectin was measured by an ELISA in which fibronectin in standard or sample that bound to gelatin that had been immobilized onto microtiter wells was detected with a monoclonal antibody raised against human plasma fibronectin (Exocell, Phila, PA). The assay was developed with horse radish peroxidase-conjugated antibody against murine IgG and TM Blue substrate, and the reaction was stopped with 2M H_2SO_4. The assay was sensitive to 1 μg/ml and showed a linear relationship between absorbance and the log of concentration between 1–50 μg/ml. Samples were appropriately diluted for assay. Intra- and inter-assay coefficients of variation were 5 and 8 percent, respectively. Absorbance in the control wells (no sample or standard, primary and/or secondary antibody present; sample or standard present, secondary but no primary antibody added) was less than 0.025.

RESULTS AND DISCUSSION

Obesity and hyperglycemia, which developed at an early age in the *db/db* mice used in these experiments, persisted throughout the study (**Table 1**). Blood glucose concentrations and body weights were unaffected by the monoclonal antibody treatment. Since A717 specifically recognizes glycated albumin and does not react with free glucose, it does not influence blood glucose or metabolic status (19–21).

Plasma fibronectin concentrations were significantly elevated in the control (vehicle-treated) diabetic *db/db* mice (**Table 2**). In contrast, mean plasma fibronectin concentrations in *db/db* mice treated with A717 did not differ significantly from those in nondiabetic *db/m* animals, but were significantly lower than those of the vehicle-treated *db/db* mice (**Table 2**). A717 did not influence plasma fibronectin levels in nondiabetic mice. Analysis of all sets of values obtained after 9 weeks showed a significant correlation ($r = 0.70$; $p < 0.001$) between plasma fibronectin and glucose concentrations. However, this most likely represents an artifact imposed by including two distinct study populations since the mean fibronectin and glucose levels were so widely disparate in nondiabetic versus diabetic animals. Separate correlative analysis was therefore performed for *db/m* controls ($r = 0.073$), *db/db* mice receiving vehicle ($r = 0.67$), and the *db/db* mice treated with A717 ($r = 0.33$). Since the mean plasma glucose concentrations in *db/db* vehicle versus *db/db* A717 treated animals were not significantly different (**Table 1**), this reduction in the correlation coefficient suggests that the lowering of fibronectin by A717 treatment resulted from influences distal to hyperglycemia *per se*. We suggest that one such influence is increased glycated albumin,

TABLE 1
Experimental Animal Data

		Body Weight (g)		Blood Glucose (mM)	
Experimental Group	Age:	10 weeks	24 weeks	10 weeks	24 weeks
db/m-vehicle (7)		26.4 ± 0.45	29.9 ± 0.86	6.7 ± 0.4	4.9 ± 0.1
db/m-A717 (7)		26.3 ± 0.33	29.3 ± 0.29	6.0 ± 0.4	4.8 ± 0.2
db/db-vehicle (6)		41.4 ± 1.50*	51.5 ± 3.20*	20.0 ± 2.1*	22.7 ± 0.9*
db/db-A717 (6)		44.1 ± 0.20*	53.7 ± 1.26*	15.3 ± 3.5*	22.5 ± 0.7*

Results given as means ± SEM. Number of animals in each experimental group given in parentheses.
*$p < 0.01$ compared with nondiabetic controls.

TABLE 2
Plasma Fibronectin Concentrations in Nondiabetic and Diabetic Mice

		Fibronectin (mg/ml)	
Experimental Group	Age:	21 weeks	24 weeks
db/m-vehicle		1.30 ± .25	1.16 ± .19
db/m-A717		1.59 ± .37	1.39 ± .16
db/db-vehicle		3.11 ± .48*	2.85 ± .15*
db/db-A717		1.97 ± .17	1.87 ± .28

Results from animals described in Table 1 given as means ± SEM. Plasma fibronectin measured by immunospecific ELISA described in Materials and Methods. *$p < 0.01$ compared with nondiabetic controls.

which is distal to but driven by hyperglycemia, and induces abnormalities in vascular endothelial cell biology that can be prevented by the A717 anti-glycated albumin monoclonal antibodies (22).

To evaluate whether normalization of plasma fibronectin with anti-glycated albumin treatment represented change in renal function (which has been shown to improve with A717 [7]), examination of the relationship between plasma fibronectin and creatinine concentrations was performed. Again, a significant correlation ($r = 0.63$; $p < 0.001$) was observed when all sets of values were included in the analysis. In part, this correlation was due to the higher mean creatinine levels in *db/db* (.92 ± .02) compared with nondiabetic (.61 ± .02; $p < 0.001$) mice, and therefore reflected the inclusion of two populations in the analysis. Separate correlative analysis revealed high correlation in vehicle-treated *db/db* mice ($r = 0.87$), but low or no correlation in A717-treated diabetic mice ($r = -0.07$) and nondiabetic *db/m* controls ($r = .10$). These relationships suggest that impaired renal function is not the dominating influence responsible for the raised fibronectin concentrations in the *db/db* mice, although renal disease and generalized vasculopathy appear to occur in tandem in this model.

Albumin modified by Amadori glucose adducts, the principal form in which glycated albumin exists *in vivo*, has been shown to modulate proliferation and basement membrane collagen synthesis by aortic endothelial cells in culture (21). These effects appear to be triggered by the interaction of glycated albumin with a dose-responsive and saturable ligand-receptor system comprised of membrane-associated polypeptides that specifically recognize fructosyllysine epitopes in glucose-modified albumin and that are distinct from receptors for proteins modified by advanced glycation end products and scavenger receptors for oxidatively-modified LDL (23–25). Receptor binding and the influence of glycated albumin on endothelial cell biology are both inhibited by the A717 monoclonal antibody, indicating that the fructosyllysine epitope is required for receptor recognition and the induction of biologic effects. The present findings demonstrate, for the first time, that increased glycated albumin *in vivo* promotes endothelial damage, and that blocking this influence with anti-glycated albumin antibodies can protect vascular integrity.

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