Diabetic Vascular Disease
An Experimental Objective

Ira J. Goldberg, Hayes M. Dansky

Abstract—It is well known that humans with diabetes have more atherosclerosis and its complications. The causes of this relationship are, however, unclear. Although recent data show that improved glycemic control reduces arterial disease in type 1 diabetes, other studies have shown that subjects with “prediabetes” have more cardiovascular disease before the development of hyperglycemia. Thus, either hyperglycemia and/or lack of insulin actions are toxic to arteries, or metabolic derangements exclusive of hyperglycemia are atherogenic. For >50 years animal models of diabetes and atherosclerosis have been used to uncover potential mechanisms underlying diabetes associated cardiovascular disease. Surprisingly, diabetes alone increases vascular disease in only a few select animal models. Increased atherosclerosis has been found in several animals and lines of genetically modified mice; however, diabetes often also leads to greater hyperlipidemia. This makes it difficult to separate the toxic effects of insulin lack and/or hyperglycemia from those caused by the lipids. These studies are reviewed, as well as more recent investigations using new methods to create diabetic-atherosclerotic models. (Arterioscler Thromb Vasc Biol. 2006;26:1693-1701.)

Key Words: macrovascular ■ lipoproteins ■ atherosclerosis ■ hyperglycemia ■ endothelial cells ■ macrophages

Epidemiological data has shown the strong association between diabetes mellitus and coronary heart disease (CHD). Patients with both type 1 and type 2 diabetes mellitus have more CHD than similar aged nondiabetic subjects. The presence of diabetes in a hypercholesterolemic Scandinavian population was associated with the same incidence of CHD events as normoglycemic patients with established CHD. This has led to clinical algorithms suggesting that risk factors in diabetic patients should be treated as aggressively as those in patients with established vascular disease. Men with CHD and diabetes had, by far, the greatest incidence of recurrent cardiac events.

The initial diagnosis of type 2 diabetes is commonly made during the presentation of a macrovascular disease complication. This is unlike the situation in type 1 diabetes, in which vascular disease complications are not manifest for, on average, over a decade. Disease and vascular calcification in patients with type 1 diabetes correlate with duration of
One hypothesis to explain the correlation of CHD and diabetes is that metabolic abnormalities associated with diabetes, and not overt hyperglycemia per se, accelerate macrovascular complications.7 Haffner et al8 reported that CHD is increased in prediabetic patients: people who have several metabolic abnormalities associated with type 2 diabetes but not fasting hyperglycemia or elevated glycylated hemoglobin. These patients are often denoted as having the metabolic syndrome.9 Support for the hypothesis that conventional risk factors, and not hyperglycemia, is the culprit has come from the failure of glucose reduction to reduce CHD, despite reduction in microvascular disease.10,11 There are several reasons why clinical trials for atherosclerosis prevention might fail: the trial period might have been too short, the interventions might be needed before the onset of disease, and the reductions in glucose may have been too meager. Finally, it is possible that a glucose threshold must be crossed to alter vascular pathology. Other clinical trials are currently underway to test the effects of better glucose control in a large population of subjects with type 2 diabetes.12

A robust intervention to specifically assess the effects of hyperglycemia on vascular disease in type 1 diabetes was accomplished via the diabetes control and complications trial (DCCT). This trial compared 2 levels of glycemic control; one group received conventional therapy and the second had more intensive treatment leading to reduced HbA1C. At the initial completion of this trial, the intensively treated group with improved glucose control had a trend to fewer vascular events.10 The subjects were then followed-up for 6 years and re-evaluated. Intensive therapy was associated with a significant decrease in carotid artery intimal/media thickness.13 Thus, as has been surmised for many years, the extent of hyperglycemia affects vascular pathology. Subsequently, it was reported that the intensive treatment group developed significantly less vascular disease; all cardiovascular disease was reduced 42%.14 Because the development of clinical disease, as opposed to asymptomatic vascular pathology, requires a threshold level of arterial alterations, it is possible that the additional years of progression at a similar rate (because the 2 groups had merged to identical glucose control) occurred on top of different basal disease. The intensive treatment group, having a reduced basal pathology at the end of the initial 6 years, would have taken longer to reach events-producing pathology. There are other possibilities; renal disease was less in the intensive treatment group. Regardless, this landmark study demonstrates that control of glucose alters macrovascular disease exclusive of changes in blood pressure or lipids. The reasons for this can be conjectured from the clinical correlations. However, only experimental evidence is likely to convincingly illustrate mechanism(s) responsible for diabetic vascular disease.

One approach to understanding the relationship of diabetes and vascular disease is to ask whether diabetic lesions are distinct from those seen in other situations. Microvascular diseases lead to characteristic pathological changes in the eyes and kidneys. Although the extent and diffuse nature of atherosclerosis in patients with diabetes is often impressive, efforts to distinguish these lesions anatomically and clinically from those of nondiabetic patients have been unsuccessful. Several recent reports imply that lesions might be more prone to rupture in the diabetic setting leaving evidence of intra-vascular thrombosis and/or repair.15,16 However, no pathological “fingerprint” has been found in the diseased diabetic artery.

What Processes Cause Diabetic Toxicity?
A large body of in vitro data using cultured vascular cells has shown multiple potentially toxic biochemical effects of hyperglycemia; detailed reviews can be found elsewhere.17–20 These processes can be cataloged as intracellular versus extracellular (Figure), and glucose toxicity versus insulin deficiency.

Intracellular processes that cause pathological events are thought to involve hyperglycemic damage to endothelial cells.17 Endothelial cells, unlike many other cells, do not downregulate their glucose transporters when exposed to...

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**Glucose-accelerated atherosclerosis**

High levels of glucose increase the production of advanced glycosylation end products (AGEs) in the circulation and on matrix proteins. AGEs are ligands for a variety of cell surface receptors including the receptors for AGE (RAGE) and scavenger receptors (SRs). Ligation of these receptors may activate inflammatory responses. Glucose might also lead to changes in the composition of matrix such as increased proteoglycans. Excess uptake of glucose, especially in endothelial cells, may generate excess reactive oxygen species (ROS). This process may be greatly increased in the presence of excess fatty acids.

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**Extracellular**
- AGEs
- RAGE
- SRs
- Matrix
- Macrophages
- SMCs

**Intracellular**
- PGs
- ROS
- AGEs
- Cytokines

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TABLE 1. Studies of Atherosclerosis in Diabetic Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Diabetes</th>
<th>Comment</th>
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<tr>
<td>Wild-type</td>
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<td>STZ</td>
<td>Small change in BalbC</td>
<td>Kunjathoor, 1996</td>
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<td>Western</td>
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<td></td>
<td>Kako, 1999</td>
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<td>Added CETP, Lpl, LpA</td>
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<td>Western</td>
<td>STZ</td>
<td>Mild hyperglycemia, increased artery AGES</td>
<td>Reaven, 1997</td>
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Diabetes increases lesions and cholesterol

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<td>Diet</td>
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<td>db/db</td>
<td>Wu, 2005</td>
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Diabetes increases lesions without increases in cholesterol

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</tr>
<tr>
<td>LDLr−/−</td>
<td>Paigen Diet</td>
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apoE−/− indicates apoE knockout; Chol, cholesterol; IR, insulin resistance; LDLr−/−, LDL receptor knockout; Paigen, cholesterol–cholic acid diet; STZ, streptozotocin; Western, high-fat and cholesterol; virus, viral destruction of islets.

Evidence for toxic effects of glucose on endothelium comes from experimental studies of endothelial function in vivo. Experimentally induced hyperglycemia and hyperinsulinemia decrease arterial vasodilation in healthy individuals.27,28,29 Hyperglycemia can directly impair arterial vasomotion vasodilation via increases in superoxide generation and subsequent decreases in endothelial nitric oxide availability29,30. Patients with obesity, insulin resistance, and diabetes have reduced endothelial function even without clinical evidence of cardiovascular disease.31,32,33 Although endothelial function measurement has not been used as a routine clinical screening tool, clinical studies have demonstrated that endothelial dysfunction is an independent predictor of future cardiovascular events.34,35 Therapeutic agents such as metformin and PPARγ agonists partially restore endothelial function in patients with diabetes.36,37

An alternative explanation for the toxic effects of hyperglycemia is that elevated plasma levels of glucose nonenzymatically glycate circulating and matrix proteins. Extracellular proteins containing advanced glycation end-products (AGEs) directly affect cell function, arterial wall stiffness, or gene expression of interacting cells. AGEs are ligands for a number of scavenger receptors including SR-A,38 SR-B1, and CD36,39 and the receptor for AGEs (RAGE). Moreover, AGE treatment will increase expression of scavenger receptors.40 RAGE ligation generates endothelial cell ROS.41 Two lines of evidence support the theory that AGEs mediate diabetic complications: (1) infusions of soluble RAGE, which is presumed to complex AGEs, reduce42 and stabilize atherosclerotic lesions,43 and inhibition of AGE formation reduces lesions;44 (2) diets enriched in AGEs promote lesions.45 Glycosylated proteins are, however, not the only ligands for RAGE and RAGE...
activation mediates multiple processes including cell differentiation, migration, and apoptosis.46

Although one would expect that AGE interaction with RAGE primarily involves an extracellular protein binding to a cell surface receptor, it has been conjectured that intracellular AGEs can be released, leading to either autocrine or paracrine cell activation.20 In addition, AGE formation on matrix proteins increases monocyte retention; glycated collagen is a ligand for the scavenger receptors.58

Could Decreased Insulin Actions Be Atherogenic?

Another hypothesis is that lack of insulin signaling, rather than hyperglycemia per se, alters cell functions. In support of this, especially in type 2 diabetes, is the association of insulin resistance with inflammatory macrophages within adipose tissues, adipocyte secretion of inflammatory cytokines or adipokines, and evidence of generalized increase in inflammatory markers in the blood.47 Several studies have associated insulin resistance, and not hyperglycemia, with elevated plasma markers of inflammation, including CRP.8 In addition, insulin resistance is associated with more conventional risk factors.7 In part, increased plasma levels of fatty acids might also play a pathologic,49 or surprisingly palliative,50 role here.

There are data implicating lack of insulin actions in abnormalities of macrophage biology: insulin receptor-deficient macrophages have greater expression of SR-A and CD36 and augmented uptake of modified lipoproteins.51 It is also reported that hyperglycemia alone will increase expression of scavenger receptors.52

How Does Diabetes Change Lipoprotein Metabolism in Humans and Animals?

Humans, like animals, require circulating apolipoprotein B (apoB)-containing lipoproteins to develop atherosclerosis. In western countries, even low average levels of cholesterol are sufficient for atherosclerosis progression. Although an occasion diabetic person has hyperlipidemia that improves with better glucose control, in general diabetes does not lead to marked elevations of blood cholesterol. Types 1 and 2 diabetes differ in their effects on plasma lipids and this has been reviewed elsewhere.53 Type 2 diabetes is associated with more hypertriglyceridemia, lower high-density lipoprotein (HDL), and more small dense low-density lipoprotein (LDL). This is because of peripheral insulin resistance and increased flux of fatty acids to the liver. There may be a reduction in total lipoprotein lipase (LpL) activity or increased concentrations of the LpL inhibiting proteins apoC3 and, perhaps, angiotensin-like peptides.55 An occasional patient with diabetes can develop severe hypertriglyceridemia caused by heterozygous LpL deficiency. More commonly, diabetes is associated with greater postprandial lipemia and circulation of more atherogenic remnant particles.56 In contrast, patients with well-controlled type 1 diabetes sometimes have increased HDL levels.

Diabetes in experimental animals often leads to major changes in plasma lipoproteins and these changes may swamp any deleterious effects of glucose/insulin resistance on the artery. In monkeys and pigs, diabetes leads to greater hyperlipidemia. Mice have a variable response. Wild-type mice, human apoE transgenics, and heterozygous LDL receptor knockout mice have minimal or no change in plasma lipids with either diet or streptozotocin (STZ)-induced diabetes. In contrast, apoE knockout mice develop elevations of cholesterol with STZ diabetes. The reasons for the hypercholesterolemia in this model were studied by Ebara et al.57 Catabolism of remnant lipoproteins was reduced in STZ-treated apoE knockout mice and this effect was attributed to a reduction in liver trapping associated with reduced proteoglycan production and loss of normal lipoprotein receptor uptake pathways.

LDL receptor knockout mice often more than double their plasma cholesterol in the setting of islet destruction58,59 or deficiency of leptin actions.60,61 The reasons for this are not known but might reflect reduced clearance pathways through LDL receptor–related protein (LRP) and/or scavenger receptors, increased lipoprotein production caused by changes in apoB production associated with intrahepatic signaling62 or fatty acid rescue of apoB from degradation,63 increased MTP expression,64 or greater ingestion of the atherogenic diet. Leptin deficient mice also have increased HDL levels65 associated with defective apoAI clearance66 and reduced scavenger receptor expression.51

Does Diabetes Increase Atherosclerosis in Animals?

Although it is expected that diabetes should increase atherosclerosis, the extrapolation of the human disease
A variety of animal models have been used to try to reproduce the relationship between diabetes and macrovascular disease. In a classic experiment, Duff et al used alloxan to produce diabetes in cholesterol-fed rabbits. In a seemingly paradoxical result, the diabetic rabbits had less atherosclerosis. This atherosclerosis was increased with insulin treatment, probably because the very large lipoproteins in diabetic rabbits that are unable to enter the artery wall are converted to smaller more atherogenic particles. Insulin infusion, by itself, is not atherosclerotic in this model.

Limited studies have been performed in monkeys made diabetic using STZ to destroy pancreatic islets. These animals develop hyperlipidemia and, of course, greater atherosclerosis. In some studies, the monkeys have increased LDL retention and reduced HDL. Diets in genetically susceptible strains and chemical destruction of islets have been used to create diabetic pigs. Alloxan-treated diabetic pigs have increased atherosclerosis; however, plasma LDL was more than doubled by the diabetes. A later study examined the effect of type 1 diabetes in high-fat diet, STZ-treated pigs. Although the induction of diabetes resulted in an increase in plasma triglyceride with no change in LDL cholesterol in the high-fat diet fed pigs, there was worsening of the extent and severity of atherosclerosis in the diabetic compared with the nondiabetic pigs. In both of these studies, the effects of diabetes cannot be discerned because increased lipoprotein levels alone should increase atherosclerosis. Genetic strains of pigs that become diabetic with a fat-rich diet are being bred and studied for atherosclerosis development.

Do Mouse Models Reproduce the Relationship Between Diabetes and Atherosclerosis?

A number of mouse models with diabetes and atherosclerosis have been created (Table 1). The most commonly used method to produce diabetes in the mouse is the injection of STZ. This insulinopenic diabetes does not precisely replicate type 1 diabetes because most mice are not ketotic and do not require insulin for survival. STZ-treated mice are also not a model of type 2 diabetes since they are not obese and insulin resistant. STZ treatment is most effective in male mice; females are more STZ-resistant. One advantage of STZ is that it is an established method and that results can be compared with those in a large body of literature. Moreover, if the mice are provided with adequate water, STZ treatment produces mice that remain hyperglycemic for many months.

High-fat diets produce insulin resistant diabetes in mice with elevated blood sugars between 180 and 300 mg/dL that are in the range seen in human type 2 diabetes. However, the response to this diet is strain-dependent; C57BL6, the strain used for most atherosclerosis and cardiomyopathy studies, is relatively resistant to diet induced hyperglycemia. Moreover, a number of genetic modifications leading to diabetes show diminished or no effect in this strain.

Mouse strains differ in their vascular response to diabetes and hyperlipidemia. Kunjathoor et al assessed atherosclerosis in several strains of STZ-treated and cholic acid-containing (Paigen) diet fed mice. Only BALB/c mice developed a small increase in lesions. Studies to assess the effects diabetes on hyperlipidemic BalbC mice are currently underway (LeBoeuf, personal communication).

Significant atherosclerosis does not develop in the mouse or other animals without plasma hyperlipidemia. Development of several lines of genetically altered mice with hyperlipidemia and vascular disease has shown that significant atherosclerosis does not occur without lipid infiltration.

The Apoe Knockout Mice Have Increased Diabetic Atherosclerosis, but This Is Often Associated With Increased Cholesterol

Atherogenic diet-fed diabetic apoE knockout mice with STZ-induced diabetes developed markedly increased circulating cholesterol levels and atherosclerosis. Lesions were reduced by infusion of soluble fragments of the receptor for advanced glycosylation endproducts (RAGE). In this model it is unclear whether hyperlipidemia from STZ-induced diabetes or the diabetes itself was the primary reason for the increased atherosclerosis. Others have reported an up to 3-fold increase in cholesterol in diabetic apoE knockout mice. With milder diabetes and a chow diet, others have reported only slight cholesterol elevations and increased lesions.

In one study diabetic 40-week-old apoE knockout mice had increased lesions in the aorta and carotids without an increase in cholesterol. In contrast, no increase in lesion size was noted in apoE knockout mice that developed obesity and diabetes caused by a hypothalamic lesion.

Accompanying increased hypercholesterolemia obscures the effects of leptin-deficient hyperglycemia and insulin deficiency in apoE knockout mice. When apoE knockout was crossed onto the db/db leptin receptor-deficient background, plasma cholesterol doubled, and this likely led to greater lesion size. A recent paper using the leptin deficient ob model showed increased aortic sinus lesions; but plasma cholesterol on this 12% fat diet increased Δ50%.

The ApoB Transgenics Do Not Normally Develop Diabetes-Induced Lipid Abnormalities or Accelerated Atherosclerosis

Transgenic expression of human apoB in western-diet fed mice allows development of atherosclerosis. Atherosclerosis was studied in STZ-induced diabetic human apoB expressing transgenic mice. Diabetes led to minor changes in plasma lipoproteins and atherosclerosis was unchanged. Lipoprotein profiles of these mice were altered to make them more human-like. Introduction of cholesterol ester...
transfer protein and deletion of one allele for LpL did not accelerate atherosclerosis with STZ-treatment. In some LpL-deficient mice, however, STZ treatment led to worse diabetes, severe hyperlipidemia, and more vascular disease. Pancreatic islet cells express LpL and lack of this enzyme leads to islet dysfunction. Therefore, partial lack of LPL in pancreatic islets may have led to greater susceptibility to STZ toxicity and dyslipidemia.

The LDL Receptor Knockout Is Probably the Best Studied Model, but Lipid Abnormalities Often Obscure Diabetic Toxicity

Genetic deficiency of LDL receptor coupled with a high fat or cholesterol-rich diet allows sufficient hyperlipidemia for atherosclerotic lesion development. Effects of diabetes superimposed on this model are variable. Reaven’s laboratory produced STZ-diabetes and reported that diabetic LDL receptor knockout mice did not have more atherosclerosis than control mice. These mice did not have an exacerbation of dyslipidemia.

This model also is amenable to diet induced insulin resistance and hyperglycemia. Insulin resistance by itself did not alter lesion area.

In other studies diabetic LDL receptor knockout mice developed more lesions associated with a marked increase in plasma cholesterol. A recent paper described experiments in which early lesions in chow fed mice were increased with viral destruction of the pancreatic islets. However, with a diet containing cholesterol and fat, diabetic mice had much greater levels of blood lipids, and a distinct effect of diabetes, rather than hyperlipidemia, was no longer apparent.

Heterozygous LDL receptor knockout mice develop atherosclerosis when placed on a cholesterol/cholic-acid diet. In one study, imposition of STZ diabetes increased lesion size at the aortic root in these mice. Are Mice Missing an Enzyme That Mediates the Pathological Effects of Glucose?

If one were convinced that hyperglycemia alone is responsible for accelerated atherosclerosis, it would appear that the mouse, despite its production of AGEs, is resistant to diabetic macrovascular disease. An alternative hypothesis that is compatible with the known human data and is consistent with the mouse models is that diabetic acceleration of vascular disease requires some additional factor(s) missing in the mouse. A summary of some differences between humans and mouse models is shown in Table 2.

One pathway leading to greater amounts of ROS is the polyol pathway mediated by AR. AR reduces the 1 carbon on glucose to create sorbitol, which then forms NADH as the sorbitol is oxidized to fructose. Downstream effects of ROS include DNA strand breaks, activation of the repair enzyme poly (ADP–ribose) polymerase (PARP), inhibition of glyceraldehyde-3 phosphate dehydrogenase, and activation of 4 potentially deleterious pathways: flux through the hexosamine pathway, AGE production, synthesis of diacylglycerol leading to activation of protein kinase C (PKC), and further shunting of glucose into the polyol pathway.

Although these consequences of hyperglycemia are felt to be especially important in endothelial cells, in other cells such as macrophages, toxic effects of glucose might also involve greater substrate flux through the polyol pathway. AR is expressed at very low levels in the mouse and mouse hearts and macrophages have much less AR expression than comparable human tissues. Moreover, investigators who have sequenced the mouse AR gene claim that a genetic alteration in mice leads to an AR with reduced activity (K. Gabbay, personal communication). Transgenic mice expressing this enzyme via mouse major histocompatibility antigen class I promoter have expression levels that are more similar to those in humans. This transgene and an AR transgene expressed via the myelin promoter increased neuropathy in diabetic mice. AR local and generalized overexpression also increased cataract formation.

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atory conditions. When, however, the model is associated with marked hyperlipidemia, plasma cholesterol, the primary driving force of atherosclerosis generation, overwhelms any toxic effects of diabetes. Perhaps dietary and genetic interventions that augment the diabetes effect or even paradoxically reduce it will uncover the factors that regulate the generation of diabetic macrovascular disease in the “outbred” human population.

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Disclosures

None.

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Pioglitazone Reduces Endothelial Microparticles in the Metabolic Syndrome
Katherine Esposito, Miryam Ciotola and Dario Giugliano

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