The Pathogenesis of Myocardial Fibrosis in the Setting of Diabetic Cardiomyopathy

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Diabetes has emerged as a major threat to worldwide health. The increasing incidence of diabetes in young individuals is particularly worrisome given that the disease is likely to evolve over a period of years. In 1972, the existence of a diabetic cardiomyopathy was proposed based on the experience with four adult diabetic patients who suffered from congestive heart failure in the absence of discernible coronary artery disease, valvular or congenital heart disease, hypertension, or alcoholism. The exact mechanisms underlying the disease are unknown; however, an important component of the pathological alterations observed in these hearts includes the accumulation of extracellular matrix (ECM) proteins, in particular collagens. The excess deposition of ECM in the heart mirrors what occurs in other organs such as the kidney and peritoneum of diabetics. Mechanisms responsible for these alterations may include the excess production, reduced degradation, and/or chemical modification of ECM proteins. These effects may be the result of direct or indirect actions of high glucose concentrations. This article reviews our state of knowledge on the effects that diabetes-like conditions exert on the cells responsible for ECM production as well as relevant experimental and clinical data. (J Am Coll Cardiol 2006;47:693–700) © 2006 by the American College of Cardiology Foundation

During the past three decades epidemiological, clinical, and laboratory studies have confirmed the existence of a cardiomyopathy associated with diabetes mellitus (DM). The diagnosis of a diabetic cardiomyopathy is made in patients in whom no other known etiological factors, such as coronary artery disease, alcoholism, or hypertension, are present (1,2). In these patients, a higher incidence of congestive heart failure is observed (3). Pathological alterations include those relevant to muscle tissue and cells, such as the presence of myocardial hypertrophy and impaired contraction, those associated with extracellular matrix (ECM) production as well as relevant experimental and clinical data. (J Am Coll Cardiol 2006;47:693–700) © 2006 by the American College of Cardiology Foundation

ORGAN FIBROSIS AS A PATHOLOGICAL OBSERVATION OF LONGSTANDING DIABETES

By far, diabetes-associated nephropathy is the pathological entity that has been best characterized and studied for any organ (6). It is well established that the chronic exposure of the kidney to hyperglycemic conditions favors the development of extensive structural damage. Indeed, diabetic nephropathy (DN) is a major cause of end-stage renal disease (7). Comparable structural changes occur in the glomeruli of either type 1 or type 2 diabetic patients (8,9). Most of these changes are associated with the ECM (10). Diabetic nephropathy is associated with hypertrophy of glomerular and tubular elements as well as the thickening of their basal membrane. In addition, there is an excessive accumulation of ECM proteins in the mesangium (glomerulosclerosis), which narrows or occludes the glomerulus lumen. Another hallmark of DN is the presence of tubulointerstitial fibrosis. The severity of changes in the mesangium and tubules ultimately correlates with loss of kidney function and reduced survival rates (10). The thickening of the glomerular basal membrane is associated with increases in collagen type IV, laminin, and proteoglycans (11,12). In the mesangium, there are increases in collagen types IV, V, VI, fibronectin, and laminin, as well as proteoglycans (12–14). In latter stages of glomerulosclerosis, increases in types I and III collagens occur (13). Animal models of diabetes mirror structural changes observed in human kidneys (10). Increases in the deposition of ECM proteins can be the result of enhanced production and/or decreased degradation. Experimental data support causative roles for altered ECM turnover as assessed through changes in messenger ribonucleic acid and/or protein levels for ECM proteins, serine proteases (e.g., plasmin), matrix metalloproteinases (MMPs), or tissue inhibitors of MMPs (10).

Organs such as the peritoneum also can develop fibrosis in diabetic patients. Many diabetic patients with end-stage
renal disease are submitted to peritoneal dialysis (15). Over the course of time, a significant proportion of patients on peritoneal dialysis develop increased permeability of small solutes, which enhances the absorption of glucose and leads to the failure of ultrafiltration by the dissipation of the osmotic gradient (15,16). Animal models of end-stage renal disease have shown that the stimulation of profibrotic growth factor release (such as transforming growth factor [TGF]-beta-1) in the peritoneum is associated with the development of submesothelial fibrosis and it has been proposed that these events may be potentiated in diabetics (17).

The chemical modification of existing proteins also can contribute to the development of fibrosis. Part of the aging process includes the loss of elasticity of the vascular system, leading to systolic hypertension secondary to enhanced arterial stiffness. Patients with diabetes manifest increased arterial stiffness at a younger age than their nondiabetic counterparts (18). These observations have led to the concept that the mechanism(s) that underlies changes in arterial stiffness associated with aging is accelerated in diabetes. Underlying the loss of tissue elasticity is a well-understood nonenzymatic reaction between glucose and proteins known as the Maillard or browning reaction (19). The glycosylation of long-lived proteins can undergo further chemical changes, yielding very stable complex products and crosslinks known as advanced glycosylation end-products (AGEs) (Fig. 1) (19). This advanced glycation of proteins is accelerated in the presence of diabetes (18). The formation of AGEs on vessels facilitates the crosslinking of long-lived collagen molecules to each other, increases resistance to proteolysis, and thus yields fibrosis (18). There is also loss of collagen elasticity and reductions in arterial compliance. Advanced glycosylation end products also are now recognized as playing important roles in cell signaling by interacting with specific receptors (e.g., RAGE) that link to the activation of adhesion molecules, proinflammatory cytokines, and growth factors, thus contributing to pathological entities such as atherosclerosis (20).

**FIBROSIS IN THE HUMAN DIABETIC HEART**

Rubler et al. (21) first recognized the existence of a diabetic cardiomyopathy in patients with congestive heart failure who had no evidence of coronary atherosclerosis. Epidemiologic evidence of an association of a specific cardiomyopathy with diabetes became evident with the Framingham study (22). Data showed a 2.4-fold greater incidence of heart failure in diabetic men and a 5.1-fold increase in women. Studies conducted in the early 1980s demonstrated changes in myocardial vasculature of diabetic subjects (5). Other studies also demonstrated evidence of left ventricular (LV) hypertrophy and fibrosis with various degrees of small vessel disease (23). Detailed pathological examinations reveal myocardial hypertrophy, interstitial fibrosis, capillary endothelial changes, and capillary basal laminae thickening (23). Microscopic alterations in small intramural coronary arteries include the narrowing of the lumen due to increased proliferation of endothelial cells, increased thickness of the arteriolar wall due to fibrosis and accumulation of mucopolysaccharides, alterations of elastic fibers, myocytolysis, and proliferation of endothelial cells. The increased accumulation of collagen types I and III primarily are found in the epicardial and perivascular regions, whereas type IV is found in the endocardial layer (25).

Echocardiography of diabetic patients indicates the development of LV hypertrophy (26). Analysis of data also indicates alterations in echocardiographic signals (backscatter reductions) in diabetic patients, which may reflect tissue fibrosis or other cardiomyopathic changes. Noninvasive studies have shown alterations in both systolic and diastolic function in diabetics and, to a greater extent, those with microvascular complications and/or hypertension (4). The impairment of LV diastolic function of patients with diabetes may be secondary to alterations in collagen structure, specifically increased collagen crosslinking or AGEs (18).
Clinical studies have yielded positive correlations between AGE serum levels and isovolumetric relaxation time and LV diameter during diastole (27).

**ANIMAL MODELS OF DIABETIC CARDIOMYOPATHY**

Animal models for the study of diabetes include those that mimic type 1 or type 2. An extensive review on the subject was published recently (28). Factor et al. (29) first evaluated the effects of hypertension and diabetes on rat myocardium in 1981 and showed hypertensive-diabetic rats develop cardiac hypertrophy and fibrosis. Subsequent results from Fein and Sonnenblick (3) indicated that diabetes alone did not yield morphological alterations in myocardium, but animals did exhibit weakened contraction. In a subsequent study by Factor et al. (30), increases in cellular lipids and myofibrinolysis were noted with patients with diabetes. The assessment of vascular lesions of hypertensive-diabetic rats indicated pronounced arteriolar constriction, which may have caused ischemic injury to the myocardium leading to replacement fibrosis (31). However, experimental studies in diabetic dogs and monkeys have demonstrated interstitial fibrosis and less compliant LVs (22). In addition, myocardial hydroxyproline content and associated crosslinks were significantly greater in the endocardium of diabetic pigs (32). Other studies also have examined the effects of diabetes on LV remodeling after infarction showing that diabetes decreases animal survival and exaggerates LV remodeling after infarction while increasing interstitial fibrosis and myocyte apoptosis (33,34).

**THE EFFECTS OF PHARMACOTHERAPY**

Pharmacologic interventions have been used experimentally in an attempt to retard or reverse the development of structural changes in the diabetic myocardium. Treating diabetic rats with the angiotensin-converting enzyme inhibitor captopril for four months prevented increases in diastolic pressure, coronary perfusion pressure, vascular resistance, and interstitial and perivascular fibrosis (35). The angiotensin-converting enzyme inhibitor enalapril can normalize glycation levels in the myocardium and reduce LV stiffness (36). The beneficial effects of enalapril may be derived at least in part by its antioxidant properties (37). Treatment of diabetic rats with the antifibrotic compound pirfenidone or the aldosterone blocker spironolactone attenuated increases in LV diastolic stiffness and reversed fibrosis without normalizing contractility (38). Evidence from clinical trials indicates that pathological cardiac remodeling can be prevented or reversed with drugs that block neurohormonal systems, including the renin angiotensin system and the sympathetic nervous system (39,40). These clinical trial results indicate that patients with diabetes benefit as much as patients without diabetes from neurohormonal-inhibition therapies (39,40). However, caution should be exercised in extrapolating pharmacologic therapy benefits demonstrated in the general population to patients with diabetes in the absence of extensive clinical data.

The observation that AGE products may be partly responsible for the structural alterations observed in various organs, in particular blood vessels, led to the development of crosslink breaker compounds such as aminoguanidine (18). The administration of these compounds to diabetic dogs or monkeys decreases the enhanced LV stiffness that develops with diabetes and improves contractile function (41,42). The use of these compounds in rats yields the restoration of LV collagen solubility, brain natriuretic peptide serum levels, and normalized gene expression for collagen type III and the profibrotic effector connective TGF (43). In a Phase 2a study completed in January of 2003, the Distensibility Improvement and Remodeling in Diastolic Heart Failure (DIAMOND) trial results indicated that treatment with the crosslink breaker alagebrium resulted in a reduction in LV mass within a 16-week treatment period, as well as an improvement in LV diastolic filling and quality of life. The compound is currently undergoing further evaluation and testing for its potential to improve diastolic function and reduce hypertrophy.

**IN VITRO MODELS OF DIABETES**

Two main methods have been developed to examine effects that diabetes-like conditions have on in vitro ECM turnover. The first uses primary cell cultures isolated from normal tissues exposed to high glucose (HG) so as to simulate the hyperglycemic milieu of diabetes. The second uses cells derived from either animal models of diabetes or diabetic humans that are cultured in HG. Differences in experimental design are important in that results derived from both types of cultures may not concur. To better simulate diabetes, most investigators also rely on protocols that include the long-term cultures of cells, typically no <2 to 3 days and up to weeks. Commonly used concentrations of D-glucose are 25 to 30 mmol/l (450 to 540 mg/dl) to represent HG and 5 mmol/l (100 mg/dl) for normal glucose. It is important to note that many culture systems routinely used to examine changes in “normal” cell function inadvertently include media with HG concentrations akin to those of diabetes-like conditions (315 to 450 mg/dl, respectively). In vitro experiments also have included the use of fluctuating levels of HG as a possible treatment scheme to better simulate diabetes spikes (44).

The in vitro assessment of hyperglycemic effects has been pursued most extensively in mesangial and glomerular epithelial cells. Published observations indicate that, in cultured mesangial cells, the presence of 30 mmol/l glucose (for four weeks) yields an increase in ECM proteins such as fibronectin, laminin, and type IV collagen production (45,46). High glucose also increased mesangial cell diacylglycerol (DAG) levels and protein kinase C (PKC) (47). To determine the mechanisms involved in enhanced ECM production, Ayo et al. (48) pursued pulse–chase experiments...
using mesangial cells cultured in normal and HG conditions. Under HG conditions, they observed a 40% to 50% increase in the synthesis of fibronectin, laminin, and type IV collagen with no apparent change in total protein synthesis or ECM protein degradation after one week. Studies by Pugliese et al. (49) indicate that in long-term cultures of rat glomerular mesangial cells, HG increases fibronectin, laminin, and collagen IV and VI by week 2, with no changes in total protein or cell proliferation. Interestingly, D-galactose and L-glucose treatment (which can induce nonenzymatic protein glycosylation) mimicked in part D-glucose–induced ECM overproduction, suggesting this process is partially responsible for the effects.

The inhibition of cell proliferation also has been reported to occur when mesangial cells were grown in the presence of glycated serum (serum exposed to 28 mmol/l of glucose for four days) whereas type IV collagen gene expression and secretion were stimulated (50). Cell proliferation also was inhibited by the use of culture plates precoated with glycated serum. The use of a monoclonal antibody immunoreactive with Amadori adducts in glycated albumin blocked these effects.

Fibroblasts from other organs can also increase ECM deposition when exposed to HG. Human skin fibroblasts cultured in HG increase collagen III synthesis both at mRNA and protein levels (51). Known complications of patients with DM also include retinopathy and neuropathy, in which excess ECM production can play a role in their pathogenesis. Long-term culture with HG of bovine retinal microvessel pericytes stimulated protein and collagen synthesis (52). In primary cell culture of sciatic nerves (a mixture of Schwann cells, perineurial cells, and fibroblasts) HG increased collagen I, collagen IV, and fibronectin mRNAs (53).

Few reports on the effects of HG on cardiac fibroblasts (CF) have been published. The culture of human cardiac myofibroblast in HG increases cell proliferation (54). In neonatal rat cardiac fibroblasts, HG and osmotic control media increase DNA and collagen synthesis as well as fibronectin and TGF-beta-1 gene expression (55). Recently, we assessed the effects that HG has on adult rat cardiac fibroblast proliferation. HG increased collagen I, collagen IV, and fibronectin mRNA (56).

We also determined whether co-treatment with Ang II yielded additive or synergistic effects with HG, as the additive effects of HG and angiotensin II on type IV collagen synthesis have been previously reported in mesangial cells (57). Our results indicated that HG-treated cardiac fibroblasts synthesized more protein and collagen, but the addition of Ang II did not further stimulate these effects. Interestingly, pretreatment with losartan blocked HG-induced increases in protein and collagen synthesis. HG also decreased total MMP activity, and the effect was blocked by losartan. Notably, AT1 mRNA levels were up-regulated with HG. These results suggest that HG may promote fibrosis by increasing CF protein and collagen synthesis and decreasing MMP activity and that HG may cause these effects via the up-regulation of AT1 receptors. As discussed in more detail to follow, the ability of HG to stimulate the production of reactive oxygen species (ROS) in cells is well established. As a result, we also determined the effects of ROS scavenger treatment on CF functions. Interestingly, although pretreatment with vitamin E blocked the effects of HG on total protein synthesis, it stimulated MMP activity without altering ECM synthesis. Thus, it is possible to speculate that vitamin E treatment may normalize HG-induced increases in ECM synthesis by enhancing its degradation.

The production/deposition of ECM induced by HG also may be organ/cell type dependent. In human gingival fibroblasts, glucose concentrations from 5 to 50 mmol/l resulted in a dose-dependent reduction of collagen synthesis. Total protein synthesis and cell number were not altered (58). In mouse proximal tubule epithelial cells, HG increases ECM and total protein synthesis (59). In human glomerular epithelial cells, HG induced the differential expression of integrins, decreased binding of the cells to type IV collagen, decreased MMP-2 gene expression, and induced up-regulation of tissue inhibitor of MMP-2 (60). In human skin fibroblasts isolated from diabetic ulcer sites cell proliferation is significantly slower versus control, and this difference persists regardless of the glucose concentration used in the media (61).

PARACRINE AND MECHANICAL FACTORS

It has been demonstrated that the exposure of ECM-producing cells to HG yields the synthesis/secretion of paracrine factors that may mediate their functional responses. For example, in cultured human mesangial cells exposed to HG, the production of Ang II was greater compared with controls and was prevented by a PKC inhibitor (62). High glucose-induced increases in TGF-beta-1, fibronectin, and type IV collagen were partially blocked by candesartan (an AT1 antagonist). Thus, HG appears to stimulate Ang II production via the activation of PKC, and Ang II partially mediates ECM synthesis (62). Human peritoneal mesothelial cells cultured in HG yield increase in basic fibroblast growth factor. Peritoneal fibroblast proliferation was noted to increase when cells were exposed to mesothelial cell HG-conditioned media, and anti–basic fibroblast growth factor neutralizing antibody blocked the effects (63). In human peritoneal fibroblasts, HG increased TGF-beta-1 and type I collagen mRNA levels in a dose-dependent manner. The use of TGF-beta-1 antibodies blocked these effects (64). In murine renal cortical fibroblasts, HG increased TGF-beta-1, -2, and -3 gene expression. High glucose and TGF-beta-1 also increased the incorporation of proline, mRNA, and protein levels for type I collagen. Pan-selective TGF-beta antibodies attenuated these effects. Therefore, HG and TGF-beta appear to elicit comparable effects on renal fibroblasts (65). Human proximal tubular cells and cortical fibroblasts that are
exposed to HG show increases in cell proliferation and secretion of TGF-beta-1 and platelet-derived growth factor. When exposed to intermittent HG, there are further increases in proliferation, TGF-beta-1 secretion, protein content, and collagen synthesis. Thus, pulsing glucose excursions may have important pathophysiologic effects on human tubulo interstitial structure (66).

Mechanical stimuli also may play roles in stimulating cell function. In isolated perfused glomeruli from diabetic rats, glomerular compliance is known to increase with time. When stretched, mesangial cells increase total collagen synthesis in both normal glucose (NG) (50%) and HG conditions (27%). However, the fraction of newly formed collagen being catabolized also increased in NG conditions, but was unchanged with HG, which results in an increase in net collagen accumulation with HG (67).

**REACTIVE OXYGEN SPECIES**

Results from in vitro experiments indicate that the generation of ROS is likely involved in inducing abnormal responses to HG (68). High glucose induces ROS generation in mesangial and tubular epithelial cells and up-regulates TGF-beta-1 and ECM synthesis (45,69,70). These effects also can be mimicked by H$_2$O$_2$ (71). The use of 3-O-methyl-D-glucose or L-glucose does not induce intracellular ROS, and cytochalasin B (a glucose transporter inhibitor) inhibits D-glucose–induced intracellular ROS, suggesting that HG-induced ROS depends on glucose uptake and metabolism (69). The source of HG-induced ROS generation appears to be linked to the NADPH oxidase system and mitochondrial metabolism (68). Further evidence of the involvement of ROS comes from the observation that the treatment of mesangial cells with antioxidants normalizes HG-induced increases in ECM synthesis (72). Reactive oxygen species also can activate cell signaling pathways such as PKC, mitogen-activated protein kinases, and JAK-STAT (73–75). Of particular interest is the activation of specific isoforms of PKC (e.g., PKC-beta). The use of PKC inhibitor compounds such as ruboxistaurin appears to prevent or reverse damage to organs such as the retina (76,77). The direct effects of these compounds on ECM production have not yet been evaluated, but their long-term use does improve renal function in diabetic mice (78).

**SIGNALING**

As can be ascertained from the topics discussed previously, the accumulation of AGEs, the activation of PKC isoforms, and HG-induced generation of ROS may explain partly how hyperglycemia mediates tissue damage and remodeling. How these events are triggered by HG is less clear. However, experimental studies have attempted to identify HG-activated cell signaling pathways associated with ECM production. In mesangial cell lines transfected with a gene expressing luciferase and murine collagen IV gene, HG stimulated luciferase activity in a dose- and time-dependent manner. The inhibition of PKC with staurosporine or calphostin C significantly reduced the activity, suggesting that HG promotes transcriptional activity of collagen IV through PKC (79). Cultured dermal fibroblasts from non–insulin-dependent DM rats have lower cell proliferation and higher L-lactate accumulation when cultured under normal glucose conditions versus controls. When incubated in HG, fibroblasts from control and DM rats decreased total DNA content and increased L-lactate levels. A PKC inhibitor reversed HG-induced changes in both cell types, as well as in the cells from DM rats cultured under NG, suggesting that PKC may play a role as a modulator of fibroblast proliferation in patients with diabetes (80). In a follow-up study by the same group, they also demonstrated the involvement of protein kinase A and cAMP (81).

An excessive accumulation of intracellular sorbitol found in diabetic animals and in cells cultured under HG conditions has been proposed as important factor in the pathogenesis of diabetic complications (82). Aldose reductase, a key enzyme of polyol pathway, catalyzes NADPH-dependent reduction of glucose to sorbitol (sorbitol pathway). To examine the role of the polyol pathway in renal ECM accumulation, Bleyer et al. (83) incubated murine proximal tubule cells in either normal or HG concentrations in the presence or absence of the aldose reductase inhibitor sorbinil. They noted that HG increased cell sorbitol levels seven-fold and the addition of sorbinil inhibited the response. In HG cells, sorbinil also reduced mRNA levels of collagen type I and IV as well as their secretion.

![Figure 2. Sorbitol signaling pathway proposed for high-glucose-mediated enhanced collagen production in fibroblasts. Increases in intracellular glucose concentrations can activate an enhancer sequence in the collagen promoter, yielding enhanced collagen production and fibrosis. Increases in diacylglycerol can occur secondary to the altered transcription of enzymes involved in its synthesis or degradation (e.g., diacylglycerol kinase). The enhanced entry of glucose into the fibroblasts can also occur independently of transporter proteins yielding the stimulation of sorbitol and the production of 3-deoxyglucosone and glyoxal intracellular advanced glycosylation end-product (AGEs), both of which are direct stimulants of protein kinase C-beta. The production of aldose reductase (AR) also can be favored by an enhanced transcription of the gene. The sorbitol pathway can also increase production of reactive oxygen species (ROS), further stimulating PKC-beta. GLUT-1 = glucose transporter-1, PKA = protein kinase A.](image-url)
The topic of HG-mediated signaling and organ pathology has been extensively reviewed elsewhere (68,77,84–87). However, it is reasonable to assume that in the development of cardiac fibrosis, HG-activated signaling pathways may parallel those known to be involved in other diabetic organs. Figure 2 illustrates one such possible scenario that involves gene transcription, effects of sorbitol, aldose reductase, ROS, and AGES. A DAG signaling pathway also can be proposed for HG-mediated enhanced production of ECM proteins (Fig. 3) (77). As we have demonstrated, Ang II AT1 receptor levels increase in fibroblasts exposed to HG conditions, and this receptor couples with the production of DAG (56). The enzyme in charge of the interconversion of this messenger, DAG kinase, allows the synthesis of other lipid mediators, which can enter the pathway for the synthesis of dihydroacetone, increasing ROS and stimulating protein kinase C (PKC)-beta and/or protein kinase A (PKA). Dihydroacetone can also generate DAG via the action of phosphatidic acid phosphatase (PAP). There also may be protein kinase-independent effects mediated via AT1 receptors, which may alter the production and/or activity of matrix metalloproteinases (MMPs) or other ECM-related genes. Phosphofructokinase (PFK₁) is a rate-limiting enzyme involved in the hexosamine pathway. GLUT-1 = glucose transporter-1; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor kappa B; PLCβ = phospholipase C beta.

load of intracellular glucose in handled by a rate limiting enzyme phosphofructokinase (Fig. 3). When the production of phosphofructokinase is halted, excess fructose-6-phosphate allows the synthesis of glucosamine-6-phosphate an activator of PKC-beta.

CONCLUDING REMARKS

Although the first reference made to the existence of a diabetic cardiomyopathy occurred in 1972, limited progress has been made in attempting to describe the underlying molecular pathology, and in particular that related to the development of myocardial fibrosis. Most of the current knowledge about how hyperglycemia may affect the deposition of ECM proteins comes from studies performed in kidney cells. Although there may be some degree of analogy between cellular responses to HG, there can be clear differences depending on the cell type studied. In the presence of an ever growing incidence of diabetes, it will become imperative to further our understanding of the underlying pathology of myocardial fibrosis so as to develop improved pharmacological therapies.

REFERENCES


