Impact of type 1 diabetes on cardiac fibroblast activation: enhanced cell cycle progression and reduced myofibroblast content in diabetic myocardium

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Diabetic patients suffer from numerous cardiovascular complications including atherosclerosis, hypertension, and myocardial fibrosis. Wound healing is impaired in diabetic patients, although the underlying causes are poorly understood. Cardiac function is also compromised during the progression of diabetes; patients with diabetes are predisposed to myocardial infarction, heart failure, and arrhythmias (1, 32). Diabetic patients are less likely to survive a myocardial infarction, perhaps because of deficits in their wound healing capabilities. Cardiac fibrosis is often associated with these cardiovascular pathologies, and recent studies have reported that streptozotocin (STZ)-induced type 1 diabetic animals develop cardiac fibrosis and left ventricular (LV) dysfunction (2, 15, 17, 28, 29).

Cardiac fibrosis is characterized by an increase in fibrillar collagen and may develop from overactive fibroblasts and aberrant remodeling, eventually leading to compromised cardiac performance (4, 30). Cardiac fibroblasts, key regulators of cardiac remodeling, comprise ~20% of the myocardial mass (9). In addition to secreting ECM components, activated cardiac fibroblasts proliferate, migrate, and differentiate to the hypersecretory myofibroblast, a cell type that is critical to the remodeling and wound healing of damaged tissue (6, 23, 26). High glucose has been shown to stimulate cardiac fibroblast and myofibroblast proliferation in vitro (3, 19). Previous studies in other tissues have demonstrated an increase in the myofibroblast population in the kidneys of diabetic rats (13, 24). Nguyen et al. (20) measured increased myofibroblast progenitor cells in the blood of type 1 diabetic patients. Interestingly, myofibroblasts have a delayed appearance in skin wounds of diabetic mice (8). The appearance of myofibroblasts in the diabetic myocardium has not been described to date.

Cardiac fibroblast activation is highly regulated by a myriad of signaling pathways. Proliferation is controlled by cell cycle regulators such as p53 and p21 (25, 31). Alterations in p53 expression have been shown in many diabetic models; p53 is increased in the kidney of type 1 diabetic rats and in myocytes and dermal fibroblasts isolated from type 1 diabetic animals cultured under hyperglycemic conditions (10, 11, 14, 27). In vitro hyperglycemia decreased p21 expression in mesangial cells but increased p21 expression in circulating progenitor cells (7, 16). The varying results indicate cell type-specific responses to high glucose.

Given the several cardiovascular complications that arise in diabetic patients, and their reduced wound healing capabilities, the aim of this study was to determine a link between diabetes and cardiac fibroblast activation with a particular focus on proliferation and differentiation to the myofibroblast phenotype and to determine whether mediators of the cell cycle are altered in the diabetic heart.

MATERIALS AND METHODS

Materials. Streptozotocin solid and anti-α-smooth muscle actin (α-SMA) were purchased from Sigma-Aldrich (St. Louis, MO). Blood glucose monitoring equipment was from One Touch II (Milpitas, CA). Anti-p53, anti-c-jun, anti-phospho-Akt, and anti-Akt antibodies were obtained from Cell Signaling Technology (Boston, MA). Anti-p21 was purchased from Santa Cruz Biototechnology (Santa Cruz, CA). Anti-desmin was obtained from BD Pharmingen (Franklin Lakes, NJ) and anti-platelet endothelial cell adhesion molecule (PECAM) from Fitzgerald Industries (Concord, MA). TRizol, DMEM, Fungizone, penicillin-streptomycin, and FBS were all purchased from Invitrogen/GIBCO (Carlsbad, CA). Collagenase type 2 and trypsin were acquired from Worthington Biochemical (Lakewood, NJ).
FIBROBLAST ACTIVATION IN DIABETIC MYOCARDIUM

Table 1. Effects of type 1 diabetes on blood glucose and body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of Diabetes, wk</th>
<th>Final Blood Glucose, mg/dl</th>
<th>Body Weight, g</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>97.5</td>
<td>288.0</td>
</tr>
<tr>
<td>Diabetic</td>
<td>42</td>
<td>550.8</td>
<td>293.1</td>
</tr>
</tbody>
</table>

Parameter values are group averages.

Induction of experimental type 1 diabetes and tissue preparation. Six-week-old adult male Sprague-Dawley rats (n = 4) received intraperitoneal injections of STZ (55 mg/kg) to induce diabetes. Age-matched control rats (n = 4) were injected with vehicle only (0.1 N sodium citrate). The development of diabetes was assessed by weekly monitoring of body weight and blood glucose levels. Six weeks after STZ administration animals were killed and LVs were isolated and divided into three sections, one for tissue lysates, the second for histology, and the third for mRNA isolation with TRZol. A second set of animals (n = 2/group) were utilized exclusively for fibroblast isolations. This study was performed under Institutional Animal Care and Use Committee approval (protocol no. 08-019) and followed American Physiological Society guidelines for the use of animals.

Isolation of cardiac fibroblasts. Cardiac fibroblasts were isolated as described previously (5, 18, 22). In brief, LVs were excised from control and diabetic animals, rinsed with cold PBS, and then placed in isolation buffer. Individual ventricles were minced and digested for 90 min in isolation buffer containing collagenase type 2 (100 U/ml) and trypsin (0.6 mg/ml). Fibroblasts were collected by centrifugation, resuspended in DMEM containing 10% FBS, and seeded onto tissue culture dishes.

Assessment of fibroblast proliferation. Proliferation of cardiac fibroblasts was assessed with the Celltiter 96 nonradioactive cell proliferation assay from Promega. Cells were grown on a 96-well plate for 24 h, followed by 4-h incubation in DMEM containing either 0% or 10% FBS. A colorimetric dye solution was added to each well for a 4-h incubation, and then a solubilization/stop solution was added for 1 h before absorbance was read on a 96-well plate reader.

Echocardiographic assessment. Overall, the diabetic rats had higher blood glucose, as expected, and did not maintain body weight compared with the control animals (Table 1). One week after STZ injection the diabetic rats displayed high levels of blood glucose averaging 564 mg/dl. Blood glucose monitoring occurred weekly, and the diabetic rats had a final average reading of 551 mg/dl (Table 1).

All groups of animals were studied by transthoracic echocardiography 6 wk after induction of type 1 diabetes. A summary of data obtained from echocardiography is given in Table 2, showing the general characteristics of animal groups included in this study.

Measurements attained in this study show a decrease (16.9%) in relative wall thickness (RWT) in diabetic rats compared with control rats, indicating wall thinning in these animals (P < 0.05). LV mass was corrected for body weight (LV Mass Corr) because of the general size differences between groups. Diabetic animals had significantly decreased (25.6%) LV Mass Corr compared with control animals (P < 0.05). Chamber dilation was evident in diabetic rats compared with normal controls.
with control rats when LV diastolic volume (LVVd) was corrected for heart size (LVVd/LV Mass Corr), 0.676 ± 0.016 vs. 0.504 ± 0.024, respectively (P < 0.01). No significant changes were observed in systolic function between groups when ejection fraction (EF) and fractional shortening (FS) were compared.

Enhanced proliferation of cardiac fibroblasts isolated from type 1 diabetic rats. Proliferation of isolated control and diabetic cardiac fibroblasts was assessed by a nonradioactive proliferation assay. Proliferation of diabetic fibroblasts increased by 15.6% versus control fibroblasts in serum-free DMEM (Fig. 1A), and on serum stimulation diabetic fibroblasts exhibited an even higher increase in proliferation of 45.0% compared with control fibroblasts stimulated with serum (Fig. 1B).

Decreased signaling gene expression in diabetic heart. We utilized microarray analysis to identify signaling genes that were differentially expressed in diabetic and control rat hearts. Surprisingly, no genes were significantly upregulated in the diabetic group compared with the control group. Eleven genes were downregulated in the STZ group; three were statistically significant: jun decreased 5.26-fold (0.19 of control), p21 decreased 7.14-fold (0.14 of control), and p53 decreased 1.85-fold (0.54 of control, P < 0.05; Fig. 2).

Inhibition of cell cycle proteins: p53, Jun, and p21. Since we detected a decrease in mRNA expression of p53, we next sought to validate the changes at the protein level by Western blot analysis. p53 protein expression significantly decreased by 1.80 ± 0.03-fold in the diabetic hearts (P < 0.05; Fig. 3A). To confirm that the changes in jun gene expression were translated to the protein level, we performed Western blot analysis on LV protein samples from each animal. We determined a slight, nonsignificant 1.24 ± 0.08-fold decrease in Jun expression in the diabetic group versus control (Fig. 3B). Since p21 mRNA levels were significantly lower in diabetic hearts versus control hearts, we measured p21 protein expression in these hearts. Surprisingly, we did not detect any statistically significant changes in p21 protein expression between groups (Fig. 3C).

Increased Akt activity in diabetic heart. Previous studies have revealed that Akt is an upstream mediator of p53 and p21; therefore, we aimed to determine whether changes in activated Akt could be detected in the diabetic hearts (12, 21, 33). LV protein lysates were collected from diabetic and control rat hearts. Western blot analysis showed a trend of increased phosphorylated (p-)Akt in diabetic hearts over control hearts (1.52 ± 0.60-fold increase; Fig. 4).

Decreased myofibroblast content in diabetic myocardium. Whole tissue lysates were obtained from the LV of diabetic and control rats. Myofibroblast content was assessed by measuring α-SMA levels, and to our surprise the diabetic myocardium contained 4.37 ± 0.16-fold less α-SMA expression (P < 0.05) (Fig. 5A). The diabetic hearts also produced 1.29 ± 0.25-fold

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**Table 1:**

<table>
<thead>
<tr>
<th>Description</th>
<th>STZ Fold Change [Vs. Control]</th>
</tr>
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<tbody>
<tr>
<td>p21</td>
<td>0.14 ± 0.17*</td>
</tr>
<tr>
<td>p53</td>
<td>0.54 ± 0.09*</td>
</tr>
<tr>
<td>Jun</td>
<td>0.19 ± 0.12*</td>
</tr>
<tr>
<td>Bmp4</td>
<td>0.37 ± 0.24</td>
</tr>
<tr>
<td>Hspa1</td>
<td>0.49 ± 0.30</td>
</tr>
<tr>
<td>Lh4ra</td>
<td>0.33 ± 0.23</td>
</tr>
<tr>
<td>Nfkbia</td>
<td>0.42 ± 0.22</td>
</tr>
<tr>
<td>Rbp1</td>
<td>0.10 ± 0.14</td>
</tr>
<tr>
<td>Rbp2</td>
<td>0.45 ± 0.24</td>
</tr>
<tr>
<td>Tmep1</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>Wisp2</td>
<td>0.10 ± 0.11</td>
</tr>
</tbody>
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**Fig. 1.** Proliferation is enhanced in isolated diabetic fibroblasts. Diabetes was induced by streptozotocin (STZ) injection compared with vehicle-treated control rats. At 6 wk after STZ administration, cardiac fibroblasts were isolated from both groups and cultured. A: proliferation of cells in serum-free media. B: fibroblasts stimulated with serum. Data are means ± SE from 4 separate samples (n = 4). *Statistical significance vs. control + serum, P < 0.05.

**Fig. 2.** Decreased gene expression of cell cycle regulators in the diabetic myocardium. Six weeks after diabetes was induced, the animals were killed and RNA was extracted from the left ventricles of animals from both groups. Microarray analysis was performed to detect changes in signal transduction genes. A: fold change of downregulated genes in diabetic vs. control heart (control values are set to 1.0). B: statistically significant genes with decreased expression in diabetic heart. Data are mean ± SE fold change of 4 animals/group. *Statistical significance vs. control, P < 0.05.
more of the muscle intermediate filament desmin and 1.59 ± 0.25-fold more of the endothelial cell marker PECAM (Fig. 5, B and C). The fact that neither desmin nor PECAM decreased indicates that the decrease in α-SMA is attributable to a decrease in myofibroblasts. In addition, we utilized isolated fibroblast cultures from both control and diabetic hearts and observed a 4.78 ± 0.01-fold reduction of α-SMA in the diabetic fibroblasts versus the control fibroblasts (P < 0.01) (Fig. 6A).

DISCUSSION

The present study presents novel data demonstrating enhanced proliferation of diabetic fibroblasts, decreased expression of cell cycle regulators, and decreased appearance of myofibroblasts in the diabetic heart. Our overall goal was to uncover altered signaling in the diabetic myocardium and to determine how this disease state affects the resident fibroblasts. Historically, research has been focused on the influence of diabetes on the cardiac myocyte, with few studies revolving around the fibroblast. Diabetic patients suffer from insufficient wound healing; therefore it is important to study how the disease influences the key mediators of wound healing, the fibroblasts and myofibroblasts. In our study we present decreased mRNA expression of cell cycle mediators p53, p21, and jun, along with decreased p53 and Jun at the protein level. Our study also reveals increased diabetic fibroblast proliferation and decreased myofibroblast content in the diabetic myocardium.

The relationship between p21 and p53 and their regulation of the cell cycle has been well studied and documented; p53 is a tumor suppressor gene that signals to p21 to inhibit cyclin-dependent kinase (CDK)/cyclin activation and prevent cell cycle progression (31). Our data support cell cycle progression in the diabetic myocardium based on the observation of decreased p21 and p53. We also observed that fibroblasts isolated from diabetic myocardium are more proliferative compared with fibroblasts from control animals, and we revealed decreased p53 expression in fibroblasts isolated from diabetic hearts compared with control fibroblasts (Fig. 6B). Upstream of p53, Akt phosphorylates Mdm2, leading to transcriptional repression and proteasome-mediated degradation of p53 (12, 21, 33). Our data reveal an increased trend of p-Akt both in diabetic whole heart tissue and in isolated diabetic fibroblasts.
Fig. 5. Myofibroblast content is significantly decreased in diabetic hearts. Whole tissue lysates were subjected to SDS-PAGE and Western blot analysis. A: representative Western blot for α-smooth muscle actin (α-SMA) and summary graph of mean ± SE fold change including at least n = 4/group. B: representative desmin Western blot and summary graph of mean ± SE fold change for at least n = 4/condition. C: representative platelet endothelial cell adhesion molecule (PECAM)/CD31 Western blot and summary graph of mean ± SE fold change including at least n = 4/group. *Statistical significance vs. control, P < 0.05.

Fig. 6. Isolated diabetic fibroblast populations contain less myofibroblasts, less p53 expression, and increased Akt activity. Cardiac fibroblasts were isolated from the 6-wk diabetic and control groups. Cell lysates were separated by SDS-PAGE and Western blot analysis for α-SMA. Displayed are representative Western blots and summary graphs of mean ± SE fold change from n = 6. A: representative Western blot and summary graph for α-SMA. B: representative Western blot and summary graph for p53. C: representative Western blot and summary graph for p-Akt/Akt. *Significance vs. control, P < 0.05; #statistical significance vs. control, P < 0.01.
suggesting that it may mediate the p53 decreased mRNA expression and its protein degradation, enhancing cell cycle progression in the diabetic heart (Fig. 6C).

The present study examined cardiac fibroblast activation in response to diabetes and demonstrated decreases in p53 and p21, whereas recent studies have reported an increase in these cell cycle regulators in diabetic myocytes (10, 11, 14). Increased cardiac fibroblast proliferation may explain the decrease in p21 and p53 expression, since p53 and p21 must be degraded for the cell cycle to progress. Other labs have shown that high glucose stimulates fibroblast proliferation (3, 19), and we observed increased proliferation of fibroblasts isolated from diabetic versus control hearts. Also, the diversity of the models may explain our results since we observed a decrease of p53 in whole heart tissue that we postulate is from fibroblast activation, whereas others observed an increase in isolated myocytes that led to apoptosis (10, 14).

We have also determined that myofibroblast differentiation is reduced in the diabetic myocardium within the first 6 wk of induction. Since other studies reported increased myofibroblasts in the kidneys and increased circulating myofibroblast progenitor cells in diabetic patients (13, 20, 24), we originally hypothesized that diabetic hearts would contain increased myofibroblast content, which would account for the development of cardiac fibrosis. To our surprise, the diabetic myocardium contained significantly less α-SMA, indicating fewer myofibroblasts in the diabetic heart. To confirm that the decrease in α-SMA was from the fibroblast population and not vascular fibroblasts in the diabetic heart. To confirm that the decrease in α-SMA was from the fibroblast population and not vascular fibroblasts in the diabetic heart.

In our study we demonstrate enhanced diabetic cardiac fibroblast proliferation, decreased cell cycle mediators, along with a reduction of myofibroblasts in the diabetic myocardium. We postulate that the increased proliferation may be explained by the decreased expression of the cell cycle inhibitors p53 and p21. Although cardiac fibroblasts are active and proliferating in the diabetic myocardium, there is a decrease in differentiation to the myofibroblast phenotype. Overall our data reveal that diabetes significantly alters cardiac fibroblast activity and may provide a better understanding of the mechanisms that lead to impaired wound healing and cardiac fibrosis in the diabetic myocardium.

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GRANTS

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REFERENCES


