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

Patricia E. Shamhart,1,2 Daniel J. Luther,1,2 Ben R. Hodson,1 John C. Koshy,1 Vahagn Ohanyan,1 and J. Gary Meszaros1,2

1Department of Integrative Medical Sciences, Northeastern Ohio Universities College of Medicine, Rootstown; and 2Graduate Program, School of Biomedical Sciences, Kent State University, Kent, Ohio

Submitted 19 May 2009; accepted in final form 18 August 2009

Shamhart PE, Luther DJ, Hodson BR, Koshy JC, Ohanyan V, Meszaros JG. Impact of type 1 diabetes on cardiac fibroblast activation: enhanced cell cycle progression and reduced myofibroblast content in diabetic myocardium. Am J Physiol Endocrinol Metab 297: E1147–E1153, 2009. First published August 25, 2009; doi:10.1152/ajpendo.00327.2009.—Diabetic patients are prone to developing myocardial fibrosis and suffer from decreased wound healing capabilities. The purpose of this study was to determine whether diabetes alters cardiac fibroblast activity in the myocardium in a 6-wk streptozotocin-induced type 1 diabetic model. In vivo echocardiography indicated significant dilation of the left ventricle (LV) in the diabetic animals, while cardiac function was comparable to that in the normal group. We isolated cardiac fibroblasts from diabetic and control hearts and observed increased proliferation of the diabetic fibroblasts. Microarray analysis using mRNA collected from whole LVs revealed downregulation of known inhibitors of proliferation, p53 and p21, in the diabetic group, consistent with our proliferation data. Western blot analysis confirmed a reduction in p53 protein expression in the diabetic hearts compared with control. We explored the potential signaling underlying the downregulation of these cell cycle mediators and determined that activated Akt, a signal that inhibits p53, was elevated in the diabetic group. Surprisingly, the hearts from the diabetic group contained lower levels of the myofibroblast marker α-smooth muscle actin (α-SMA) and higher levels of desmin and platelet endothelial cell adhesion molecule (PECAM). The isolated fibroblasts from the diabetic group also contained significantly less α-SMA. These data suggest that early-stage diabetic hearts contain highly proliferative fibroblasts, which predisposes the diabetic myocardium to fibrosis, but have fewer myofibroblasts, which may compromise wound healing.

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 Biotechnology (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).

Address for reprint requests and other correspondence: J. G. Meszaros, Northeastern Ohio Universities College of Medicine, Dept. of Integrative Biomedical Sciences, 4209 State Route 44, Rootstown, OH 44272-0095 (e-mail: jgmeszar@neoucom.edu).

http://www.ajpendo.org 0193-1849/09 $8.00 Copyright © 2009 the American Physiological Society E1147
**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 TRIzol. 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 190 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 viability 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.

**Western blot analysis.** Hearts were removed from animals in all groups 6 wk after diabetic induction. Protein samples were collected and quantified with the bichinchoninic acid (BCA) method as previously described (5, 18). Equal amounts of protein were mixed with 2× sample buffer (100 mM Tris base, 20% glycerol, 2% SDS, and 0.01% bromophenol blue), boiled for 5 min, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were stained with naphthol blue to confirm even loading of protein. Next the membranes were blocked in 0.1% Tween 20-Tris-buffered saline (TBS) containing either BSA or milk for 1 h at room temperature, incubated in primary antibody overnight, and washed three times in 0.1% Tween 20-TBS. The membranes were incubated in secondary antibody for 1 h at room temperature and washed five times in 0.1% Tween 20-TBS, and protein signals were detected with ECL Supersignal (Pierce). Band intensity was quantified by densitometric scanning with a Kodak 1D Digital Science Imaging System and was normalized to bands from the naphthol blue stain of the same membrane.

**Microarray analysis.** Total RNA was extracted from the heart with TRIZol. Total RNA was quantified, and 2 μg of total RNA was utilized to make biotin-labeled cRNA according to the SuperArray Turolabeling-AMP 2.0 protocol. We utilized the signal transduction Oligo GEArray HybTube microarray from SuperArray Bioscience according to the protocol provided. Briefly, the array membranes were prehybridized for 2 h at 60°C and then hybridized with 2 μg of biotin-labeled cRNA overnight at 60°C with gentle agitation. The array membranes were washed in an SDS solution with medium agitation and visualized on X-ray film via chemiluminescence. The images were quantitatively analyzed with GEArray Expression Analysis Suite software.

**Echocardiographic assessment.** In vivo heart function was assessed with a Vevo 770 system (VisualSonics; Toronto, ON, Canada) with a 710B-075 transducer (20–30 MHz) designed specifically for small-animal studies at a frame rate of 40–60 Hz. Animals were anesthetized with 2–2.5% sevoflurane via nose cone, hair was removed from the chest, and the animal was situated in the supine position on an adjustable platform equipped with ECG electrodes to monitor heart rate and respiratory rate. A rectal temperature probe was placed, and body temperature was carefully maintained between 37.0 and 37.5°C. Two-dimensional (2D), M-mode and pulsed-wave (PW) Doppler echocardiography images were obtained in parasternal short- and long-axis views and apical four-chamber view with standard techniques. M-mode and 2D images at the midpapillary level were obtained from the parasternal short-axis view. Mitral valve (MV) inflow was obtained from the apical four-chamber view followed by PW Doppler interrogation of MV inflow at the tips of MV leaflets. All measurements were averaged from at least three cardiac cycles. Calculations were completed offline with Vevo 770/3.0 software.

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism 4.0 statistical software. Statistical significance (P < 0.05) between groups was determined by one-way ANOVA.

**RESULTS**

**Blood glucose, body weight monitoring, and 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

---

**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>
</thead>
<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.

---

**Table 2. 2D echocardiographic measurements of left ventricular function and dimensions of rats 6 wk after induction of type 1 diabetes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWT</td>
<td>0.243±0.012</td>
<td>0.202±0.000*</td>
</tr>
<tr>
<td>LVVd, μl</td>
<td>399.31±30.87</td>
<td>398.66±20.62</td>
</tr>
<tr>
<td>LV Mass Corr, mg</td>
<td>791.75±49.25</td>
<td>588.75±18.50*</td>
</tr>
<tr>
<td>EF, %</td>
<td>72.08±2.09</td>
<td>72.77±2.02</td>
</tr>
<tr>
<td>FS, %</td>
<td>42.81±1.83</td>
<td>43.40±1.70</td>
</tr>
<tr>
<td>LVVd/LV Mass Corr</td>
<td>0.504±0.024</td>
<td>0.676±0.016†</td>
</tr>
</tbody>
</table>

Values are means ± SE. 2D, 2-dimensional; RWT, relative wall thickness; LV, left ventricular; LVVd, LV diastolic volume; LV Mass Corr, LV mass corrected for body weight; EF, ejection fraction; FS, fractional shortening. *Statistical significance, P < 0.05; †statistical significance, P < 0.01.
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 group versus control (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

<table>
<thead>
<tr>
<th>Description</th>
<th>STZ Fold Change</th>
</tr>
</thead>
<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>Hspca</td>
<td>0.49 ± 0.30</td>
</tr>
<tr>
<td>L14ra</td>
<td>0.33 ± 0.23</td>
</tr>
<tr>
<td>Nkbbia</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>Tmem41</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>Wisp2</td>
<td>0.10 ± 0.11</td>
</tr>
</tbody>
</table>

*Statistical significance vs. control, P < 0.05.

---

**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 en-
hanced proliferation of diabetic fibroblasts, decreased expres-
sion 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 de-
creased 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 prolifera-
tion and decreased myofibroblast content in the diabetic myo-
cardium.

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 de-
creased p21 and p53. We also observed that fibroblasts isolated
from diabetic myocardium are more proliferative compared
with fibroblasts from control animals, and we revealed de-
creased 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), 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 smooth muscle cells, we isolated fibroblasts from control and diabetic hearts and discovered decreased α-SMA in the diabetic fibroblast lysates, indicating fewer resident myofibroblasts from the diabetic hearts. This reduction of myofibroblasts may contribute to the insufficient wound healing ability of diabetic hearts and begin to explain why diabetic patients are less likely to survive a myocardial infarction (1, 32).

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.

ACKNOWLEDGMENTS
We thank Dr. Erik R. Olson and Dr. G. Timothy Bowden for generously providing the Akt antibodies.

GRANTS
This work was supported by the Northeastern Ohio Universities College of Medicine (NEOUCOM) Foundation DiYorio Diabetes Fund.

REFERENCES


