Heart Failure

Endothelial Cell–Derived Endothelin-1 Promotes Cardiac Fibrosis in Diabetic Hearts Through Stimulation of Endothelial-to-Mesenchymal Transition

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Background—Persistently high plasma endothelin-1 (ET-1) levels in diabetic patients have been associated with the development of cardiac fibrosis, which results from the deposition of extracellular matrix and fibroblast recruitment from an as-yet unknown source. The underlying mechanism, however, remains elusive. Here, we hypothesize that ET-1 might contribute to the accumulation of cardiac fibroblasts through an endothelial-to-mesenchymal transition in diabetic hearts.

Methods and Results—We induced diabetes mellitus in vascular endothelial cell–specific ET-1 knockout [ET-1f/f;Tie2-Cre (+)] mice and their wild-type littermates using the toxin streptozotocin. Gene expression and histological and functional parameters were examined at 8, 24, and 36 weeks after the induction of diabetes mellitus. Diabetes mellitus increased cardiac ET-1 expression in wild-type mice, leading to mitochondrial disruption and myofibril disarray through the generation of superoxide. Diabetic mice also showed impairment of cardiac microvascularization and a decrease in cardiac vascular endothelial growth factor expression. ET-1 further promotes cardiac fibrosis and heart failure through the accumulation of fibroblasts via endothelial-to-mesenchymal transition. All of these features were abolished in ET-1f/f;Tie2-Cre (+) hearts. Targeted ET-1 gene silencing by small interfering RNA in cultured human endothelial cells ameliorated high glucose–induced phenotypic transition and acquisition of a fibroblast marker through the inhibition of transforming growth factor-β signaling activation and preservation of the endothelial cell-to-cell contact regulator VE-cadherin.

Conclusions—These results provide new insights suggesting that diabetes mellitus–induced cardiac fibrosis is associated with the emergence of fibroblasts from endothelial cells and that this endothelial-to-mesenchymal transition process is stimulated by ET-1. Targeting endothelial cell–derived ET-1 might be beneficial in the prevention of diabetic cardiomyopathy. (Circulation. 2010;121:2407-2418.)

Key Words: cardiomyopathy ■ diabetes mellitus ■ endothelin ■ fibrosis ■ heart failure

Diabetes mellitus can affect cardiac structure and function, and this may lead to heart failure in the absence of coronary atherosclerosis and hypertension. However, despite the importance of this clinical entity, the multifactorial nature of the disease remains incompletely understood. Three decades have passed since Rubler et al first described patients with diabetic cardiomyopathy, and to date, a number of epidemiological, clinical, and experimental studies have confirmed the existence of this unique cardiomyopathy. Several mechanisms are considered to be important in the development of cardiac structural and ultrastructural changes, with hyperglycemia and altered cardiac metabolism being proposed as central to the pathophysiology of this disorder. Given the increased risk of heart failure and cardiovascular events in diabetic patients, a better understanding of the underlying mechanisms and additional therapeutic strategies would be of considerable value.

Clinical Perspective on p 2418

Endothelin-1 (ET-1), a 21–amino acid peptide, is known to be the most potent vasoconstrictor. In addition to its important role in regulating vascular tone, this peptide shows strong...
proliferative and mitogenic properties. Twenty years of efforts in translating endothelin research into clinical practice has resulted in the application of ET-1 receptor antagonist as one of the first-line therapies for patients with pulmonary arterial hypertension, and ET-1 blockade is currently also being considered as a potential strategy for the treatment of certain cardiovascular-, metabolic-, and neoplastic-related diseases. Persistently high plasma ET-1 levels in diabetic patients are associated with the development of cardiac fibrosis, which results from excess deposition of extracellular matrix and fibroblast recruitment. Recently, the benefit of endothelin receptor antagonist in attenuating diabetes mellitus–induced myocardial dysfunction in experimental studies was reported. Most of the aforementioned studies demonstrate attenuation of fibrosis, restoration of vascular hyperactivity, and improvement of cardiac function as the important features of ET-1 receptor blockade; however, such pharmacology-based studies are not able to identify the molecular mechanism linking ET-1 to diabetic cardiomyopathy.

Homozygous deletion of ET-1 in mice is a useful approach for investigating ET-1 signaling in embryonic development; however, early postnatal lethality caused by craniofacial abnormalities precludes the use of these mice in exploring the role of ET-1 in adult physiology and pathophysiology. Accordingly, to investigate the role of ET-1 in adult physiology and pathophysiology, we believe that these mice are suitable for investigating the role of ET-1 in adult physiology and pathophysiology. Accordingly, to investigate the precise role of ET-1 in mediating diabetes mellitus–induced cardiac injury, we developed a type 1 diabetes model in endothelial cell–specific ET-1 knockout mice and made the novel observation that diabetes mellitus–induced cardiac fibrosis is promoted by endothelial cell–derived ET-1 through stimulation of fibroblast accumulation via an endothelial-to-mesenchymal transition (EndMT).

Methods
Further detailed methodology is provided in the online-only Data Supplement.

Development of the Diabetes Model
In this study, we used 12- to 14-week-old heterozygous ET-1<sup>loxP</sup>;Tie2-Cre (++) mice and their wild-type (WT) littermates [ET-1<sup>loxP</sup>;Tie2-Cre (−/−)] as controls. A detailed description of how we generated the ET-1<sup>loxP</sup>;Tie2-Cre (++) mice will be published elsewhere (Y.Y.K. and M.Y., submitted manuscript, 2009). These mice have undetectable serum ET-1 levels and are resistant to neointimal formation after blood flow cessation through the attenuation of vascular inflammation. Because ET-1 is produced mainly in endothelial cells, we believe that these mice are suitable for investigating the role of ET-1 in adult physiology and pathophysiology. Accordingly, to investigate the precise role of ET-1 in mediating diabetes mellitus–induced cardiac injury, we developed a type 1 diabetes model in endothelial cell–specific ET-1 knockout mice and made the novel observation that diabetes mellitus–induced cardiac fibrosis is promoted by endothelial cell–derived ET-1 through stimulation of fibroblast accumulation via an endothelial-to-mesenchymal transition (EndMT).

Data Analysis
Results are presented as mean±SEM. Statistical analyses were performed with a paired or unpaired Student t test for direct 2-group comparisons and the Tukey-Kramer test after significant 1-way ANOVA F test for multiple-group comparisons.

Results
Characteristics of Mice With Streptozotocin-Induced Diabetes Mellitus
Injection of streptozotocin induced moderate to severe hyperglycemia in both mouse genotypes [ET-1<sup>loxP</sup>;Tie2-Cre (+) and WT mice], whereas the blood glucose in nondiabetic mice was maintained at a normal level (Table 1). We found that under baseline nontreatment conditions, ET-1<sup>loxP</sup>;Tie2-Cre (+) mice exhibit slight but significantly lower blood pressure than their WT littermates (110±1 versus 116±3 mm Hg for systolic blood pressure, respectively; P<0.01), which confirms our previous report. Hyperglycemia did not increase blood pressure in the early stage (8 weeks) of the disease. However, after 24 weeks of diabetes mellitus, we observed an increase in systolic blood pressure, with no significant statistical difference between the 2 genotypes. Diabetes mellitus also reduced the heart rate in both genotypes (Table 1), although no other abnormalities were observed on ECGs (data not shown).

Diabetes Mellitus Uregulates ET-1 Expression in the Heart
We investigated the effect of diabetes mellitus on cardiac ET-1 expression. Eight weeks of hyperglycemia increased ET-1 mRNA expression by 1.5-fold in the hearts of ET-1<sup>loxP</sup>;Tie2-Cre (−) mice, thereby exaggerating the
differences between these mice and the ET-1tif f;Tie2-Cre (+) mice (Figure 1A). Immunohistochemistry revealed an enhanced ET-1 signal in the epicardial coronary arterioles of diabetic ET-1tif f;Tie2-Cre (−) mice, which is consistent with the increase in cardiac ET-1 peptide levels measured by ELISA (445.67±62.48 versus 209.49±43.53 pg/g heart weight; respectively; Figure 1B and 1C).

Ultrastructural Changes in the Early Stage of Diabetes Mellitus

Electron microscopic observations revealed a significant disruption of mitochondria and myofibril disarray in the hearts of ET-1tif f;Tie2-Cre (−) mice. These abnormalities were not as pronounced in ET-1tif f;Tie2-Cre (+) hearts (Figure 2A and C, left). Because oxidative stress is proposed to be involved in high glucose–induced inhibition of myofibrillar formation and mitochondrial damage, we examined superoxide production by dihydroethidium staining.

Diabetes mellitus increased superoxide production in ET-1tif f;Tie2-Cre (−) hearts, whereas the lack of ET-1 in endothelial cells surprisingly suppressed diabetes mellitus–induced superoxide production, as shown by lower dihydroethidium intensity in ET-1tif f;Tie2-Cre (+) mice (Figure 2B and 2C, right). To confirm this finding, we also performed lucigenin-enhanced chemiluminescence assay (using 10 μmol/L per 1 L lucigenin concentration). The result shows that superoxide production in the diabetes ET-1tif f;Tie2-Cre (+) heart is increased significantly compared with the nondiabetes heart, but the level is still half that of WT mice (data not shown).

We further attempted to quantify the effect of oxidative stress in causing global cardiac DNA damage and observed slight (but significant) differences in the proportion of nuclei positive for 8-hydroxydeoxyguanosine (8-OHdG), which is higher in diabetes WT hearts compared with ET-1tif f;Tie2-Cre (+) hearts (Figure 2B, left, and 2C, middle).

### Table 1. Blood Glucose, Blood Pressure, and Heart Rate of Streptozotocin-Induced Diabetic Mice

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose, mg/dL</th>
<th>SBP, mm Hg</th>
<th>MBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ET-1tif f;Tie2-Cre (−)</td>
<td>129±11</td>
<td>116±3</td>
<td>90±7</td>
<td>77±8</td>
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<tr>
<td></td>
<td>ET-1tif f;Tie2-Cre (+)</td>
<td>126±5</td>
<td>110±1*</td>
<td>84±7</td>
<td>68±10</td>
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<tr>
<td>Diabetes after 8 wk</td>
<td>ET-1tif f;Tie2-Cre (−)</td>
<td>542±10*</td>
<td>113±2</td>
<td>83±3</td>
<td>72±5</td>
</tr>
<tr>
<td></td>
<td>ET-1tif f;Tie2-Cre (+)</td>
<td>533±16†</td>
<td>107±2</td>
<td>80±2</td>
<td>67±4</td>
</tr>
<tr>
<td>Diabetes after 24 wk</td>
<td>ET-1tif f;Tie2-Cre (−)</td>
<td>549±22*</td>
<td>131±2*</td>
<td>104±3*</td>
<td>91±4</td>
</tr>
<tr>
<td></td>
<td>ET-1tif f;Tie2-Cre (+)</td>
<td>544±25†</td>
<td>126±1†</td>
<td>91±2†</td>
<td>74±2</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; and HR, heart rate. n=8 mice for each group. All results are presented as mean±SEM. Data for the control (nondiabetic) mice were obtained after 24 weeks.

*P<0.01 versus ET-1tif f;Tie2-Cre (−) control group; †P<0.01 versus ET-1tif f;Tie2-Cre (+) control group. ‡P=NS versus ET-1tif f;Tie2-Cre (−) after 24 weeks of diabetes.
Attenuation of Cardiac Fibrosis and Restoration of Cardiac Microvascularization in ET-1f/f;Tie2-Cre (+) Mice

Diabetes mellitus induces cardiac fibrosis in the absence of coronary arteriole stenosis. Staining with Masson trichrome revealed marked interstitial and perivascular fibrosis in the late stage of diabetes mellitus in ET-1f/f;Tie2-Cre (+/H11002) mice compared with ET-1f/f;Tie2-Cre (+/H11001) mice (Figure 3A and B).

To address whether cardiac microvascularization is impaired in diabetic hearts, we examined capillary density by CD31 immunohistochemistry. We observed a significant reduction in microvascularization at both 8 and 24 weeks of diabetes mellitus. However, ET-1f/f;Tie2-Cre (+/H11001) mice showed a higher number of capillaries than their WT littermates (Figure 3A and B).

Figure 2. Cardiac abnormality in the early stage of diabetes mellitus (after 8 weeks of disease). Mitochondrial damage, myofibril disarray, and oxidative stress are pronounced in diabetic ET-1f/f;Tie2-Cre (−/H11002) hearts compared with ET-1f/f;Tie2-Cre (+/H11001) hearts. A, Representative transmission electron photomicrographs showing cardiac ultrastructural changes. Scale bars = 2 μm; High Voltage (HV) = 80 kv; direct magnification, ×1500. B, left, Detection of oxidative DNA damage with 8-OHdG immunostaining in diabetic heart. Nuclei positive for 8-OHdG were stained brown. Scale bars = 20 μm. Right, Detection of superoxide production by dihydroethidium (DHE) fluorescence staining. Superoxides are stained bright red. C, left, Quantification of ultrastructural abnormalities. Data are represented as an abnormality score ± SEM; n = 3 mice for each group; *P < 0.05 vs diabetic ET-1f/f;Tie2-Cre (−/H11002). ND indicates not determined (see the Methods section in the online-only Data Supplement for details). Middle, Quantification of 8-OHdG–positive nuclei (percent to total nuclei per field); n = 6 mice for each group; **P < 0.005 vs diabetic ET-1f/f;Tie2-Cre (−/H11002). Right, Quantification of DHE intensity; n = 6 to 8 mice for each group; *P < 0.001 vs diabetic ET-1f/f;Tie2-Cre (−/H11002).

We also evaluated the expression of profibrotic genes and observed upregulation of TGF-β and connective tissue growth factor (CTGF) mRNA expressions in ET-1f/f;Tie2-Cre (−/H11001) but not in ET-1f/f;Tie2-Cre (+/H11002) hearts (Figure 3G). Protein expression of TGF-β was also confirmed by Western blot (Figure 3H). These results suggest that diabetes mellitus–induced cardiac fibrosis is mediated in part by ET-1 through the activation of TGF-β and CTGF signaling pathways.

Cardiac Function Is Decreased in Diabetic Mice

We assessed cardiac function sequentially and observed an increase in left ventricular end-diastolic and end-systolic dimensions and a decrease in systolic function starting from 24 weeks of diabetes mellitus (Table 2). After 36 weeks of diabetes mellitus, ET-1f/f;Tie2-Cre (+/H11001) mice exhibited significantly smaller left ventricular end-diastolic dimension and higher fractional shortening than ET-1f/f;Tie2-Cre (−/H11002) mice (43.72 ± 0.35% versus 37.58 ± 1.11%, respectively; Figure 4A through 4C).
Fibrotic Areas Consist of ET-1–Producing Cells

We further investigated the precise mechanism that might explain how a lack of ET-1 could attenuate the development of cardiac fibrosis. Fibrosis in diabetic hearts might occur by the accumulation of fibroblasts and collagen or result in a form of replacement fibrosis characterized by necrotic tissue in the very late stage. Our first clues to the underlying mechanism of fibrosis were the marked presence of ET-1 and collagen in fibrotic areas (Figure 5A), which led us to speculate that it is ET-1– and collagen-producing cells that form the fibrotic areas. We then attempted to confirm the presence of fibroblasts using fibroblast-associated protein S100A4 (also called FSP1) as a marker for fibroblasts. Immunofluorescence staining showed that S100A4+ cells were present in fibrotic areas and that they were colocalized with staining for ET-1 and collagen-1 (Figure 5B).

EndMT as a Source of Fibroblast Accumulation in Diabetic Hearts

We next investigated the source of the fibroblasts observed in the fibrotic areas. We performed double immunofluorescence staining with antibodies to CD31 (as an endothelial cell marker) and S100A4/FSP1 (as a fibroblast marker). We observed colocalization of CD31 and S100A4/FSP1 expression in the endothelium layer of epicardial coronary arteries in ET-1f/f;Tie2-Cre mice, as well as in the interstitial tissue and microcapillary vessels. Other mesenchymal cells markers, α-smooth muscle actin (α-SMA) and vimentin, were also showing colocalization with CD31+ cells (Figure 6A). The double-positive immunofluorescence staining was examined by z-stack image analysis, which confirmed the specific overlay of CD31+/S100A4+ cells (Figure 6B). This suggests that endothelial
cells are undergoing phenotypic conversion and are beginning to acquire the fibroblast marker, a process referred to as EndMT. We further observed significantly higher S100A4/H11001 and S100A4/H11001 CD31/H11001 cells among total S100A4/H11001 cells in ET-1f/f;Tie2-Cre (H11002) hearts (Figure 6C and 6D). S100A4 and collagen 1/H9251 protein expressions were also upregulated; in contrast, endothelial cell adherent junction VE-cadherin protein was reduced in ET-1f/f;Tie2-Cre (H11002) hearts (Figure 6E and 6F).

Targeted ET-1 Gene Silencing Inhibits High Glucose–Induced EndMT in Cultured Endothelial Cells

To examine the molecular mechanism linking ET-1 to EndMT, we used HUVECs and human aortic endothelial cells for in vitro study and performed siRNA transfection to knockdown ET-1 expression in cultured endothelial cells. Treatment with 33 mmol/L glucose for 6 days induced morphological changes of HUVECs into spindle-shaped fibroblast-like cells (Figure 7A, top). Control (5 mmol/L glucose-treated) endothelial cells expressed VE-cadherin and CD31, whereas long-term high-glucose treatment reduced VE-cadherin and CD31 signals. Endothelial cells then began to express S100A4/FSP1 protein (Figure 7A, middle and bottom). All of these features were abolished when ET-1 was knocked down by siRNA transfection before high-glucose treatment. Optimal transfection of ET-1 siRNA resulted in an up to 70% reduction in ET-1 mRNA expression in HUVECs (Figure 7B); 100% knockdown of ET-1 may cause endothelial cell death in vitro (data not shown). Similar results were observed with human aortic endothelial cells (Figure I in the online-only Data Supplement), suggesting that EndMT may occur in both arterial and vein endothelial cells.
ET-1-Induced EndMT Is Mediated by Activation of TGF-β Signaling in Endothelial Cells

A recent study reported the involvement of TGF-β in EndMT and cardiac fibrosis.26 We evaluated TGF-β expression and observed its upregulation after treatment with high glucose or ET-1. Interestingly, knockdown of ET-1 inhibited the stimulation of TGF-β by high glucose (Figure 7C); conversely, treatment with TGF-β also increased ET-1 expression by almost 3-fold (Figure 7D), suggesting reciprocal regulation of ET-1 and TGF-β in endothelial cells. Further investigation of TGF-β downstream signaling pathways revealed an increase in the phosphorylation state of Smad3 and Akt and stabilization of Snail protein expressions in diabetes ET-1f/f;Tie2-Cre hearts (Figure 7E and 7F) and in high glucose–treated endothelial cell culture, which are abolished by knockdown of TGF-β with neutralizing antibody. Pretreatment with siRNA ET-1 was also effective in inhibiting activation of TGF-β and the aforementioned downstream signaling proteins. However, phosphorylation of Smad3 was not affected by the lack of ET-1 (Figure 7G). In addition, inhibition of Akt by the Akt inhibitor LY294002 was sufficient to prevent stabilization of Snail transcription factor (Figure 7H). We further observed that both knockdown of TGF-β and inhibition of Akt also prevented acquisition of mesenchymal markers in high glucose–treated HUVECs (Figure II in the online-only Data Supplement).

High Glucose–Induced Transitioned Endothelial Cells Are Able to Migrate to Surrounding Tissue

We attempted to confirm whether the transitioned endothelial cells migrated to surrounding tissue as we observed in the diabetic heart. We performed an in vitro scratch assay and discovered that endothelial cells in high glucose–treated HUVECs migrated faster than those in high glucose–treated cells with knockdown of ET-1 (Figure 8A). We further observed that cells with faster migration capacity are the transitioned endothelial cells (CD31+/S100A4+ cells; Figure 8B).

Discussion

Here, we report the novel insight that diabetes mellitus–induced cardiac fibrosis is associated with the emergence of fibroblasts from endothelial cells and that this EndMT is stimulated in part by endothelial cell–derived ET-1. In the early stage of diabetes mellitus, we documented pathological changes in the heart initiated by the stimulation of cardiac ET-1 expression mainly from endothelial cells, which induces oxidative stress and leads to ultrastructural abnormalities. In the late stage, upregulation of profibrotic gene expression and impairment of cardiac microvascularization by ET-1 contribute to the development of cardiac fibrosis and accelerate the progression to heart failure. All of these features were attenuated in mice lacking ET-1 in vascular endothelial cells. This attenuation is mediated via inhibition of TGF-β–induced EndMT, which is preceded by the loss of the cell-to-cell contact regulator VE-cadherin.

In normal hearts, ET-1 is produced by endothelial cells, cardiomyocytes, fibroblasts, and vascular smooth muscle cells.28 The upregulation of cardiac ET-1 expression in diabetes mellitus is consistent with other reports, whereas the unchanged ET-1 expression in ET-1f/f;Tie2-Cre (+) hearts suggests that diabetes mellitus stimulates ET-1 release only from endothelial cells. The latter observation may reflect the occurrence of endothelial dysfunction in an early stage of the
disease. Although the mechanism by which endothelial cell ET-1 is upregulated by diabetes mellitus remains elusive, a previous study suggests that chronic hyperglycemia may lead to protein kinase C activation, which in turn enhances ET-1 production.

High glucose has been reported to inhibit myofibrillar formation and to cause mitochondrial damage through excessive generation of oxidative stress, which is abolished by antioxidant treatment. In the present study, the myofibril disarray and mitochondrial disruption observed in diabetes

Figure 6. EndMT in cardiac fibrosis. In vivo evidence of endothelial cells undergoing phenotypic transition into mesenchymal cells was observed in ET-1f/f;Tie2-Cre (-) hearts, accompanied by a reduction in VE cadherin and upregulation of S100A4 protein. A, Double immunofluorescence staining of antibodies to CD31 (red) with antibodies to S100A4, vimentin, and α-SMA (green) in diabetic hearts. Colocalization of CD31 with S100A4, vimentin, and α-SMA expression in coronary arterioles is shown in yellow. Scale bars = 20 μm. B, Representative z-stack image analysis shows specific overlay of double immunostaining. CD31/S100A4- cells in specific ordinate were analyzed in z stack with optimal interval range of 0.8 μm. C and D, Quantification of S100A4, vimentin, and percentage of S100A4+/VE-cadherin+ cells in diabetic hearts; n = 6 to 8 mice for each group; *P < 0.01 vs ET-1f/f;Tie2-Cre (-). E and F, Western blot analysis shows S100A4, VE-cadherin, and collagen 1α protein in hearts normalized with β-actin; n = 4 to 6 mice for each group; *P < 0.01 vs ET-1f/f;Tie2-Cre (-).
WT hearts were mostly prevented in ET-1\textsuperscript{f/f},Tie2-Cre (+) mice, which may be associated with lower superoxide production in ET-1\textsuperscript{f/f},Tie2-Cre (+) hearts, as we observed by dihydroethidium staining and lucigenin-enhanced chemiluminescence. However, detection of DNA damage by 8-OHdG staining revealed the possibility of various types of reactive oxygen species other than superoxide alone. On the basis of this finding, we can only suggest that paracrine action of endothelial cell–derived ET-1 to cardiomyocytes may contribute to superoxide production, at least in very early stage of diabetes mellitus when endothelial dysfunction is present before pathological changes in cardiomyocytes.

Patients with diabetes mellitus have been shown to have poorer coronary collateral vessels,\textsuperscript{30} which are associated with impairment of the angiogenic response to ischemia as a result of a decrease in VEGF expression.\textsuperscript{31,32} This condition has been associated with a worsened prognosis in diabetic patients suffering from myocardial infarction.\textsuperscript{33} We observed a significant reduction in capillary density in all diabetic groups. Diabetic ET-1\textsuperscript{f/f},Tie2-Cre (+) mice exhibit a higher capillary density than their WT littermates, which is positively correlated with better preservation of VEGF expression. However, the definite proximate regulators linking ET-1 and VEGF signaling in diabetic hearts have not yet been identified.

In this study, development of cardiac fibrosis was attenuated in mice lacking ET-1 in endothelial cells. Initially, we observed that the upregulation of profibrotic gene expression
(TGF-β and CTGF) in the hearts of diabetic WT mice is inhibited in ET-1f/f;Tie2-Cre (+) mice. These growth factors have previously been reported to be regulated by protein kinase C-β2 in stimulating extracellular matrix accumulation and to induce collagen production from fibroblasts in diabetic hearts.34 Furthermore, in concordance with our data, treatment with an endothelin receptor antagonist was shown to attenuate the upregulation of TGF-β in diabetes experimental studies.12.15.16 Thus, ET-1 may act as an upstream regulator of TGF-β and CTGF expression in diabetic hearts.

Our finding relative to the enhanced ET-1 and collagen expressions in fibrotic areas is consistent with a previous study12 and leads us to speculate that it is ET-1– and collagen-producing cells that form the fibrotic tissue in diabetic hearts. We further observed that these particular cells are endothelial cells that are undergoing phenotypic transition and beginning to acquire fibroblast characteristics, a process referred to as EndMT. EndMT is widely known to be important in embryonic development of the heart because the primary source of mesenchyme in the atrioventricular canal is thought to be the endocardium.35 Studies using transgenic mice in which the endothelial cell lineage can be traced have confirmed this hypothesis.36.37 Recently, Zeisberg et al26 showed for the first time in adult tissues that endothelial cells can undergo EndMT and that these cells made up 27% to 35% of the total fibroblast pool in pressure overload–induced cardiac fibrosis. Similarly, we observed that 15% to 20% of fibroblasts coexpressed both the endothelial marker CD31 and the fibroblast marker S100A4/FSP1 in the hearts of diabetic WT mice, whereas CD31+/S100A4+ cells were rarely detected in the hearts of diabetic ET-1f/f;Tie2-Cre (+) mice, suggesting the significant role of endothelial cell–derived ET-1 in mediating EndMT. This CD31+/S100A4+ double labeling, as well as the presence of CD31+ /α-SMA+ and CD31+/vimentin+ cells, allowed characterization of the intermediate but not the late stage of EndMT.38.39 Thus, our observations may underestimate the actual number of fibroblasts that are of endothelial origin because in the late stage of diabetes mellitus, most of the EndMT-derived fibroblasts may have migrated into the surrounding tissue and lost their endothelial marker. The origin of such cells can be traced by lineage analysis using endothelial cell reporter gene constructs. Accordingly, this could be considered one of the limitations of the present study.

Although we did not evaluate the exact contribution of proliferating resident mesenchymal cells and circulating bone marrow–derived fibroblasts to cardiac fibrosis, our observations imply that the fibroblasts of endothelial cells origin play a significant role in the development of diabetes mellitus–induced cardiac fibrosis.

In our diabetes mellitus–induced cardiac fibrosis model, ET-1–induced EndMT is preceded by the loss of endothelial cell-to-cell contact, as shown by the decreased VE-cadherin expression. The disaggregated endothelial cells then start to alter their morphology, migrate to surrounding sites, exhibit endothelial marker depression, and acquire mesenchymal characteristics. This cascade of events reveals the importance of the preservation of endothelial cell integrity via the suppression of ET-1, thereby preventing the initiation of diabetes mellitus–induced EndMT.

As for the mechanistic insight, previous studies report the role of ET-1 in mediating the epithelial-to-mesenchymal transition–induced progression of ovarian cancer40 and alveolar-epithelial-to-mesenchymal transition in idiopathic pulmonary fibrosis41 and demonstrate the regulation of TGF-β and Smad signaling as the downstream signaling pathway by ET-1 in epithelial cells. Our results, which underline the importance of TGF-β in EndMT, are consistent with those of other studies; however, to the best of our knowledge, this is the first study to demonstrate the involvement of ET-1 in EndMT-induced fibrosis in diabetic hearts, in part by mediating high glucose–induced TGF-β expression and activation of Akt-Snail signaling, independently of Smad signaling pathway. For the decrease in phosphorylation of Smad3 protein that is observed in diabetes ET-1f/f;Tie2-Cre (+) heart but is not affected by knockdown of ET-1 in high glucose–treated
endothelial cells, we speculate that Smad3 transcription factors might be important in reciprocal regulation of ET-1 by TGF-β autocrine action in endothelial cells, as previously described by Rodríguez-Pascual et al.42

The other interesting phenomenon that we observed is that the high glucose–induced transitioned endothelial cells (CD31+/S100A4− cells) possess faster migration capacity compared with CD31+ endothelial cells with knockdown of ET-1. This in vitro result may provide a more detailed mechanism of how transitioned endothelial cells contribute to the accumulation of fibroblasts in perivascular areas that we observed in diabetic heart. Thus, we proposed the cascade mechanism of high-glucose ET-1–induced EndMT in Figure III in the online-only Data Supplement.

In addition, these findings provide evidence to support the supposition that the cause of cardiac microvascularization impairment in our model may extend beyond its relationship to the decrease in VEGF expression. EndMT may contribute significantly to the loss of microvascular endothelial cells, which in turn leads to rarefaction and a reduced capillary density in diabetic hearts. This is in agreement with the hypothesis suggested by Goumans et al.43

Conclusions

We report novel findings indicating the involvement of endothelial cell–derived ET-1 in promoting diabetes mellitus–induced cardiac fibrosis through the stimulation of EndMT. We accordingly suggest that targeting endothelial cell–derived ET-1 might be beneficial for preventing diabetic cardiomyopathy.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Despite optimal treatment with current standard therapy, the high risk of heart failure and major cardiovascular events remains an unresolved problem for patients with diabetic cardiomyopathy. Thus, a better understanding of the underlying mechanisms and additional therapeutic strategies is needed. A persistently high plasma endothelin-1 (ET-1) level in diabetic patients is associated with the development of cardiac fibrosis. Considering the unfavorable results from clinical trials using ET-1 receptor blockade in heart failure patients—in contrast to its successful application in pulmonary arterial hypertension patients—we conducted more detailed investigation into the role of specific cell-derived ET-1. The importance of endothelial cells as a major source of ET-1 and the contribution of endothelial dysfunction in the natural history of diabetes cardiovascular complications led us to use endothelial cell–specific ET-1 knockout mice that we generated previously. In a type 1 diabetes model, we made the novel observations that diabetes mellitus–induced cardiac fibrosis is associated with the emergence of fibroblasts from endothelial cells and that this so-called endothelial-to-mesenchymal transition is stimulated by endothelial cell–derived ET-1. This cardiac fibrosis, along with ET-1-induced ultrastructural abnormalities, oxidative stress, and impairment of cardiac microvascularization, contributes to the acceleration of heart failure, which is ameliorated in mice lacking ET-1 in endothelial cells. We therefore suggest that targeting endothelial cell–derived ET-1 might be beneficial in the prevention of diabetic cardiomyopathy.
Endothelial Cell–Derived Endothelin-1 Promotes Cardiac Fibrosis in Diabetic Hearts Through Stimulation of Endothelial-to-Mesenchymal Transition
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SUPPLEMENTAL MATERIAL
Supplemental Methods

Non-invasive analysis of blood pressure and cardiac function

Blood pressure was measured using the tail cuff method in conscious mice (Muromachi Kikai, Japan). Mean values were determined from at least 5 measurements for each mouse. Left ventricular dimension and cardiac function were assessed over several time courses by echocardiography (Envision, Philips). Two-dimensional parasternal short-axis images were obtained, and targeted M-mode tracings at the level of the papillary muscles were recorded (sweep speed of 150 mm/s). Examinations were performed under light anesthesia induced by pentobarbital 50 mg/kg BW. Electrocardiograms were recorded using an AD instrument (Nihon Kohden, Tokyo, Japan).

Histological analysis

After euthanization, hearts were harvested, embedded in optimum cutting temperature (OCT) compound, and directly snap frozen in liquid N₂ or dry ice-cooled acetone. Frozen-section slides (4 µm) were stained with hematoxylin-eosin, Masson’s trichrome, and Sirius red. Photomicrographs were analyzed using NIH image software version CIRCULATIONAHA/2009/907386/S2
1.37 (http://rsb.info.nih.gov/ij/). Perivascular fibrosis was calculated as the ratio of the fibrotic area surrounding the vessels to the total vessel area. Quantifications of other fibrosis-related markers were made within the fibrotic areas.

**Electron microscopy**

Fresh heart tissues were cut into 1–2-mm slices, fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M cacodylate buffer solution for 2 h at room temperature, and then subjected to a standard block preparation for transmission electron microscopy. For quantification of morphological abnormalities; 9 samples were randomly selected from each animal group, and 2 pathologists blindly examined the ultrastructural features of the whole area under an electron microscope. Morphological abnormalities were classified into the following 3 groups: 0, not significant; 1, slight; and 2, marked. When there was discordance between the observers, the sample was immediately re-evaluated.

**Immunostaining**

Expression and localization of various target genes were observed using appropriate immunohistochemical, immunofluorescence, and immunocytochemical methods. The
primary antibodies used in this study were as follows: ET-1 (Peninsula Laboratories),
CD31 (BD Pharmingen), S100A4/FSP1 (Abcam), α-SMA clone 1A4 (Sigma),
dyhydroethidium (Molecular Probes, Invitrogen), VE-cadherin (Santa Cruz
Biotechnology), collagen 1A1 (Santa Cruz Biotechnology), and vimentin (Santa Cruz
Biotechnology). 4′-6-Diamidino-2-phenylindole (DAPI; Invitrogen) was used for nuclear
staining, and CD31/PECAM-1 (Santa Cruz Biotechnology) was used for
immunocytochemistry in human umbilical vein endothelial cells (HUVECs) and human
aortic endothelial cells (HAECs).

Real-time PCR

RNA was isolated from heart tissue, HUVECs and HAECs using Isogen (Nippon Gene).
Quantitative real-time PCR was performed using Superscript™ III Platinum® One-Step
Quantitative RT-PCR (Invitrogen), a One Step SYBR® Primescript™ RT-PCR Kit II
(TaKaRa), and an ABI 7500 RT PCR System.

Western blotting

Proteins extracted from heart tissue and cultured endothelial cells were separated on
SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) transfer membrane, and
probed with VE-cadherin polyclonal antibody (Santa Cruz Biotechnology), S100A4 polyclonal antibody (Abcam), TGF-β polyclonal antibody (Cell Signaling), phospho-Smad3 polyclonal antibody (Cell Signaling), Smad3 polyclonal antibody (Cell Signaling), phospho-Akt Ser 473 (Cell Signaling), Akt (BD Pharmingen), Snail monoclonal antibody (Cell Signaling) and anti-β-actin antibody (Invitrogen). Proteins were visualized using an ECL Plus WB detection system (Amersham-GE Healthcare). Blots were quantified by densitometry using ImageJ (NIH Image).

*Measurement of ET-1 peptide*

ET-1 peptide was extracted from plasma and heart tissue and measured using an ELISA kit (R&D Systems).

*Reverse-transcriptase PCR*

cDNA was obtained from heart tissue RNA, and semiquantitative RT-PCR was performed using the Superscript First-Strand Synthesis System (Invitrogen).
Targeted ET-1 gene silencing by siRNA and knockdown of TGF-β inhibits high glucose-induced EndMT in HAECs. Figure shows double immunofluorescence staining with antibodies to CD31 and S100A4. Results are from 3 independent experiments.
Supplemental Figure 2. Involvement of TGF-β - Akt signaling in EndMT

Knockdown of TGF-β and treatment with Akt inhibitor LY294002 inhibits high glucose-induced EndMT in HUVECs. Figure shows double immunofluorescence staining with antibodies to CD31 (red) and S100A4 (green). Nuclei were counterstained with DAPI (blue). Results are from 3 independent experiments.
Supplemental Figure 3. Proposed mechanism of ET-1 induced EndMT in diabetic heart

The scheme illustrates our proposed mechanism that hyperglycemia-induced ET-1 production in ECs stimulates activation of TGF-β signaling and promotes EndMT.