Molecular Cardiology

Thioredoxin-1 Gene Therapy Enhances Angiogenic Signaling and Reduces Ventricular Remodeling in Infarcted Myocardium of Diabetic Rats

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Background—The present study evaluated the reversal of diabetes-mediated impairment of angiogenesis in a myocardial infarction model of type 1 diabetic rats by intramyocardial administration of an adenoviral vector encoding thioredoxin-1 (Ad.Trx1). Various studies have linked diabetes-mediated impairment of angiogenesis to dysfunctional antioxidant systems in which thioredoxin-1 plays a central role.

Methods and Results—Ad.Trx1 was administered intramyocardially in nondiabetic and diabetic rats immediately after myocardial infarction. Ad.LacZ was similarly administered to the respective control groups. The hearts were excised for molecular and immunohistochemical analysis at predetermined time points. Myocardial function was measured by echocardiography 30 days after the intervention. The Ad.Trx1-administered group exhibited reduced fibrosis, oxidative stress, and cardiomyocyte and endothelial cell apoptosis compared with the diabetic myocardial infarction group, along with increased capillary and arteriolar density. Western blot and immunohistochemical analysis demonstrated myocardial overexpression of thioredoxin-1, heme oxygenase-1, vascular endothelial growth factor, and p38 mitogen-activated protein kinase-β, as well as decreased phosphorylated JNK and p38 mitogen-activated protein kinase-α, in the Ad.Trx1-treated diabetic group. Conversely, we observed a significant reduction in the expression of vascular endothelial growth factor in nondiabetic and diabetic animals treated with tin protoporphyrin (SnPP, a heme oxygenase-1 enzyme inhibitor), even after Ad.Trx1 therapy. Echocardiographic analysis after 4 weeks of myocardial infarction revealed significant improvement in myocardial functional parameters such as ejection fraction, fractional shortening, and E/A ratio in the Ad.Trx1-administered group compared with the diabetic myocardial infarction group.

Conclusions—This study demonstrates for the first time that impairment of angiogenesis and myocardial dysfunction can be regulated by Ad.Trx1 gene therapy in streptozotocin-induced diabetic rats subjected to infarction. (Circulation. 2010; 121:1244-1255.)

Key Words: myocardial infarction ■ diabetes mellitus ■ angiogenesis ■ oxidative stress ■ thioredoxins ■ gene therapy ■ echocardiography

Diabetic individuals often experience complications such as coronary artery disease, peripheral artery disease, and stroke that contribute to the morbidity and mortality of these subjects. This higher incidence of cardiovascular complications and the unfavorable prognosis among diabetic individuals who develop such complications has been correlated to diabetes-mediated impairment of angiogenesis. Inhibited angiogenesis is known to contribute to impaired coronary collateral vessel formation and wound healing. Various studies have linked diabetes-mediated impaired angiogenesis to improper degradation of the basement membrane, alterations to the delicate balance of angiogenic growth factors and cytokines that regulate vascular stability, problems in signal transduction, and, to a large extent, diabetes-mediated oxidative stress. Evidence suggests that reactive oxygen species (ROS)–mediated oxidative stress is the primary contributor to vascular and myocardial dysfunction, compromising recovery from such conditions.

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Maintenance of a balanced redox status in the microenvironment might aid in alleviating many diabetes-mediated complications.
complications, especially those related to impaired angiogenesis. Balanced and responsive antioxidant systems are vital for proper regulation of the redox status of the cell. Cells are normally able to defend themselves against oxidative stress–induced damage by use of several antioxidant systems.6 The thioredoxin system is one of the main ubiquitously expressed thiol-reducing antioxidant systems. The classic 12-kDa cytosolic thioredoxin-1 (Trx1) is the most studied of the different forms of thioredoxin.5,6 Trx1 has been described as a growth regulator, a transcriptional factor regulator, and a cofactor, apart from its very important antioxidative role.7

Key biological activities of Trx1 include antioxidative, antiapoptotic, and growth-stimulatory properties through its interactions with nuclear factor-κB, apoptosis signal regulation kinase-1, and hypoxia inducible factor-1α.8–10 Induction of apoptosis signal regulation kinase-1 is known to activate the proapoptotic JNK and p38 mitogen-activated protein kinase (MAPK) signaling pathways.11 In addition, it was reported that transgenic mouse hearts overexpressing Trx1 displayed significantly improved posts ischemic ventricular recovery, reduced myocardial infarct size, and resistance to ischemic reperfusion injury compared with hearts from wild-type mice.7,12 Inhibition of Trx1 expression in the heart has been associated with increased oxidative stress and apoptosis.13 Several reports suggest the contribution of the thioredoxin system in the upregulation of heme oxygenase-1 (HO-1) protein levels and HO-1 promoter activity under conditions associated with inflammation and increased oxidative stress.14,15 HO-1 is known to be a stress-response protein that has important redox regulatory functions.15,16 Some of our recent studies have demonstrated that the mechanism responsible for the enhanced angiogenesis and the cardioprotective effect of resveratrol and sildenafil operates through Trx1, HO-1, and vascular endothelial growth factor (VEGF).16–18 We also observed that resveratrol alleviates cardiac dysfunction in streptozotocin-induced diabetic rats through the induction of Trx1, HO-1, and VEGF.17 We recently reported the possible role of decreased VEGF in the pathogenesis of diabetes-mediated impairment of angiogenesis in the myocardium.19 The process of angiogenesis was shown to be highly impaired in diabetic mouse models of hindlimb ischemia and wound healing owing to decreased expression of VEGF.20,21 Moreover, Trx1 has been shown to promote the expression and activity of hypoxia inducible factor-1α, which leads to elevated expression of VEGF and thus enhanced vascular growth.10

There have been several attempts at the preclinical and clinical levels to induce myocardial angiogenesis by overexpressing angiogenic factors such as VEGF in the peri-infarct zone after myocardial infarction (MI).20,21 Recently, we showed that intramyocardial coadministration of a combination of adenoviral vectors encoding VEGF and angiopoietin-1 induced and stabilized the process of angiogenesis in ischemic diabetic myocardium and reduced ventricular remodeling, which led to cardioprotection.19

In view of the increased functional severity of cardiac failure in diabetic post-MI subjects, the given role of oxidative stress–mediated reduction in myocardial angiogenesis, and the antioxidative, growth regulatory, and angiogenic potential of Trx1 via induction of VEGF, we hypothesized that overexpression of Trx1 alone in the myocardium could prove to be useful for prophylaxis of subsequent heart failure after diabetes-associated MI. Therefore, to address the importance of Trx1 in angiogenesis in vivo during myocardial ischemia in a diabetic scenario and identify a potential therapeutic tool, we investigated the effect of Trx1 gene therapy with an adenoviral vector carrying the Trx1 gene in the MI model of streptozotocin-induced diabetic rats. In the present study, we investigated the effect of Trx1 gene delivery on myocardial fibrosis, oxidative stress, cardiomyocyte and endothelial cell apoptosis, capillary and arteriolar density, and left ventricular remodeling in diabetic rats. We demonstrate for the first time that the myocardium can be rescued from diabetes-related impairment of angiogenesis, severity of functional disorder, and subsequent heart failure by Trx1 gene therapy in streptozotocin-induced diabetic rats.

Methods

Experimental Animals

The present study was performed in accordance with the principles of laboratory animal care formulated by the National Society for Medical Research and in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (publication No. 85–23, revised 1985). The experimental protocol was approved by the Institutional Animal Care Committee of the University of Connecticut Health Center (Farmington, Conn). Male Sprague-Dawley rats (weight 300 to 325 g) were randomly separated into normal and diabetic groups as they received an intraperitoneal injection of vehicle (0.1 mol/L citrate buffer, pH 4.5) alone or streptozotocin at a dosage of 65 mg/kg body weight dissolved in 0.1 mol/L citrate buffer. Five days after streptozotocin injection, hyperglycemia was documented by measuring the glucose content of tail-vein blood with the FreeStyle Flash glucose-monitoring system (Abbott Diabetes Care, Alameda, Calif). Rats with blood glucose concentrations ≥300 mg/dL were used for the study.

Experimental Design

MI was induced in the diabetic animals 30 days after the induction of diabetes, as described previously.16 Age-matched nondiabetic animals were used as comparable controls. Sham-operated rats underwent thoracotomy and pericardectomy without MI. The rats were randomized into 8 groups: (1) Control sham (CS), (2) control plus MI (CMI), (3) CMI plus Ad.LacZ (CMI-AdLacZ), (4) CMI plus Ad.Trx1 (CMI-AdTrx1), (5) diabetic sham (DS), (6) diabetic plus MI (DMI), (7) DMI plus Ad.LacZ (DMI-AdLacZ), and (8) DMI + Ad.Trx1 (DMI-AdTrx1).

The adenoviral vector encoding Trx1 (Ad.Trx1, 1 × 10⁹ plaque-forming units)22 was administered intramyocardially (in 100 μL of PBS, with a 30-g needle) at 4 different sites into the left anterior free wall adjacent to the point of ligation (border zone surrounding the infarct) immediately after an MI was induced. Adeno-LacZ (Ad. LacZ, 1 × 10⁹ plaque-forming units)22 was used as the control. Ad.Trx1 and Ad.LacZ were generous gifts from Dr J. Sadoshima, New Jersey Medical School, Newark, NJ.15,23

In the present study, we used streptozotocin-induced diabetic rats, and MI was induced 30 days after the induction of hyperglycemia. These rats tend to lose weight, become weak, and show less physical activity and their vital sign indices deteriorate even before the first surgical intervention. There was a significant reduction in heart rate in these animals in the sham group and after MI.19 Overall, there was a higher rate of mortality in the diabetic animals after MI (45% to 52%) than in the nondiabetic MI controls (8% to 12%). Although we considered a second thoracotomy for gene delivery at a later time after the induction of MI, because of the higher rate of mortality and...
the stress and pain that the diabetic animals have to undergo, we
decided to administer the adenoviral vectors immediately after MI.

Another group of nondiabetic and diabetic animals were treated with
tin protoporphyrin IX (SnPP, an inhibitor of HO-1 activity; 50 μmol/kg IP) to determine whether HO-1 plays an important role in
Trx1-mediated VEGF expression.8 These SnPP-treated animals were
then subjected to MI and treated with Ad.Trx1 as described above.

For detailed descriptions of the materials and methods and details
on the time point of harvesting the tissue for various experiments,
please refer to the online-only Data Supplement.

Statistical Analysis
All data were analyzed with the statistical software GraphPad Prism
5.0’ (San Diego, Calif). Statistical analysis was performed with
1-way ANOVA. Post hoc comparisons between groups were per-
formed by Newman-Keuls multiple comparison test. Results are
presented as mean±SEM, with P≤0.05 used to indicate statistical
significance.

Results
Transfection Efficiency of Ad.LacZ and Ad.Trx1 in
Human Umbilical Vein Endothelial Cells
To examine the transfection efficiency of Ad.LacZ and
Ad.Trx1, cytochemistry was performed after 48 hours to examine the extent of β-galactosidase (β-gal) and immuno-
cytochemistry for Trx1 expression in the cells. Figure 1A and
1B represent expression of β-gal and Trx1, respectively, in
Ad.LacZ- and Ad.Trx1-treated and untreated human umbilical
vein endothelial cells (HUVECs). Significant β-gal staining was
also observed in Ad.LacZ-treated HUVECs (Figure 1A). A
significant increase in expression of Trx1 was observed in
Ad.Trx1-treated cells compared with untreated HUVECs
(Figure 1B).

Effect of Ad.Trx1 Gene Transfection on
Tubulogenesis in HUVECs
To determine whether Ad.Trx1 gene transfection has angi-
genic potential, we performed an in vitro Matrigel assay with
HUVECs to analyze the extent of tube formation, as shown in
Figure 1C. We observed a significant increase in tubu-
genesis in the Ad.Trx1-treated HUVECs compared with the
Ad.LacZ-treated control. This Ad.Trx1-mediated tubulogen-
esis was found to be significantly abolished when HUVECs
were transfected with Ad-sh-Trx1 (adenovirus vector-sh-Trx1
encoding small hairpin RNA that inhibits Trx1 expression)
along with Ad.Trx1 (Figure 1C), thereby documenting the
angiogenic potential of Trx1.

Ad.Trx1 Transfection Increased the Expression of
Trx1, HO-1, and VEGF in HUVECs
The present in vitro Matrigel assay experiments documented the
angiogenic potential of Trx1. To examine the molecular basis of the angiogenic potential of Ad.Trx1 treatment in
HUVECs, we examined the effect of Ad.Trx1 transfection on
the expression levels of HO-1 and VEGF (n=3 per group).
We observed a significant increase in the expression of Trx1
(8.7-fold; Figure 1D and 1E), HO-1 (9.2-fold; Figure 1D and
1F), and VEGF (1.8-fold; Figure 1D and 1G) in HUVECs
treated with Ad.Trx1 compared with Ad.LacZ-treated cells.
However, when the cells were transfected with Ad.Trx1 and
with SnPP, we observed a significant reduction in

VEGF expression (2-fold) even though there was no signif-
icant change in Trx-1 or HO-1 expression compared with the
Ad.Trx1-treated group (Figure 1D through 1G).

Histochemistry for the Transfection Efficiency of
Ad.LacZ and Ad.Trx1 (In Vivo)
After we verified the angiogenic potential of Trx1 and the
molecular basis of this angiogenic capacity, we intended to
study the effect of Ad.Trx1 gene therapy in the infarcted
diabetic myocardium. To examine the efficiency of our gene
transfer technique in vivo, we evaluated adenoviral gene expres-
sion 4 days after intramyocardial adenoviral gene administration
in the sham-operated nondiabetic control groups. We injected the
adenoviral vector encoding β-gal (Ad.LacZ) and Trx1 (Ad.Trx1)
at 4 different sites (in 100 μL of PBS, using a 30-g
needle) into the left anterior wall of the myocardium in
nondiabetic sham animals (n=3 per group). We observed
robust infection and expression of the respective proteins in
the myocardium as assessed by X-gal staining (Figure 1H)
and Trx1 immunohistochemical staining (Figure 1I) in the
myocardium surrounding the sites of gene transfer. β-Gal
expression was not evident in remote areas such as the
septum and the right ventricular muscles.

Biological Effects of Trx1 Gene Therapy in
Diabetic Ischemic Myocardium
Trx1 Gene Therapy Reduced Myocardial Fibrosis
Four days after MI and gene therapy, hearts (n=3 per group)
were harvested, and Masson trichrome staining was per-
formed on paraffin-embedded tissue sections to visualize the
infarct scar (Figure 2A). There was no evident difference in
infarct scar extension or thickness in Ad.LacZ-treated CMI-
AdLacZ and DMI-AdLacZ compared with their respective
nontreated controls (CMI and DMI, respectively). After Trx1
gene therapy, the nondiabetic CMI-AdTrx1 group showed a
thicker, more muscular infarct, a smaller scar region, and
more islands of viable cardiac tissue than the CMI group.
Similarly, the diabetic MI group that received Trx1 gene
therapy (DMI-AdTrx1) demonstrated an obviously smaller
scar region and more islands of viable cardiac tissue than the
DMI group.

Thirty days after MI and gene therapy, hearts were har-
vested, and Masson’s trichrome staining was performed on
paraffin-embedded tissue sections to visualize the extent of
collagen deposition and myocardial fibrosis (Figure 2B).
Infarct scar extension and thinning of the infarct were evident
30 days after MI in the DMI groups compared with the CMI
group. There was no marked difference in the extent of
collagen deposition or myocardial fibrosis in Ad.LacZ-treated
CMI-AdLacZ and DMI-AdLacZ rats with their respective
nontreated controls (CMI and DMI, respectively). There was a less prominent scar extension, less collagen
deposition, and less fibrosis in Ad.Trx1-treated nondiabetic
(CMI-AdTrx1) and diabetic (DMI-AdTrx1) rats than in CMI
and DMI rats, respectively.

Trx1 Gene Therapy Reduced Oxidative Stress
in Myocardium
The amount of ROS-mediated oxidative stress was deter-
mined 4 days after MI and gene therapy by evaluation of
superoxide anion (O$_2^-$) formation by means of dihydroethidium staining (n = 3 to 4 per group; Figure 2C and 2D). MI alone increased levels of ROS in the nondiabetic MI (CMI) and nondiabetic Ad.LacZ-treated MI (CMI-AdLacZ) groups compared with the nondiabetic sham (control sham) group. However, this increase in myocardial ROS was exacerbated in all of the diabetic MI groups compared with their respective nondiabetic controls. Myocardial ROS levels increased significantly in the diabetic sham group compared with the nondiabetic sham (control sham) group. The induction of MI significantly intensified the formation of ROS in the DMI and DMI-AdLacZ groups compared with the diabetic sham group. Although there was a reduction in MI-induced ROS levels in the CMI-AdTrx1 group compared with the CMI and CMI-AdLacZ
groups, the change was not significant. However, Ad.Trx1 gene therapy significantly reduced ROS levels in the treated DMI-AdTrx1 myocardium compared with the DMI and DMI-AdLacZ myocardium, thereby reducing ROS-mediated oxidative stress (Figure 2C and 2D).

**Trx1 Gene Therapy Significantly Reduced Cardiomyocyte and Endothelial Cell Apoptosis**

To understand whether Trx1 gene therapy was paralleled by a reduction in cardiomyocyte and endothelial cell apoptosis, we performed a terminal dUTP nick end-labeling (TUNEL) assay followed by antibody staining with either anti-α-sarcomeric actin or anti-von Willebrand factor (Figure 3A through 3D) to measure cardiomyocyte and endothelial cell apoptosis, respectively (n=4 per group). We observed a significant increase in apoptotic cardiomyocytes (520±92.6 versus 306±16.3 cells/100 HPF) and endothelial cell (650±90.5 versus 369±38.7 cells/100 HPF) apoptosis in the DMI group compared with the CMI group. There was no significant difference in the number of apoptotic cardiomyocytes or endothelial cells between the Ad.LacZ-treated CMI-AdLacZ or DMI-AdLacZ group and their respective nontreated controls (CMI or DMI). However, Trx1 gene therapy significantly reduced cardiomyocyte (119±21.5 versus 306±16.3 cells/100 HPF) and endothelial cell (163±15.4 versus 369±38.7 cells/100 HPF) apoptosis in the CMI-AdTrx1 group compared with the CMI group. Similarly, Trx1 gene therapy in the DMI-AdTrx1 group significantly reduced cardiomyocyte (204±32.8 versus 520±92.6 cells/100 HPF) and endothelial cell (208±32.8 versus 650±90.5 cells/100 HPF) apoptosis compared with the DMI group (Figure 3A through 3D).

**Trx1 Gene Therapy Increased Capillary and Arteriolar Density**

We performed immunohistochemical analysis of CD31/PECAM-1 (platelet and endothelial cell adhesion molecule, an endothelial cell marker) for capillary density (counts/mm²) and -smooth muscle actin for arteriolar density (counts/mm²) (n=4 per group; Figure 3E through 3H). There was a significant reduction in capillary density (1773±126 versus 2415±105; Figure 3E and 3F) and arteriolar density (13.4±1.4 versus 23.7±1.3; Figure 3G and 3H) in the DMI group compared with the CMI group. There was no significant difference in capillary or arteriolar density between the Ad.LacZ-treated CMI-AdLacZ or DMI-AdLacZ group and their respective nontreated controls (CMI or DMI). However, Trx1 gene therapy significantly increased capillary density (13.4±1.4 versus 23.7±1.3; Figure 3G and 3H) in the DMI group compared with the CMI group (Figure 3E through 3H).
and arteriolar (38.4±1.7 versus 23.7±1.3; Figure 3G and 3H) density in the CMI-AdTrx1 group compared with the CMI group. Similarly, a significant increase in capillary density (2450±153 versus 1773±126; Figure 3E and 3F) and arteriolar density (30.6±1.5 versus 13.4±1.4; Figure 3G and 3H) was observed with Trx1 gene therapy in the DMI-AdTrx1 group compared with the DMI group.
Molecular Basis for the Angiogenic and Cardioprotective Effect of Trx1 Gene Therapy in Diabetic Ischemic Myocardium

Trx1 Gene Therapy Significantly Increased Expression of Trx1, HO-1, and VEGF

Immunohistochemical analysis was performed for protein expression of Trx1, HO-1, and VEGF 4 days after MI and gene therapy (n=3 to 4 per group). We observed an evident reduction in the expression of Trx1 (Figure 4A), HO-1(Figure 4B), and VEGF (Figure 4C) in diabetic sham-operated and MI-induced (DMI and DMI-AdLacZ) groups compared with their respective nondiabetic sham-operated (control sham) and MI-induced (CMI and CMI-AdLacZ) controls. However, there was an evident increase in the expression of Trx1, HO-1, and VEGF in the Ad.Trx1-administered CMI-AdTrx1 group compared with the CMI or CMI-AdLacZ controls. Similarly, Trx1 gene therapy resulted in increased expression of these proteins in the DMI-AdTrx1 group compared with the DMI and DMI-AdLacZ groups (Figure 4A through 4C).

Western blot analysis 4 days after gene therapy (n=3 to 4 per group) also revealed that Trx1 gene therapy in the CMI-AdTrx1 group significantly increased the expression of Trx1 (1.8-fold; Figure 5A and 5B), HO-1 (2.2-fold; Figure 5A and 5C), and VEGF (1.9-fold; Figure 5A and 5D) compared with the CMI group. Ad.LacZ treatment in the CMI-AdLacZ or DMI-AdLacZ group did not reveal any significant difference in the expression of these proteins compared with their respective nontreated controls (CMI and DMI). The expression of Trx1 (2.4-fold), HO-1 (2.5-fold), and VEGF (2.3-fold) was found to be decreased significantly in the DMI group compared with the CMI group. However, Trx1 gene therapy significantly increased the expression of Trx1 (2.8-fold), HO-1 (3.2-fold), and VEGF (2.2-fold) in the DMI-AdTrx1 group compared with the DMI group (Figure 5A through 5D).

SnPP Treatment Significantly Reduced Trx1-Mediated VEGF Expression In Vivo

To examine whether HO-1 mediated the Trx1-induced VEGF expression in Ad.Trx1-treated animals, we intramyocardially administered Ad.Trx1 after MI to SnPP-treated nondiabetic and diabetic animals (n=3 per group). There was no signif-
icant difference in the expression of Trx1 and HO-1 between the SnPP-treated and nontreated animals (in both the nondiabetic and diabetic groups) that received Ad.Trx1 gene therapy. However, we observed significant downregulation of VEGF (2.7- and 2.2-fold, respectively) despite the increase in the expression of Trx1 and HO-1 in the DMI and DMI-AdLacZ groups compared with the CMI and CMI-AdLacZ groups, respectively. There was no significant difference in the expression of these proteins in the Ad.LacZ-treated group compared with the respective nontreated MI groups. Trx1 gene therapy significantly increased the expression of Trx1, HO-1, and VEGF compared with nontreated and Ad.LacZ-treated MI groups in both nondiabetic and diabetic myocardium. SnPP treatment significantly reduced the Trx1 gene therapy-mediated increase in VEGF in both nondiabetic and diabetic myocardium, whereas there was no significant difference in the expression of Trx1 and HO-1 in these groups. Values are expressed as mean±SEM (n=3 to 4 per group). CMI-AdTrx1 + SnPP indicates nondiabetic animals that were treated with SnPP and then subjected to MI and Ad.Trx1 treatment; DMI-AdTrx1 + SnPP, diabetic animals that were treated with SnPP and then subjected to MI and Ad.Trx1 treatment. Other abbreviations as in Figure 2. *P≤0.05 vs CMI; #P≤0.05 vs CMI-AdLacZ; †P≤0.05 vs DMI; ‡P≤0.05 vs DMI-AdLacZ; ††P≤0.05 vs CMI-AdTrx1; and ¥P≤0.05 vs DMI-AdTrx1.

Figure 5. Effect of Trx1 gene therapy on Trx1, HO-1, and VEGF expression (Western blot analysis). A, Representative Western blots show expression of Trx1, HO-1, and VEGF. Bar graphs in B, C, and D represent the quantitative difference in expression of Trx1, HO-1, and VEGF, respectively, in arbitrary units. There was a significant decrease in the expression of Trx1, HO-1, and VEGF in the DMI and DMI-AdLacZ groups compared with the CMI and CMI-AdLacZ groups, respectively. There was no significant difference in the expression of these proteins in the Ad.LacZ-treated group compared with the respective nontreated MI groups. Trx1 gene therapy significantly increased the expression of Trx1, HO-1, and VEGF compared with nontreated and Ad.LacZ-treated MI groups in both nondiabetic and diabetic myocardium. SnPP treatment significantly reduced the Trx1 gene therapy-mediated increase in VEGF in both nondiabetic and diabetic myocardium, whereas there was no significant difference in the expression of Trx1 and HO-1 in these groups. Values are expressed as mean±SEM (n=3 to 4 per group). CMI-AdTrx1 + SnPP indicates nondiabetic animals that were treated with SnPP and then subjected to MI and Ad.Trx1 treatment; DMI-AdTrx1 + SnPP, diabetic animals that were treated with SnPP and then subjected to MI and Ad.Trx1 treatment. Other abbreviations as in Figure 2. *P≤0.05 vs CMI; #P≤0.05 vs CMI-AdLacZ; †P≤0.05 vs DMI; ‡P≤0.05 vs DMI-AdLacZ; ††P≤0.05 vs CMI-AdTrx1; and ¥P≤0.05 vs DMI-AdTrx1.

Trx1 Gene Therapy Increased Expression of Antiapoptotic p38MAPKβ While Reducing Expression of Proapoptotic Phosphorylated JNK and p38MAPKα

In the present study, we evaluated the expression of phosphorylated JNK (p-JNK) and levels of p38MAPK (α and β) proteins (n=3 to 4 per group; Figure 6). Trx1 gene therapy in the CMI-AdTrx1 group significantly increased the expression of p38MAPKβ (1.9-fold; Figure 6A and 6C) and decreased the expression of p38MAPKα (2.3-fold; Figure 6A and 6B) and p-JNK (2.3-fold; Figure 6A and 6D) compared with the CMI group. Ad.LacZ treatment in the CMI-AdLacZ and DMI-AdLacZ groups did not yield any significant difference in the expression of these proteins compared with their respective nontreated controls (CMI and DMI). A significant increase in activation of proapoptotic p-JNK (1.5-fold) and p38MAPKα (1.5-fold) expression and downregulation of antiapoptotic p38MAPKβ (2.5-fold) was observed in the DMI group compared with the CMI group. However, Trx1 gene therapy increased the expression of p38MAPKβ (3.1-fold) and decreased the expression of p-JNK (6.4-fold) and p38MAPKα (2.1-fold) in the DMI-AdTrx1 compared with DMI group (Figure 6A through 6D).

Preservation of Myocardial Function After Acute MI in Diabetic Myocardium Through Trx1 Gene Therapy

Echocardiographic assessment of myocardial function was performed 30 days after surgical intervention, MI, and gene therapy in the experimental animals (n=4 to 5 per group). The representative pictures (Figure 7A through 7F) show the parasternal short-axis view in M-mode. Quantitative representations of echocardiographic measurements are shown in Figure 7B through 7F. There was significant functional disorder in all of the diabetic groups compared with their respective non-diabetic controls. Trx1 gene therapy significantly improved myocardial function in both the nondiabetic
and diabetic groups compared with their respective non-treated or Ad.LacZ-treated controls.

Improved contraction along with improved end-systolic and end-diastolic dimensions were observed with Trx1 treatment, which preserves the contractile function of the heart and suggests attenuated remodeling in the treated animals. Increased left ventricular internal diameter in systole (8.7 ± 0.1 versus 7.1 ± 0.1 mm) and left ventricular internal diameter in diastole (10.9 ± 0.1 versus 9.3 ± 0.1 mm) were observed in DMI rats compared with CMI rats (Figure 7B and 7C). Trx1 gene therapy reduced the left ventricular internal diameter in systole (6.6 ± 0.2 versus 8.6 ± 0.1 mm) and diastole (8.6 ± 0.2 versus 10.9 ± 0.1 mm) in the DMI-AdTrx1 group compared with the DMI group, although a significant difference was not observed in the DMI-AdLacZ group, which shows the functional recovery of the ischemic myocardium after Trx1 gene delivery (Figure 7D).

Discussion

The present study is the first to document that induction of the expression of Trx1 can rescue diabetic myocardium from diabetes/oxidative stress–related impairment of myocardial angiogenesis by reducing oxidative stress and enhancing the expression of HO-1 and VEGF. In addition, we observed decreased expression of proapoptotic proteins such as p-JNK and p38MAPKα and increased expression of the antiapoptotic protein p38MAPKβ while decreasing expression of p38MAPKα and p-JNK compared with the nontreated and Ad.LacZ-treated MI groups in both nondiabetic and diabetic myocardium. Values are mean ± SEM (n = 3 to 4 per group). Abbreviations as in Figure 2. *P < 0.05 vs CMI; #P < 0.05 vs CMI-AdLacZ; †P < 0.05 vs DMI; and ‡P < 0.05 vs DMI-AdLacZ.

There was also significant diastolic dysfunction as evidenced by a higher E/A ratio in the diabetic groups compared with the respective nondiabetic controls. After 30 days of MI, the DMI-AdTrx1 group showed a significant improvement in diastolic function, as evidenced by the E/A ratio (3.1 ± 0.1 versus 4.3 ± 0.1) compared with the DMI group (Figure 7D).
HO-1 promotes neovascularization in the ischemic heart via angiogenesis in CD1 diabetic mice. VEGF could promote wound healing via the induction of endothelial cell apoptosis and reduction of antioxidative and angiogenic activities. In the present study, we observed that Trx1 gene therapy in ischemic diabetic myocardium was able to significantly reduce oxidative stress, as evidenced by the reduction in ROS generation. This reduction in ROS generation can be correlated to the reduction in myocyte and endothelial cell apoptosis and the increase in the expression of angiogenic VEGF.

The proangiogenic effect of resveratrol through the activation of Trx1, HO-1, and VEGF was blocked by SnPP, a specific inhibitor of HO-1 activity. HO-1 is a stress-inducible enzyme system that catalyzes the breakdown of prooxidant heme into biliverdin, carbon monoxide, and iron and is well known for its antiapoptotic and antiinflammatory activities. The expression and activity of HO-1 have been shown to be impaired in a diabetic milieu. It was reported that HO-1 can inhibit postmyocardial infarction remodeling and restore ventricular function. Evidence suggests that HO-1 promotes neovascularization in the ischemic heart via the induction of VEGF. Rivard and colleagues reported that the impairment in new blood vessel formation in a hindlimb ischemia model of diabetic mice was a result of reduced expression of VEGF and that VEGF gene therapy could induce neovascularization in this diabetic mouse model. It was also shown that adenovirus-mediated gene transfer of VEGF could promote wound healing via the induction of angiogenesis in CD1 diabetic mice.

With Trx1 gene therapy, we observed a significant increase in the expression of Trx1, HO-1, and VEGF in the DMI-AdTrx1 group compared with the DMI group, which suggests that Trx1 gene therapy is effective in inducing the expression of these proteins, which were downregulated in a diabetic condition. However, in the SnPP-treated, Ad.Trx1-injected nondiabetic and diabetic MI animals, there was significant downregulation of VEGF even though there was no significant difference in the expression of Trx1 and HO-1 compared with the CMI-AdTrx1 and DMI-AdTrx1 groups, respectively, which suggests that the Trx1-induced expression of VEGF is mediated by HO-1.

The fact that Trx1 gene therapy significantly reduced the number of apoptotic cardiomyocytes and endothelial cells in the DMI-AdTrx1 group compared with the DMI group suggests that Trx1 gene therapy significantly aids in the rescue of diabetic myocardium from MI-induced degeneration and also might support the neovascularization process. The decrease in the number of apoptotic cardiomyocytes and endothelial cells in the treatment group can be correlated with the increase in expression of Trx1 and HO-1, both of which have been shown to be antioxidative, antiapoptotic, and growth stimulatory.

We also observed decreased expression of p-JNK and p38MAPK (antiapoptotic signal) in the DMI-AdTrx1 rats that also might have resulted in decreased cardiomyocyte and endothelial cell apoptosis. Several reports have shown that ischemia and doxorubicin-induced cardiomyocyte apoptosis are attenuated by pharmacological p38 inhibition. Four splice variants of the p38 family (p38MAPKα, p38MAPKβ, p38MAPKγ, and p38MAPKδ) were identified. Differential activation of p38MAPKα and p38MAPKβ has been reported. Although p38MAPKα has been shown to contribute to the proapoptotic pathway, p38MAPKα activation through ischemic preconditioning has been shown to decrease infarct size, thus demonstrating activation of the antiapoptotic pathway. Moreover, p38MAPKα dominant-negative mice were reported to be less susceptible to ischemia-reperfusion injury and subsequent apoptosis. In the present study, we observed increased activation of p38MAPKβ after Ad.Trx1 treatment that might have resulted in decreased apoptosis and a subsequent reduction in myocardial fibrosis.
We also documented that capillary and arteriolar density after 30 days of MI was increased significantly with Trx1 gene therapy in the DMI-AdTrx1 group compared with the DMI group. This can be correlated to the increase in expression of Trx1, HO-1, and VEGF in the treatment group. The antiapoptotic and growth-stimulatory Trx1 and HO-1 might have reduced endothelial cell apoptosis, whereas the increase in VEGF should have stimulated the neovascularization process in the treatment group.

The observed reduction in ROS and apoptosis and the improvement in the vascular density in the treatment group might have led to a reduction in infarct scar extension and fibrosis in the Ad.Trx1 treatment groups. This ultimately could explain the fact that there was significant improvement in left ventricular myocardial function (assessed by echocardiogram) in areas such as left ventricular internal diameter in systole or diastole, E/A ratio, ejection fraction, and fractional shortening in the DMI-AdTrx1 group compared with the DMI group. Reports have linked type 1 diabetes mellitus to early diastolic dysfunction, progressive systolic dysfunction, and subsequent heart failure. The E/A ratio, an index of diastolic dysfunction, has been shown to be reduced significantly in patients with type 1 diabetes mellitus. However, in diabetes-mediated advanced heart failure, the E/A ratio tends to increase. In the present study, the severity of heart failure could be assessed by the significantly higher E/A ratio in all diabetic groups compared with the respective nondiabetic controls. However, Trx1 gene therapy significantly reduced the magnitude of diastolic dysfunction in the diabetic rats. The magnitude and duration of hyperglycemia in a hypoinsulinemic setting are known to determine the extent of progressive systolic dysfunction in subjects with type 1 diabetes mellitus. There was significant systolic functional disorder in the diabetic myocardium after MI; however, Trx1 gene therapy preserved systolic functional parameters significantly in treated diabetic MI rats compared with nontreated diabetic MI rats.

In conclusion, the present preclinical findings indicate that a precisely balanced antioxidant system is essential for the induction of a functional neovascularization in diabetic ischemic myocardium. The present data are supportive of the concept that in a diabetic milieu, impaired angiogenesis is not only caused by downregulation of angiogenic growth factors but also is dependent on excessive oxidative stress and the lack of responsive antioxidant systems. Therefore, for the first time, we propose to treat diabetes-mediated impairment of angiogenesis by normalizing and stabilizing the redox microenvironment in the myocardium by means of Ad.Trx1 gene therapy, thereby reducing oxidative stress and cell death and inducing neovessel formation and maturation, subsequently reducing ventricular remodeling after an MI. Our novel and unique approach of Trx1 gene delivery could prove to be a strategic therapeutic modality in the treatment of diabetes-related cardiac failure and may ultimately improve quality of life and mitigate disease progression in affected subjects.

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Disclosures
None.

References

**CLINICAL PERSPECTIVE**

The higher incidence of post–myocardial infarction angina, larger infarcts, severity of myocardial functional disorder, and unfavorable prognosis among diabetic individuals who develop such complications has been correlated with oxidative stress–mediated impairment of angiogenesis. Moreover, diabetes reflects a challenging condition in which the usual revascularization techniques such as coronary artery bypass surgery and percutaneous transluminal coronary angioplasty tend to fail, thereby leaving many diabetic patients with ischemic heart disease with no options for treatment. Maintenance of a balanced redox status in the microenvironment might aid in alleviating many diabetes-mediated complications, particularly those related to impaired angiogenesis. Our preclinical findings are supportive of the concept that in a diabetic milieu, impaired angiogenesis is not only caused by the downregulation of angiogenic growth factors but is also dependent on excessive oxidative stress and the lack of responsive antioxidant systems. For the first time, our unique results have identified the in vivo angiogenic potential of thioredoxin-1 and substantiated its antioxidative and antiapoptotic properties in the setting of myocardial infarction in a diabetic milieu. We therefore propose to treat diabetes-mediated impairment of angiogenesis by normalizing and stabilizing the redox microenvironment in the myocardium by means of thioredoxin-1 gene therapy, thereby reducing oxidative stress and cell death and inducing neovessel formation and maturation, subsequently reducing ventricular remodeling after a myocardial infarction. Our novel approach of Trx1 gene delivery may prove to be a strategic therapeutic modality in the treatment of diabetes-related cardiac failure and may ultimately improve quality of life and mitigate disease progression in affected individuals.
Thioredoxin-1 Gene Therapy Enhances Angiogenic Signaling and Reduces Ventricular Remodeling in Infarcted Myocardium of Diabetic Rats
Samson Mathews Samuel, Mahesh Thirunavukkarasu, Suresh Varma Penumathsa, Srikanth Koneru, Lijun Zhan, Gautam Maulik, Perumana R. Sudhakaran and Nilanjana Maulik

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SUPPLEMENTAL MATERIAL

Supplemental Methods

*In vitro Experiments*

**Cell culture**

Human Umbilical Vein Endothelial Cells (HUVECs) was obtained from Lonza, Walkersville, MD, USA and they were serially passaged. Cells were maintained in EGM-2 culture medium supplemented with growth factors and antibiotics according to company specifications.

**Cytochemistry for transfection efficiency of Ad.LacZ:**

To test the transfection efficiency of *Ad.LacZ in vitro*, cultured HUVECs were treated with *Ad.LacZ* (1 x 10^7 pfu) and then stained for β-galactosidase (β-gal) activity. In brief, the cells were cultured in 8 well chamber slides and the cells were treated with *Ad.LacZ* and were compared with untreated cells. After 48 hours (hr) of treatment the cells were fixed with 4% paraformaldehyde, washed with PBS thrice for 5 min each. The slides were air dried for 20 min and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 30 min and stained for β-galactosidase activity using X-gal substrate. The cells were incubated with X-gal solution (0.1% X-gal, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆ and 2mM MgCl₂ in PBS, pH-7.0) overnight at 30°C. After incubation, the slides were mounted and the images were digitally captured using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.
**Immunocytochemistry for transfection efficiency of Ad.Trx1:**

To test the transfection efficiency of Ad.Trx1 in vitro, cultured HUVECs were treated with Ad.Trx1 (1 x 10^7 pfu) and stained for Trx1. In brief, the cells were cultured in 8 well chamber slides and the cells were treated with Ad.Trx1 and were compared with untreated cells. After 48hr of treatment the cells were fixed with 4% paraformaldehyde, washed with PBS thrice for 5 min each and blocked with 1% BSA for 1 hr. After blocking the cells were again washed with PBS for three times 5 min each and incubated with primary antibody for rabbit anti-Trx1 (1:100 in PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After incubation the slides were again washed with PBS thrice for 5 min each and incubated with secondary anti-rabbit Alexa Fluor 488 (1:200 in PBS, Invitrogen, Carlsbad, CA, USA) for 1 hr. After incubation the cells were again washed in PBS and stained with TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA) for nuclear staining. After nuclear staining the slides were mounted with using Vectashield mounting medium (Vector laboratories, Burlingame, CA). The sections were observed and images were acquired using Zeiss LSM510 Meta confocal microscope and stored as digital TIFF file format.

**Matrigel Tube Formation Assay:**

HUVECs were cultured in 12-well cell culture plates and were transfected with Ad.Trx1 (1 x 10^7 pfu) or adeno-sh-Trx-1 (Ad.sh.Trx1, 1 x 10^7 pfu) 48 hr prior to matrigel (BD Bioscience, Bedford, MA, USA) assay. These cells were then used for matrigel assay. In brief, 250μl of ice cold matrigel was coated on a 24 well cell culture plate as a base for tube formation. After allowing the gel to settle for 30min in a 37°C, 5% CO₂ incubator, the endothelial cells (4 x 10^4) from normal and adeno pre-transfected cells were seeded onto the matrigel and incubated
overnight at 37°C in a 5% CO₂ incubator. After 18h, the extent of tube formation was recorded (200X) using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

**Effect of Adeno-Trx-1 gene transfection on Trx1, HO-1 and VEGF expression in HUVECs:**

HUVECs were maintained in EGM-2 culture medium supplemented with growth factors and antibiotics according to company specifications. HUVECs were subcultured in 12 well cell culture plates and were transfected with Ad.LacZ (1 x 10⁷ pfu), Ad.TrxI (1 x 10⁷ pfu) and Ad.TrxI + 10 μM tin-protoporphyrin IX (SnPP) (HO-1 activity inhibitor) (Porphyrin Products, Logan, Utah, USA). Following 48 hr of adeno transfection the total cell lysate protein was isolated for Western blot analysis of Trx-1, HO-1 and VEGF. The Trx1 antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, HO-1 antibody was purchased from Stressgen Bioreagents, Ann Arbor, MI, USA and VEGF antibody was obtained from R & D systems Inc. Minneapolis, MN, USA. Primary antibody binding was visualized by respective horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

**In vivo Experiments**

**Induction of Diabetes:**

Male SD rats (300-325gm) were randomly separated into normal and diabetic rats as they received an (i.p.) injection of vehicle (0.1mol/l citrate buffer, pH 4.5) alone or streptozotocin (STZ) at a dosage of 65mg/kg body weight dissolved in 0.1mol/l citrate buffer. Five days after STZ injection, hyperglycemia was documented by measuring the glucose content of tail vein blood with Freestyle Flash blood glucose monitoring system. Rats with blood glucose concentrations ≥ 300mg/dl were used for the study. MI was induced in these animals 30 days
after the induction of diabetes. Age matched non-diabetic animals were used as comparable controls.

**Surgical Procedure:**

Myocardial Infarction (MI) was induced by and permanent Left Anterior Descending (LAD) coronary artery ligation, as previously described \(^1,2\). The rats were randomized into eight groups: 1) Non-diabetic Control Sham (CS), 2) Non-diabetic Control MI (CMI), 3) Non-diabetic Control MI + *Ad.LacZ* (1 x10\(^9\) pfu; CMI-AdLacZ), 4) Non-diabetic Control MI + *Ad.Trx1* (1 x10\(^9\) pfu; CMI-AdTrx1), 5) Diabetic Sham (DS), 6) Diabetic MI (DMI), 7) Diabetic MI + *Ad.LacZ* (1 x10\(^9\) pfu; DMI-AdLacZ), and 8) Diabetic MI + *Ad.Trx1* (1 x10\(^9\) pfu; DMI-AdTrx1).

Briefly, the rats were anesthetized with ketamine HCl (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Cefazolin (25 mg/kg i.p.) was administered as a preoperative antibiotic cover. After endotracheal intubation and initiation of ventilation (Harvard Apparatus Rodent Ventilator, model 683), the heart was exposed through a left lateral thoracotomy (fourth intercostal space) \(^1\). A 6-0 polypropylene suture was passed with tapered needle under the LAD just below the tip of the left atrium and MI was induced by permanent LAD occlusion in the MI groups \(^1,3\).

Immediately after LAD ligation the adenoviral vector encoding Trx1 (*Ad.Trx1*, 1 x 10\(^9\) pfu) \(^4\) was intramyocardially administered (in 100μl of PBS, using a 30g needle) at 4 sites at the border zone of the infarct in the CMI-AdTrx1 and DMI-AdTrx1 groups. Adeno-LacZ (*Ad.LacZ*, 1 x10\(^9\) pfu) \(^4\) was used as the control adenoviral vector encoding β-galactosidase in the CMI-AdLacZ and DMI-AdLacZ groups, in order to nullify any possible effects exerted by the vector itself. The animals in the Sham groups underwent the same time matched surgical procedure but for fact that the 6-0 suture was passed beneath the LAD and was removed without
ligation. After application of buprenorphine (0.1 mg/kg s.c.) and weaning from the respirator, the rats were placed on a heating pad while recovering from anesthesia

Kloner RA, et al. have assessed and measured the efficiency of adenoviral vector mediated gene transfer and expression in the infarcted myocardium. In their study they have placed the animals in 4 groups based on time interval between induction of MI and the Adenovirus mediated transgene delivery (Ad.LacZ). Ad.LacZ was administered either immediately or after 7, 22 and 30 days of MI and the expression of the reported gene was assessed 7 days after gene delivery in each case. The rats those were administered Ad.LacZ, 7, 22 and 30 days after MI underwent a second thoracotomy. They have observed that the expression of transgene was significant in the rats which received the therapy either immediately or 22 and 30 days after MI, while it was reduced in the rats those received Ad.LacZ, 7 days after MI.

In our study, we have used STZ induced diabetic rats and MI was induced 30 days after the induction of hyperglycemia. These rats even before the first surgical intervention tend to lose weight, become weak, shows lesser physical activity and vital sign indices deteriorate. There was significant reduction in the heart rate in these animals in the sham group as well as after MI. Overall there was a higher rate of mortality in the diabetic animals after MI (45-52%) than compared to the non-diabetic MI controls (8-12%). Though we considered a second thoracotomy for gene delivery at a later time after the induction of MI, owing to the higher rate of mortality and the stress and pain that the diabetic animals have to undergo, we have administered the adenoviral vectors immediately after MI.

For molecular analysis the left ventricular tissue sections from the border zone/risk area surrounding the infarct were harvested and flash frozen in liquid nitrogen and stored at -80°C.
while for histochemical analysis the hearts were removed and the tissue was horizontally sectioned between the point of ligation and the apex and fixed overnight in 4% paraformaldehyde for paraffin sections or directly embedded in Optimum Cutting Temperature (OCT) medium for frozen sectioning. The heart tissue was harvested after 4 days for analysis of Dihydroethidium (DHE) staining, Masson’s trichrome staining, cardiomyocyte and endothelial cell apoptosis, for immunohistochemistry and Western blot analysis. Echocardiography was performed to assess the ventricular functions 30 days after gene therapy after which the hearts were harvested for immunohistochemical analysis of capillary and arteriolar density and Masson’s trichrome staining.

**Effect of SnPP on the expression of VEGF**

Alternatively, another group of non-diabetic and diabetic animals were treated with tin-protoporphyrin IX (SnPP, an inhibitor of HO-1 activity, 50μmol/kg, i.p.) to determine whether HO-1 plays an important role in Trx1 mediated VEGF expression *in vivo*. Each animal in this group received five i.p. injections of 50μmol/kg SnPP, spaced on alternated days, 2 injections prior to MI and Ad.Trx1 gene therapy, 1 injection on the day of the intervention and 2 injections after the MI and gene therapy. The hearts were harvested 4 days after the surgery for Western blot analysis.

**Histochemistry for the transfection efficiency of Ad.LacZ and Ad.Trx1:** In order to examine the efficiency of our gene transfer technique, *in vivo*, we have evaluated the adenoviral gene expression 4 days after intramyocardial adenoviral gene administration in the Sham operated non-diabetic control groups. The heart was exposed through a left lateral thoracotomy as described above and we injected the adenoviral vector encoding β-galactosidase (β-gal, Ad.LacZ) at 4 different sites (in 100μl of PBS, using a 30g needle) on the left anterior wall of the
myocardium. The rats were sacrificed 4 days after gene transfer, hearts were removed and the tissue was horizontally sectioned and embedded in OCT medium. In brief, the slides were air dried for 20 min and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 30 min and stained for β-galactosidase activity using X-gal substrate. The sections were incubated with X-gal solution (0.1% X-gal, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆ and 2mM MgCl₂ in PBS, pH-7.0) overnight at 30°C. After incubation, the slides were mounted and the images were digitally captured at (400X) using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

Similarly, Ad.Trx1 was injected at 4 different sites (in 100µl of PBS, using a 30g needle) on the left anterior wall of the myocardium. The rats were sacrificed 4 days after gene transfer, hearts were removed and fixed overnight in 4% paraformaldehyde. Horizontal sections of the heart tissue were embedded in paraffin and 5µm sections were prepared. Briefly, the tissue sections were then cleared in 2 washes of histoclear and rehydrated in gradients of alcohol (twice in 100%, once each in 90%, 80% and 70% alcohol for 5 min each). The tissue sections were washed three times in PBS and antigen retrieval was performed in antigen retrieval buffer (Dako). Following endogenous peroxidase blocking with 0.3% H₂O₂ in PBS for 30min, non-specific binding sites were blocked with buffered casein solution (Power Block Universal Blocking Reagent; Bio Genex, San Ramon, USA) for 10 min at room temperature. After PBS wash (3 times) the sections were stained for Trx1 using mouse monoclonal anti-Trx1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation in biotinylated anti-mouse secondary antibody using the Vectastain Elite ABC Kit-Mouse IgG (Vector Laboratories, Inc, Burlingame, CA, USA) and bound antibody was visualized with 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc,
Assessment of Myocardial Fibrosis (Masson’s Trichrome staining):

To determine the effect of Trx1 gene therapy on myocardial fibrosis (4 days and 30 days after MI/gene therapy, respectively), the paraffin embedded sections of the hearts from each group were stained as per the Masson’s Trichrome staining protocol, as previously described 6. In brief, the paraffin sections were deparaffinized in two washes of Histoclear rehydrated through alcohol gradients (100%, 90%, 80% and 70%) at room temperature. The slides were then washed thrice in distilled water and fixed in Bouin’s fixative overnight. The slides were then stained with Weigert’s iron hematoxylin (10 min) and placed under running tap (10 min). The sections were again washed in distilled water and then stained with Biebrich scarlet-acid fuschin solution for 15 min. Following another wash in distilled water the sections were further differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min. The sections were then directly transferred to aniline blue solution and stained for 10 min, followed by a brief wash in distilled water and was then treated with 1% acetic acid solution for 5 min. The sections were then dehydrated quickly in 90% and 100% alcohol, cleared in Histoclear and mounted using Permount. The heart tissue sections were digitally imaged in high pixel resolution on an Epson Scanner and the images were stored as JPEG file format 6.

Reactive Oxygen Species (ROS) detection in the myocardium:

4 days after MI and gene therapy the animals were sacrificed and heart tissue sections were harvested and directly embedded in OCT medium as described above. Superoxide production in the hearts 4 days after MI and gene therapy was detected by dihydroethidium (DHE) staining.
(Invitrogen, Carlsbad, CA, USA). 10μm frozen heart sections were incubated with 30μM DHE for 45min at 37°C in a humidified chamber protected from light. Fluorescent images were obtained using Zeiss LSM510 Meta confocal laser scanning microscopy. All images were treated equally and background corrections were performed. The average fluorescent intensity of the nuclei were then analyzed using AutoQuant X2 software (MediaCybernetics, Bethesda, MD, USA).

**Determination of cardiomyocyte and endothelial cell apoptosis:**

The rats were sacrificed after 4 days of MI and gene therapy, hearts were removed and fixed overnight in 4% paraformaldehyde and then preserved in 70% ethanol. The heart tissue was horizontally sectioned between the point of ligation and the apex and the sections were embedded in paraffin to prepare the block from paraffin embedded tissue sections were made. The heart tissue sections (5μm) were then cleared in 2 washes of histoclear, rehydrated in gradients of alcohol (twice in 100%, once each in 90%, 80% and 70% alcohol for 5 min each) and washed thrice in 1X PBS. Immunohistochemical detection of apoptotic cells was carried out using TUNEL reaction using In Situ Cell Death Detection Kit, Fluorescein as per the kit protocol (Roche Diagnostics, Mannheim, Germany). In brief, the TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis which can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction catalysed by Terminal deoxynucleotidyl transferase (TdT). Fluorescein labels incorporated in nucleotide polymers are detected by fluorescence microscopy. The sections were washed in PBS three times, blocked with 1X Power Block for 10min and incubated with cardiomyocyte specific mouse monoclonal anti-α sarcomeric actin (1:100 in PBS; Sigma Aldrich, St. Louis, MO, USA) and endothelial cell specific rabbit monoclonal anti-von-Willebrand factor (anti–vWF, 1:100 in PBS; Santa Cruz
Biotechnology, Santa Cruz, CA, USA) followed by staining with the respective secondary Alexa Flour-555 antibodies (1:200 in PBS; Invitrogen, Carlsbad, CA, USA). After incubation, the sections were rinsed thrice in PBS and mounted with Vectashield fluorescent mounting medium (Vector, Burlingame, CA, USA). The sections were observed and images were captured using a confocal laser Zeiss LSM 510Meta microscope. For the quantitative purpose, the number of TUNEL-positive cardiomyocytes and endothelial cells were counted from the endocardium through the epicardium of the mid portion of the left ventricular free wall in from each heart 3, 8.

**Immunohistochemistry for capillary and arteriolar density:**

The rats were sacrificed 30 days after MI and gene therapy and hearts were removed and paraffin embedded tissue sections were prepared as described previously and were used for capillary and arteriolar density staining and analysis.

Briefly, the sections were stained for capillary density using goat polyclonal anti-CD31/PECAM-1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with secondary anti-goat ImmPRESS reagent as per manufacturer’s specifications (ImmPRESS Anti-Goat Ig (peroxidase) Kit, Vector Laboratories, Inc, Burlingame, CA, USA). The antigen antibody interaction was visualized using 3,3’-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA). The images were digitally acquired at 400 x magnification using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope and were used for CD-31 counting. Counts of capillary density per square millimeter of the area at risk from the endocardium through the epicardium of the mid portion of the left ventricular free wall were counted after superimposing a calibrated morphometric grid on each digital image using Adobe Photoshop CS4 Software 9.
For arteriolar density the vascular smooth muscle cells were labeled using mouse monoclonal anti-smooth muscle actin (1:100 in PBS; Abcam Inc, Cambridge, MA) followed by incubation in donkey anti-mouse Alexa Flour 488 (1:200 in PBS; Invitrogen, Carlsbad, CA, USA). To-Pro 3 iodide (Invitrogen, Carlsbad, CA, USA) was used as nuclear stain. After incubation, the sections were again washed in PBS three times and mounted with coverslip using a Vectashield fluorescent mounting medium (Vector, Burlingame, CA, USA). The images were captured using Zeiss LSM510 Meta confocal microscope and the arteriolar density was assessed and was expressed in per square millimeter of the area at risk \(^3\).\(^9\).

**Immunohistochemistry for expression of Trx-1, HO-1 and VEGF:**

The rats were sacrificed 4 days after MI and gene therapy and hearts were removed and paraffin embedded tissue sections were prepared as described previously and were used for immunohistochemical analysis of Trx1, HO-1 and VEGF.

Briefly, the separate sections were stained for Trx1 and HO-1 using mouse monoclonal anti-Trx1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-HO-1 (1:100 in PBS; Abcam Inc, Cambridge, MA), respectively. This was followed by incubation in biotinylated anti-mouse secondary antibody using the Vectastain Elite ABC Kit-Mouse IgG (Vector Laboratories, Inc, Burlingame, CA, USA) and bound antibody was visualized with 3,3’-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA).

For VEGF staining, paraffin embedded tissue sections were stained for VEGF using rabbit polyclonal anti-VEGF (Lab Vision Products, Thermo Fisher Scientific, Fremont, CA, USA) followed by incubation with secondary anti-rabbit ImmPRESS reagent as per manufacturer’s
specifications (ImmPRESS Anti-rabbit Ig (peroxidase) Kit, Vector Laboratories, Inc, Burlingame, CA, USA). The antigen antibody interaction was visualized using 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA). The images for Trx1, HO-1 and VEGF staining were digitally acquired at 400 x magnification using Olympus QCColor 3TM digital camera mounted on an Olympus BH2 microscope.

**Western blot analysis for Trx1, HO-1, VEGF, p-JNK, JNK, p38MAPK (α and β):**

The rats were sacrificed 4 days after MI and gene therapy and the hearts were removed and left ventricular risk area sections were frozen in liquid nitrogen and stored at -80°C. To quantify the Trx1 (12kDa), HO-1 (32kDa), VEGF (46kDa), p38MAPKα (38kDa), p38MAPKβ (38kDa) and p-JNK (54kDa and 46kDa) standard SDS/PAGE Western blot technique was performed. Heart tissue sections from each treatment group were homogenized and suspended (50 mg/ml) in sample buffer (10 mM Tris.HCl, pH 7.3, 11.5% sucrose, 1mM EDTA, 10µg/ml pepstatin A, 10 µg/ml leupeptin, 10 µg/ml aprotinin). The cytosolic protein was isolated and total protein concentration was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL). The cytosolic proteins were run on polyacrylamide electrophoresis gels (SDS-PAGE) typically using 12% for Trx1 & 10% for HO-1, VEGF, p38MAPKα, p38MAPKβ, p-JNK and JNK. GAPDH was used as loading control for Trx1, HO-1, VEGF, p38MAPKα, and p38MAPKβ, while JNK was used as loading control for p-JNK. The Trx1 and p38MAPKβ antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, HO-1 antibody was purchased from Stressgen Bioreagents, Ann Arbor, MI, USA, VEGF antibody was obtained from R & D systems Inc. Minneapolis, MN, USA, and the p38MAPKα, p-JNK (Thr183/Tyr185) and JNK antibodies were purchased from Cell Signaling, Danvers, MA, USA. Primary antibody
binding was visualized by respective horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

**Echocardiography:**

Echocardiogram analysis was carried as previously described. After 30 days of MI each rat was sedated using isoflurane (3%, inhaled). When adequately sedated, the rat was secured with tape in the supine position in a custom-built mold designed to maintain the rat's natural body shape after fixation. The hair on the chest wall was removed with a chemical hair remover. Ultrasound gel was spread over the precordial region, and ultrasound biomicroscopy (Vevo 770, Visual-Sonics Inc., Toronto, ON, Canada) with a 25-MHz transducer was used to visualize the left ventricle. The left ventricle was analyzed in apical, parasternal long axis, and parasternal short axis views for LV systolic function, LV cavity diameter, wall thickness, diastolic function, and LV end-systolic and end-diastolic volume determination. MI segments were determined according to the kinetics: hypokinetic (reduction in wall motion), akinesis (no wall motion), and dyskinesis (unsynchronized movement of segment with normal myocardium). Two-dimensional directed M-mode images of the LV short axis were taken just below the level of the papillary muscles for analyzing ventricular wall thickness and chamber diameter. All the LV parameters were measured according to the modified American Society of Echocardiography–recommended guidelines. Ejection fraction and fractional shortening were assessed for LV systolic function. Diastolic function was assessed by measuring mitral peak flow velocity of the E-wave and A-wave in centimeters per second (cm/s), as was the ratio between the two waves (E/A). All the measurements represent the mean of at least three consecutive cardiac cycles. Throughout the procedure, ECG, respiratory rate, and heart rate were monitored.
References


