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Original article

Thioredoxin 1 enhances neovascularization and reduces ventricular remodeling during chronic myocardial infarction: A study using thioredoxin 1 transgenic mice

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ABSTRACT

Oxidative stress plays a crucial role in disruption of neovascularization by alterations in thioredoxin 1 (Trx1) expression and its interaction with other proteins after myocardial infarction (MI). We previously showed that Trx1 has angiogenic properties, but the possible therapeutic significance of overexpressing Trx1 in chronic MI has not been elucidated. Therefore, we explored the angiogenic and cardioprotective potential of Trx1 in an *in vivo* MI model using transgenic mice overexpressing Trx1. Wild-type (W) and Trx1 transgenic ($Trx1^{Tg/+}$) mice were randomized into W sham (WS), $Trx1^{Tg/+}$ sham (TS), WMI, and TMI. MI was induced by permanent occlusion of LAD coronary artery. Hearts from mice overexpressing Trx1 exhibited reduced fibrosis and oxidative stress and attenuated cardiomyocyte apoptosis along with increased vessel formation compared to WMI. We found significant inhibition of Trx1 regulating proteins, TXNIP and AKAP 12, and increased *p*-Akt, *p*-eNOS, *p*-GSK-3β, HIF-1α, β-catenin, VEGF, Bcl-2, and survivin expression in TMI compared to WMI. Echocardiography performed 30 days after MI revealed significant improvement in myocardial functions in TMI compared to WMI. Our study identifies a potential role for Trx1 overexpression and its association with its regulatory proteins TXNIP, AKAP12, and subsequent activation of Akt/GSK-3β/β-catenin/HIF-1α-mediated VEGF and eNOS expression in inducing angiogenesis and reduced ventricular remodeling. Hence, Trx1 and other proteins identified in our study may prove to be potential therapeutic targets in the treatment of ischemic heart disease.

1. Introduction

Myocardial infarction (MI) remains as a major cause of morbidity and mortality worldwide. Although MI triggers a spontaneous angiogenic response, which aims at reestablishing myocardial blood flow, this protective response is usually not sufficient to restore physiological levels of coronary perfusion. Hence, therapeutic angiogenesis by using biological factors such as vascular endothelial growth factor (VEGF) or angiopoietin-1 has emerged as an attractive treatment option to restore blood flow and thus the supply of oxygen and nutrients to the ischemic regions of the heart after MI [1,2]. Accumulating evidence supports the notion that thioredoxin 1 (Trx1), with its antioxidant, growth stimulatory, anti-inflammatory, and pro-angiogenic properties, could play a role in modulating the pathological processes involved in cardiovascular disease progression and therefore might represent a promising therapeutic target for the prevention and treatment of the disease [3,4].

Trx1 is a cytosolic 12-kDa redox protein with important antioxidative and cell signaling functions that undergoes reversible oxidation of its two active site cysteine residues at its active site where reduction of which is catalyzed by the NADPH-dependent flavoenzyme thioredoxin reductase [5]. The activity of Trx1 is found to be regulated through its interaction with Thioredoxin Interacting Protein (TXNIP). Thus, the ratio of Trx1 to TXNIP expression is an important factor in redox-sensitive regulation of myocardial angiogenesis [6,7]. We have previously reported that the *Ad-Trx1* gene therapy is cardioprotective in the ischemic myocardium of streptozotocin-induced diabetes rat by reducing oxidative stress and apoptosis with a corresponding improvement in myocardial functions [3]. Furthermore, other studies demonstrated that elevated Trx1 levels attenuate myocardial damage induced by ischemia–reperfusion

Abbreviations: AKAP12, A kinase anchoring protein 12; HIF-1α, hypoxia inducible factor alpha; LAD, left anterior descending coronary artery; LVIDd, left ventricular internal diameter at diastole; LVIDs, left ventricular internal diameter at systole; MI, myocardial infarction; SEM, standard error mean; TBARS, thiobarbituric acid reactive substances; TMI, $Trx1^{Tg/+}$ MI; Trx1, thioredoxin-1; TS, $Trx1^{Tg/+}$ sham; TXNIP, thioredoxin interacting protein; VEGF, vascular endothelial growth factor; WMI, wild-type MI; WS, wild-type sham.

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injury while the overexpression of TXNIP has been shown to increase cardiomyocytes sensitivity to oxidative stress-induced apoptosis [7].

A kinase anchoring proteins (AKAPs) compose a growing list of diverse but functionally related proteins defined by their ability to bind to the regulatory subunit of protein kinase A. AKAPs perform an integral role in the spatiotemporal modulation of a multitude of cellular signaling pathways [8]. Among all the AKAPs found so far, AKAP12 was found to be critical regulator of angiogenesis [9]. Exposure of endothelial cells to AKAP12 significantly reduced VEGF mRNA and increased anti-angiogenic proteins such as thrombospondin-1 [10]. AKAP12 is also known to cause significant downregulation of hypoxia-inducible factor (HIF)-1α and thereby reduce the hypoxia-induced VEGF expression [11]. Furthermore, Trx1 may play a crucial role in regulating Akt signaling [12]. The Akt survival pathway is a central signaling node involved in cell growth, proliferation, differentiation, apoptosis, and angiogenesis [13]. Akt also affects cell survival by phosphorylating and inactivating GSK-3β, which in turn results in the release, stabilization, and subsequent accumulation of β-catenin in the cytosol, followed by its translocation into the nucleus [14,15]. Conversely, the phosphorylation of β-catenin marks this protein for ubiquitination and subsequent degradation by the proteosomal pathway [16]. Therefore, when phosphorylation of β-catenin is blocked, it stabilizes, accumulates, and translocates into the nucleus, where it forms a complex with T-cell transcription factors/lymphoid-enhancer binding factor and is able to activate or repress several important angiogenic target genes, such as c-Myc, cyclin D1, fibronectin, VEGF, eNOS, Bcl-2, and survivin [15,17].

We hypothesized that myocardial angiogenesis is regulated by a complex interplay between pro-angiogenic signaling and anti-angiogenic signaling under the redox control by Trx1. Therefore, the aim of the present study was to establish the role of Trx1 as therapeutic angiogenic growth factor by investigating the possible role of Trx1 overexpression in attenuating oxidative stress, TXNIP and AKAP12 expression, and inducing Akt/GSK-3β/HIF-1α/β-catenin-mediated VEGF and eNOS expression during myocardial infarction by using Trx1 transgenic mice.

2. Methods

2.1. 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 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 examined and approved by the Institutional Animal Care Committee of the Connecticut Health Center (Farmington, CT, USA). Eight-week-old male $Trx^{Tg/+}$ overexpressing C57BL/6 background mice and respective wild-type mice were used for the study. The generation, characterization, and maintenance of $Trx^{Tg/+}$ mice have been described in supplemental material (Suppl. Fig. 1). Further, the genotype of each animal was confirmed by polymerase chain reaction analysis on purified ear DNA. Moreover, the overexpression of Trx1 in transgenic mice is also shown by Western blot in Fig. 3A.

2.2. Experimental design

Eight-week-old male $Trx1^{Tg/+}$ and respective wild-type mice were randomized into four groups: (1) wild-type sham (WS); (2) $Trx1^{Tg/+}$ sham (TS); (3) wild-type MI (WMI); and (4) $Trx1^{Tg/+}$ MI (TMI). MI was induced by permanent left anterior descending (LAD) coronary artery ligation. Sham groups underwent the same time-matched surgical procedure without ligation. The apoptosis and β-catenin nuclear translocation were measured 24 h after surgical intervention, whereas the protein expression profile for the Trx1, TXNIP, AKAP12, VEGF, Bcl-2, and survivin and oxidative stress index was measured in the left ventricular tissue (risk area) 4 days after MI. The extent of phosphorylation of eNOS, Akt and GSK-3β, expression of β-catenin, and HIF-1α DNA-binding activity was observed 8 h after MI. The immunohistostaining was performed to reflect the degree of angiogenesis by calculating the capillary and arteriolar density after 7 days of surgical intervention. The cardiac function was assessed by echocardiogram 30 days after MI.

2.3. Surgical procedures

Mice were anesthetized with ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip) dissolved in physiological saline, then orally intubated with a 22-G IV catheter, and ventilated with a rodent respirator (Harvard Apparatus, Hilliston, USA). Hearts were then exposed through the left lateral thoracotomy. MI was created by permanent LAD ligation with 8–0 polypropylene suture viewed under a stereo zoom dissection microscope. The lungs were inflated by positive end-expiratory pressure and the chest was closed with 6.0 nylon suture. After surgery, the analgesic buprenorphine (0.1 mg/kg, sc) was given, and the animals were weaned from the respirator and then placed on a heating pad for recovery [18,19].

2.4. Assessment of cardiac fibrosis (Masson's trichrome staining)

To determine the effect of Trx1 overexpression on cardiac fibrosis, the collagenous fibrotic area of the heart was stained by Masson's Trichrome staining in paraffin embedded sections (of 4 μm thick) [20]. In brief, the sections were deparaffinized in histoclear and rehydrated using sequential passage through 100 to 70% ethanol for 6 min each followed by washing in distilled water three times. The slides were then stained with Weigert's iron hematoxylin for 10 min and washed under tap water for 10 min. The sections were washed again in distilled water and then stained with Biebrich scarlet-acid fuschin solution for 15 min, in phosphomolybdic–phosphotungstic acid solution for 15 min, and aniline blue solution and stained for 10 min. The sections were rinsed briefly in distilled water and were treated with 1% acetic acid solution for 5 min. After a final wash in distilled water, the sections were dehydrated through sequential gradient of 70–100% alcohol followed by histoclear wash and then mounted using Permount. The heart tissue sections were digitally imaged in high pixel resolution on an Epson Scanner.

2.5. Assessment of oxidative stress by TBARS estimation

The extent of oxidative stress in the heart was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS) (Niehaus and Samuelsson [21] method). To 0.5 ml of tissue homogenate (known weight of the myocardium tissue from risk area was homogenized at 3000 rpm in 0.5 ml of 0.025 M Tris–HCl, pH 7.5), 1 ml of TBA:TCA:HCl in the ratio of 1:1:1 (0.37% thiobarbituric acid:15% trichloroacetic acid:0.25 N HCl) was mixed and placed in a boiling water bath for 15 min, cooled and centrifuged at room temperature for 10 min at 1000 rpm. A series of standard solutions (tetramethoxy propane, Sigma-Aldrich, St. Louis, MO) in the range of 2–10 nmol were treated in a similar manner. The absorbance of the clear supernatant was measured against a reference blank at 535 nm. Results were expressed as nM/mg tissue.

2.6. Cardiomyocyte apoptosis

Formaldehyde-fixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick), and deparaffinized with a graded series of histoclear and ethanol solutions. Immunohistochemical detection of apoptotic cells was carried out using TUNEL