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Thioredoxin-1 augments wound healing and promote angiogenesis in a murine ischemic full-thickness wound model☆,☆☆

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ABSTRACT

Background: Nonhealing wounds are a continuing health problem in the United States. Overproduction of reactive oxygen species is a major causative factor behind delayed wound healing. Previously we reported that thioredoxin-1 treatment could alleviate oxidative stress under ischemic conditions, such as myocardial infarction and hindlimb ischemia. In this study, we explored the potential for thioredoxin-1 gene therapy to effectively aid wound healing through improved angiogenesis in a murine ischemic wound model.

Methods: Full-thickness, cutaneous, ischemic wounds were created in the dorsum skin flap of 8- to 12-week-old CD1 mice. Nonischemic wounds created lateral to the ischemic skin flap served as internal controls. Mice with both ischemic wounds and nonischemic wounds were treated with Adeno-LacZ (1×10^9 pfu) or Adeno-thioredoxin-1 (1×10^9 pfu), injected intradermally around the wound. Digital imaging was performed on days 0, 3, 6, and 9 to assess the rate of wound closure. Tissue samples collected at predetermined time intervals were processed for immunohistochemical analysis.

Results: No significant differences in wound closure were identified among the nonischemic wounds control, nonischemic wounds-LacZ, and nonischemic wounds–thioredoxin-1 groups. Hence, only mice with ischemic wounds were further analyzed. The ischemic wounds–thioredoxin-1 group had significant improvement in wound closure on days 6 and 9 after surgery compared with the ischemic wounds control and ischemic wounds-LacZ groups. Immunohistochemical analysis indicated increased thioredoxin-1, vascular endothelial cell growth factor, and β-catenin levels in the ischemic wounds–thioredoxin-1 group compared with the ischemic wounds control and ischemic wounds-LacZ groups, as well as increased capillary density and cell proliferation, as represented by Ki-67 staining.

Conclusion: Taken together, thioredoxin-1 gene therapy promotes vascular endothelial cell growth factor signaling and re-epithelialization and activates wound closure in mice with ischemic wounds.

Introduction

Wound treatment in the United States constitutes a significant proportion of health care spending because of the patient morbidities that acute and chronic wounds portend. The term *wound* may refer to a wide variety of pathologic conditions, including surgical or traumatic wounds, chronic ulcers (pressure, diabetic, venous, arterial, etc), and skin grafts or flaps. It is estimated that total health care spending on chronic wounds alone exceeds $25 billion per year.[1–3] The healing of acute wounds can be divided into 3 phases: the inflammatory phase, the fibroproliferative phase, and the maturation/remodeling phase.[4] Failure of a wound to progress in a normal fashion through any of these stages can delay healing and lead to a chronic wound.[5] The molecular events that take place during the wound-healing process promote acute inflammation and contribute to the active proliferation of cells for the

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formation of granulation tissue and re-epithelialization and tissue remodeling.[6]

Wound healing in an acute setting differs from that observed under chronic conditions. The progression of an acute wound to a chronic wound is largely driven by an imbalance between extracellular matrix–disrupting matrix metalloproteinases and tissue-remodeling growth factors.[7] During wound healing, lack of tissue-remodeling growth factors at the ischemic region causes cessation of active cell proliferation, resulting in the development of chronic wounds. Among the proangiogenic growth factors that have been explored to date, vascular endothelial cell growth factor (VEGF) appears to be most active in the repair of ischemic dermal wounds.[8] However, the free-radical–mediated inflammatory response that prevails at the site of ischemic wounds can lead to failure of angiogenesis because of a decrease in proangiogenic growth factors during long-term therapy. Thus, active redox molecules that decrease the formation of reactive oxygen species in ischemic wounds may augment healing and improve clinical outcomes. The thioredoxin (Trx) antioxidant system is a major intracellular regulator of the oxidation-reduction balance.[9] Thioredoxin-1 (Trx-1) is a cytosolic, 12-kDa redox protein.[10] In addition to the oxidative stress–induced expression of Trx-1, the activity of Trx-1 is found to be regulated by post-translational modifications and through its interaction with thioredoxin-interacting protein. Binding of Trx-1 to thioredoxin-interacting protein reduces the ability of Trx-1 functions.[11,12] Trx-1 scavenges reactive oxygen species effectively during oxidative stress and rescues injured cells from hypoxia to maintain homeostasis.[9]

Previous studies in our laboratory using preclinical animal models have found that Trx-1 gene therapy upregulates important signaling molecules in the VEGF pathway to promote neovascularization and angiogenesis.[13–15] We have also found that transgenic overexpression of Trx-1 during acute myocardial infarction reduces ischemic stress and preserves cardiac function.[16] In line with the above said findings, our present study investigate the therapeutic role of Trx-1 in wound healing process using a murine, full thickness ischemic wound model. Our *in vitro* experimental results found that pretreatment with Trx-1 rescued cells from hypoxic stress and helped to maintain cellular function. The *in vivo* results indicated that Trx-1 could serve as a future therapeutic solution for the treatment of ischemic wound disorders because of its rapid wound-healing function and proangiogenic characteristics.

Materials and Methods

In vitro cell-culture studies

To study the *in vitro* efficacy of Trx-1, human umbilical vein endothelial cells (HUVECs) at passage 2–3 (Lonza, Walkersville, MD) were used. The cells were maintained in endothelial cell growth medium with growth supplements added according to manufacturer's instructions. Cells were used for experimental analysis after reaching 80%–90% confluence.[17]

In the present study, the *in vitro* studies pertaining to proliferation and migration were carried out in HUVECs and not in smooth muscle cell or fibroblasts, because the isolation from tissue source is tedious and results in heterogeneous mixture of cells. Moreover the biological response by HUVECs toward Trx-1 to initiate angiogenesis has been extensively characterized and is well established.[14,17]

HUVECs were divided into 2 groups: Adeno-LacZ (Ad.LacZ) and Adeno–Trx-1 (Ad.Trx-1). After attaining 90% confluence, the cells were treated with Ad.LacZ (1×10^9 pfu) or Ad.Trx-1 (1×10^9 pfu) for 48 hours. After 48 hours, the cells were washed with 1X Hanks' balanced salt solution (Gibco, Waltham, MA) and used in proliferation or scratch assays.

In vitro Cell-Proliferation Analysis

For the prolferation assay, cells treated with Ad.LacZ or Ad.Trx-1 for 48 hours were trypsinized, counted, and uniformly reseeded in 96-well plates and allowed to adhere overnight. After 48 hours, the cells were subjected to normoxia and hypoxic environments. For the hypoxic group, Ad.LacZ- or Ad.Trx-1-treated cells were maintained in a 3-tray modular incubator chamber (Billups-Rothenberg Inc, San Diego, CA) for 24 hours. For the normoxic group, Ad.LacZ- or Ad.Trx-1-treated cells were maintained at 37 °C with CO_2 for 24 hours. The baseline for the proliferation assay was calculated from the total number of cells after 24 hours of seeding. After 24 hours of normoxic/hypoxic treatment, the culture medium was aspirated from the wells and the plates were frozen at –80°C until DNA quantification was performed. A flourescence-based DNA-quantification assay was performed to calculate the number of cells proliferated using a CyQUANT cell-proliferation assay kit (C7026, Life Technologies, Carlsbad, CA) per manufacturer's instructions.[17]

Briefly, the 96-well plates were thawed to room temperature and 200 µL of the 1X CyQUANT dye was added to the wells. The plates were then wrapped with aluminum foil and incubated at room temperature for 60 to 90 minutes. The development of the flourescent signal was measured using a multiplate reader (SpectraMax, M3 multimode microplate reader, Molecular Devices, Sunnyvale, CA) by setting excitation and emission wavelengths at 480 and 520 nm, respectively.

In vitro migration analysis through the scratch assay

After treatment with Ad.LacZ or Ad.Trx-1 for 48 hours, the HUVECs were allowed to reach 90% confluence in 12-well plates and a migration assay was performed. Uniform scratch wounds were created in the HUVEC cell monolayer with a sterile 200 µL pipette tip. The migration of cells at the wound edges during wound closure was captured at 0, 6, 12, and 24 hours using the 10X objective of an inverted microscope (Olympus Inc, Waltham, MA), fitted with an external Uranus CMOS-series digital camera operated through ToupView 3.2 imaging software. The wound area at various time points was measured from the acquired microscopic images and wound closure percentage was calculated using ImageJ software (National Institutes of Health, Bethesda, MD), as previously reported[17–19]:

$$\text{Wound closure } (\%) = (\text{Initial wound area} - \text{Final wound area})/(\text{Initial wound area}) \times 100$$

In vivo experimental design

This study was performed according to the principles of laboratory animal care set forth 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 published by the National Institutes of Health (Publication No. 85-23, revised 1985). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health (Farmington, CT). Male CD1 mice aged between 8 to 12 weeks were purchased from Envigo (Indianapolis, IN) and housed under a 12-hour light/dark cycle. All the animals were allowed ad libitum access food and water. The viral vectors Ad.Trx-1 and Ad.LacZ were generous gifts from Dr J. Sadoshima, New Jersey Medical School, Newark, NJ.[14]

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