Edaravone, a Free Radical Scavenger, Accelerates Wound Healing in Diabetic Mice

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ABSTRACT

Refractory wound healing is a major complication of diabetes. Diabetes restricts wound healing by interfering with the inflammatory response, decreasing granulation, causing peripheral neuropathy, and inhibiting angiogenesis. Oxidative stress has been proposed as an important pathogenic factor in diabetic wound complications. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a strong free radical scavenger that suppresses the effect of oxidative stress. Streptozotocin-induced diabetes was established in adult C57BL/6 mice, and full-thickness skin was then removed from the dorsomedial back using an 8-mm biopsy punch. Edaravone or vehicle alone was applied to the wound on days 0 and 4 after wound creation. The wound was monitored with a digital camera and analyzed on days 0, 4, and 7 after wound creation. We investigated whether accelerated wound closure occurred in the edaravone group (n = 24) compared with the vehicle-alone group (n = 15). On day 7, wound closure between the two groups was statistically different (p = 0.0019). Angiogenesis and lymphangiogenesis were markedly promoted. We explored the possibility of an inhibitory effect of edaravone characterized by suppression of oxidative stress. Edaravone induced upregulation of endothelial nitric

oxide synthase (eNOS) mRNA expression, and eNOS protein was immunohistochemically detected. Edaravone upregulates eNOS expression and accelerates wound healing.

INTRODUCTION

The number of patients with diabetes is multiplying worldwide [1]. Accordingly, impaired wound healing, one of its complications, has been receiving increasing attention. Following skin injury, the wound goes through four often overlapping phases: coagulation, inflammation, new tissue formation, and tissue remodeling [2]. This wound healing process requires orderly events among cells, inflammatory mediators, cytokines, hormones, and growth factors. In patients with diabetes, however, this integrated progression is impaired because of several pathogenic abnormalities, including dysfunction of neovascularization. Neovascularization occurs by two processes: angiogenesis and vasculogenesis. In angiogenesis, new vessels are formed from preexisting mature vessels. In contrast, vasculogenesis requires both adjacent existing blood vessels and bone marrow-derived endothelial progenitor cells (EPCs) [3].

Both type 1 and type 2 diabetes are characterized by hyperglycemia, which is accountable for intracellular hyperglycemia in susceptible cells, such as endothelial cells. Although hyperglycemia causes tissue damage through five pathways, it has recently been suggested that mitochondrial overproduction of reactive oxygen species (ROS) is

the single upstream event common to these five pathways. ROS are necessary in wound healing for efficient defense against invading pathogens and successful cellular signaling. However, if produced excessively or not properly detoxified, this oxidative stress leads to cell damage. In the diabetic microvasculature, overproduction of ROS is suggested to impair angiogenesis, resulting in delayed wound healing [4]. In addition, lymphangiogenesis is reduced in diabetic wounds [5]. Lymphatic vessels are important for the maintenance of proper interstitial fluid pressure and serve as the primary passage of lymph fluid as well as a location for immune functions to take place. Lymphangiogenesis in adulthood occurs under pathological conditions, including tissue repair [6]. In diabetic mice, reduced lymphangiogenesis and weakened macrophage function have been demonstrated to contribute to impaired wound healing [7].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is an antioxidant that is used to treat acute cerebral infraction, and it was initially approved in Japan [8]. Edaravone also reportedly reduces the increased amount of ROS induced by postischemic reperfusion [9] and induces endothelial nitric oxide synthase (eNOS) in the ischemic spinal cord in rabbits [10].

This study was performed to investigate whether topical application of edaravone

accelerates wound healing in diabetic mice, mainly focusing on neovascularization.

MATERIALS AND METHODS

Animals

C57BL/6 male mice aged 9 to 10 weeks were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). At 10 to 11 weeks of age, the mice were rendered diabetic by intraperitoneal injection of 60 mg/kg of streptozotocin (Sigma-Aldrich, St. Louis, MO) in 0.05 M sodium citrate (pH 4.5) per day for 5 consecutive days. Mice whose blood glucose level reached >350 mg/dl twice were used for further study. All works were carried out in accordance with the Fukuoka University animal welfare committee.

Wound creation and drug application

Two days before wound creation, the backs of the mice were depilated with wax

(SUPER wacs; HOLLYWOOD COSMETICS, Tokyo, Japan), and mice with pink skin (in the telogen phase) were investigated for further study. After induction of deep anesthesia by isoflurane inhalation, a single wound was created on the dorsum of the mice using an 8-mm skin biopsy punch (Kai Industries Co., Ltd., Gifu, Japan). Immediately after the wound creation, 10 mg of petroleum jelly with or without edaravone (Toronto Research Chemicals Inc., Toronto, Canada) comprising a mixture of 30 mg of edaravone and 970 mg of petroleum jelly was applied to the wounds and covered with a semipermeable polyurethane dressing (Opsite; Smith & Nephew, Massillon, OH). Edaravone or petroleum jelly alone was applied to the wound on days 0 and 4 after wound creation.

Wound closure measurements

Wound healing was recorded by a digital camera (Nikon D3200; Nikon, Tokyo, Japan) on days 0, 4, and 7 after wound creation. The images were analyzed using ImageJ software (NIH, Bethesda, MD) by tracing the wound margin and calculating the pixel area individually. Wound closure was calculated as follows: Wound closure (%) = [(Area

on day 0 – Area on indicated day) / Area on day 0] × 100.

Histological study

The wound was fixed in 10% formaldehyde solution, embedded in paraffin, cut into 3-μm sections, and stained with hematoxylin and eosin.

Wound vascularity and lymphangiogenesis

On day 7, the wounds were harvested, fixed in 10% formaldehyde solution, embedded in paraffin, and stained with anti-vWF antibody (Abcam, Cambridge, UK) or LYVE-1 antibody (ReliaTech GmbH, Wolfenbüttel, Germany). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO). Images were captured using a fluorescence microscope (All-in-One Fluorescence Microscope, BZ-9000 series [Biorevo]; Keyence, Osaka, Japan). Cells positive for both vWF/LYVE-1 and DAPI and that formed lumens were counted at high-power (×400).

Immunohistochemistry

On day 7, the wounds were harvested, fixed in 10% formaldehyde solution, and embedded in paraffin. Immunohistochemistry was performed for sections with polyclonal rabbit anti-eNOS/NOS type III (BD, Franklin Lakes, NJ) and secondary antibodies using avidin-biotin complex/diaminobenzidine histochemistry.

RNA isolation and microarray analysis for NO signaling pathway

On day 7, the wounds were excised and total RNA was homogenized in TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from the isolated total RNA, and microarray analysis was performed using a Nitric Oxide Signaling Pathway RT^2 Profiler™ PCR Array (PAMM-062Z; QIAGEN, Valencia, CA).

Validation of expression by quantitative real-time PCR

On day 7, total RNA was isolated in TRIzol and cDNA was synthesized by a High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed using SYBR Premix Ex Taq™ II (Takara, Shiga, Japan) and LightCycler (Roche, Basel, Switzerland). The following oligonucleotides were used: β-actin (Takara, Shiga, Japan): 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, 5′-ATGGAGCCACCGATCCACA-3′; Nos3 (Takara, Shiga, Japan): 5′-ATTCTGGCAAGACAGACTACACGA-3′, 5′-TCCCGGTAGAGATGGTCCAG-3′; VEGF-A (Sigma-Aldrich, St. Louis, MO): 5′-AAAGGCTTCAGTGTGGTCTGAGAG-3′, 5′-GGTTGGAACCGGCATCTTTATC-3′; VEGF-C (Sigma-Aldrich, St. Louis, MO): 5′-CCAGCACAGGTTACCTCAGCAA-3′, 5′-TAGACATGCACCGGCAGGAA-3′; C-X-C motif chemokine ligand 12 (Takara, Shiga, Japan): 5′-CCCGAAATTAAAGTGGATCCAAGAG-3′, 5′-GCGAGTTACAAAGCGCCAGAG-3′; and fibroblast growth factor 2 (Sigma-Aldrich, St. Louis, MO): 5′-CCTCTCAGAGACCTACGTTCAA-3′, 5′-GGAGGTCAAGGCCACAAT-3′.

Statistical analysis

Statistical analysis was carried out using Prism 5 software (GraphPad Software, Inc., La Jolla, CA). A two-tailed Student's t-test and the Mann–Whitney U-test were used for statistical analysis. A p value of <0.05 was considered statistically significant.

RESULTS

Topical application of edaravone accelerates wound healing in diabetic mice

Full-thickness wounds were created on the backs of streptozotocin-induced diabetic mice, and edaravone (n = 24) or petroleum jelly (n = 15) was applied on days 0 and 4 after wound creation. Wound healing was examined on days 0, 4, and 7 (Figure 1a). On day 7, the rate of wound closure was significantly greater in mice treated with edaravone than in mice treated with petroleum jelly ($p < 0.01$) (Figure 1b). Histologically, more abundant blood vessels were observed in the edaravone-treated wound sites than in the control wound sites (Figure 1c, magnified yellow squares).

Edaravone promotes neovascularization and lymphangiogenesis

To further investigate the number of blood vessels, wound angiogenesis was assessed by immunostaining of the endothelial cell-specific marker von Willebrand factor (vWF) on day 7. Some small vessels were found in the margin of the wound in petroleum jelly-treated mice. However, many more vessels were found in edaravone-treated mice, and they were located in the center of the wound (Fig. 2a). Edaravone significantly promoted wound vascularity (Fig. 2b). Lymphangiogenesis was also assessed by immunostaining of the lymphatic endothelial cell-specific marker lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) on day 7 (Fig. 3a). Edaravone significantly promoted lymphangiogenesis as indicated by LYVE-1 immunostaining (Fig. 3b).

Topical application of edaravone induces eNOS expression in endothelial cells

Edaravone reportedly induces eNOS in the ischemic spinal cord in rabbits [10]. Therefore, comparative analysis of nitric oxide (NO) signaling gene expression was performed to further investigate the effect of edaravone on neovascularization. The microarray results revealed that seven genes showed higher expression in edaravone-treated skin on day 7

than in controls, including eNOS (Table 1). In quantitative polymerase chain reaction (PCR), there was a statistically significant difference in the relative expression of eNOS on day 7 between the edaravone and control groups. Moreover, edaravone induced more eNOS-positive cells, which form lumen constructs (Fig. 4).

Edaravone induces expression of stromal cell-derived factor 1α and vascular endothelial growth factor C, but not vascular endothelial growth factor A

We next analyzed the inducers of edaravone in vasculogenesis and lymphangiogenesis. Quantitative PCR was performed on day 7 to investigate candidate angiogenesis and lymphangiogenesis genes that resulted in the upregulation of eNOS in edaravone-treated wounds. Edaravone induced the expression of stromal cell-derived factor 1α (SDF-1α), a chemokine that promotes the homing of EPCs, and vascular endothelial growth factor C (VEGF-C). VEGF-A was expressed at a lower level in edaravone-treated wounds on day 7 (Fig. 5).

DISCUSSION

Our principal finding is that edaravone promotes neovascularization and increases the expression of eNOS mRNA and protein in wounds of diabetic mice. As previously reported, edaravone is a free radical scavenger that possesses antioxidant characteristics [11-14]. In addition, it upregulates eNOS [10,15,16] and NO [17]. These reports indicate that neovascularization occurs by 1) quenching the ROS in wounds and 2) increasing the NO concentration, leading to more rapid wound healing in our study.

Vasculogenesis in wounds requires the homing of EPCs to the wound followed by mobilization of EPCs from the bone marrow [18]. Reduced numbers and impaired functions of EPCs have been described in patients with both type 1 and type 2 diabetes [19,20]. The mechanisms responsible for damage to EPCs have not been elucidated, but Gallagher *et al.* showed that the levels of both phosphorylated eNOS protein in bone marrow and SDF-1α expression in epithelial cells and myofibroblasts were decreased in diabetic mice [21]. Once in circulation, homing occurs by binding between the C-X-C chemokine receptor 4 on EPCs with SDF-1α in the peripheral area. Furthermore, hypoxic microenvironments such as wounds are suggested to induce the production of hypoxia-inducible factor 1 in endothelial cells, resulting in the expression of SDF-1 α in

epithelial cells [22,23]. In diabetic mice, mobilization and homing are typically disrupted because phosphorylation of eNOS in bone marrow EPCs is impaired and expression of SDF-1 α is decreased [21]. We demonstrated that edaravone increases SDF-1 α expression in wounds, and this may enhance homing. Similar to vasculogenesis, eNOS is indispensable for angiogenesis. Angiogenesis of $eNOS^{-/-}$ mice is reportedly impaired in ischemic environments [24]. Moreover, NO produced by eNOS was demonstrated to play a crucial role in endothelial migration and proliferation *in vitro* [25]. We believe edaravone modulates angiogenesis by increasing NO production via eNOS.

VEGF is a potent inducer of angiogenesis. The angiogenic effect of VEGF can be modified by VEGF-activated eNOS in endothelial cells [24,26,27]. Moreover, phosphorylation of VEGF receptor 2 reportedly activates eNOS [28-31], and several pathways are involved, including Akt/PKB, Ca^{2+}/calmodulin, and protein kinase C [32]. In contrast, among a number of angiogenic stimuli, NO regulates VEGF expression. Some groups have reported that NO downregulates VEGF expression [33-36], while others demonstrated upregulation [37-39]; it has been suggested that the amount of released NO, environmental oxygen tension, and cell types contribute to these conflicting results [32]. Thus, the lower level of VEGF expression in edaravone-treated mice in our study does

not necessarily reflect lower angiogenesis activity, which could have been the result of reciprocal regulation between NO produced by eNOS and VEGF. Although the association between NO and neovascularization has been extensively investigated, less information is available about the effects of NO on other facets of wound healing (i.e., inflammation, granulation tissue formation, epithelialization, collagen synthesis, and contraction) [40]. Cytokines represent other candidates for future investigation because similar to VEGF-A, cytokines can induce NO production, and NO can in turn modulate cytokine production [41].

Notably, topical application of edaravone promoted lymphangiogenesis in this study. Although NO derived from eNOS had a direct lymphatic effect, its role in lymphatic endothelial cells is unclear [42]. Stimulation of VEGF receptor 2 and/or 3 in lymphatic endothelial cells can lead to PI3K-dependent activation of eNOS. Conversely, NO reportedly stimulates lymphatic endothelial cell proliferation and/or survival *in vitro* [43]. Statins (HMG CoA reductase inhibitors) have been shown to have beneficial effects (e.g., stimulation of angiogenesis and activation of eNOS) that are independent of their cholesterol-lowering effects [44]. Interestingly, topical simvastatin stimulates lymphangiogenesis by recovering macrophage function and preventing apoptosis [45].

VEGF-C, produced mainly by macrophages, has an important effect on lymphangiogenesis in ischemic and diabetic conditions [5,46]. From the results of the previous reports and the finding that edaravone increased VEGF-C expression in the present report, it is tempting to surmise that edaravone provides favorable environment for lymphangiogenesis (e.g., affecting macrophage functions).

In one study, streptozotocin-induced diabetic mice demonstrated increased levels of superoxide [47]. It was proposed that increased cutaneous superoxide levels contribute to reduced NO bioavailability and loss of functional wound healing [48]. In accordance with this, edaravone accelerates wound healing. However, whether edaravone simultaneously scavenges superoxide and induces increased eNOS expression or the increased eNOS expression is dependent upon achieving balance of the redox state of the wound environment remains to be elucidated.

CONCLUSION

The simple application of edaravone could accelerate wound healing via angiogenesis and lymphangiogenesis in patients with diabetes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Table 1. Fold change of NO signaling pathway of edaravone- and petroleum jelly-treated wounds 7 days after treatment

Symbol	Description	Fold change (Edaravone/Control)
Epx	Eosinophil peroxidase Gene involved in Response to Oxidative Stress	2.10
Gpx5	Glutathione peroxidase 5 Gene involved in Response to Oxidative Stress	2.27
Mpo	Myeloperoxidase Gene involved in Response to Oxidative Stress	2.11
Nos 3	Nitric oxide synthase 3, endothelial cell Gene involved in Nitric Oxide (NO) Biosynthesis	2.55
Rpm	Reprimo, TP 53 dependent G2 arrest mediator candidate Gene Induced by Nitric Oxide	2.11
Tpo	Thyroid peroxidase Gene involved in Response to Oxidative Stress	7.95
Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier) Gene involved in Response to Oxidative Stress	3.80

Figure legends

Figure 1. Wound closure in diabetic mice. Full-thickness wounds were created on the backs of streptozotocin-induced diabetic mice. 10 mg of edaravone (30 mg of edaravone in 970 mg of petroleum jelly) or petroleum jelly alone was applied on day 0 and 4 after wound creation. The wounds were examined 0, 4, and 7 days after wound creation. **(a)** The time courses of the wounds were digitally recorded. Bar = 5 mm. **(b)** The extent of wound closure was calculated as the percentage decrease in the wound area. **$p < 0.01$. **(c)** Representative sections of a wound harvested 7 days after treatment; the boxed regions in the upper panels (×20 magnification) are shown at higher magnification (×400) in the lower panels.

Figure 2. Effects of edaravone on vascularity at the wound margin. (a) Neovascularization in edaravone- or petroleum jelly-treated diabetic mice after 7 days (×400 magnification). Red and blue fluorescence correspond to vWF-positive cells and DAPI-labeled nuclei, respectively. Bar = 50 μm. **(b)** The numbers of vWF-positive cells forming lumens per wound area were counted for petroleum jelly-treated mice (n = 3)

and edaravone-treated mice (n = 4). *p < 0.05.

Figure 3. Effects of edaravone on lymphangiogenesis at the wound margin. (a) Lymphangiogenesis in edaravone- or petroleum jelly-treated diabetic mice after 7 days (×400 magnification). Red and blue fluorescence correspond to LYVE-1-positive cells and DAPI-labeled nuclei, respectively. Bar = 50 μm. **(b)** The numbers of LYVE-1-positive cells forming lumens per wound area were counted for petroleum jelly-treated mice (n = 3) and edaravone-treated mice (n = 4). *p < 0.05.

Figure 4. Effects of edaravone on eNOS expression at the wound site. Relative quantification of eNOS expression at the wound site 7 days after treatment (left panel). **p < 0.01. Representative sections of eNOS-positive cells forming lumen constructs 7 days after treatment; the boxed regions in the upper panels (×20 magnification) are shown at higher magnification (×400) in the lower panels. Bar = 50 μm.

Figure 5. Relative quantification of VEGF-A, VEGF-C, SDF-1α, and fibroblast growth factor 2 expression at the wound site 7 days after treatment. *p < 0.05, **p <

0.01, ***p < 0.001.

Abbreviations:

EPC, endothelial progenitor cell; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; vWF, von Willebrand factor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; PCR, polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole