Maggot debridement therapy promotes diabetic foot wound healing by up-regulating endothelial cell activity

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ABSTRACT

To determine the role of maggot debridement therapy (MDT) on diabetic foot wound healing, we compared growth related factors in wounds before and after treatment. Furthermore, we utilized human umbilical vein endothelial cells (HUVECs) to explore responses to maggot excretions/secretions on markers of angiogenesis and proliferation. The results showed that there was neo-granulation and angiogenesis in diabetic foot wounds after MDT. Moreover, significant elevation in CD34 and CD68 levels was also observed in treated wounds. In vitro, ES increased HUVEC proliferation, improved tube formation, and increased expression of vascular endothelial growth factor receptor 2 in a dose dependent manner. These results demonstrate that MDT and maggot ES can promote diabetic foot wound healing by up-regulating endothelial cell activity.

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1. Introduction

Diabetes mellitus (DM) is one of the most prevalent diseases that affected more than 371 million people worldwide in 2012. By 2030, this number is expected to rise to 552 million (Waniczek et al., 2013). Of all diabetic complications, diabetic foot ulcer (DFU) is one of the most devastating and costly syndromes. DFS is defined as any necrosis, gangrene, or full-thickness skin defect occurring distal to the ankle in a diabetic patient (Schaper et al., 2012). The lifetime risk of developing a foot ulcer in a patient with diabetes can be as high as 25% (Boulton, Schmidt, Hartmann, & Fassler, 2002). MDT is believed to have an effect on at least three components of wound bed preparation: it effectively removes non-viable tissue, it helps combat infection by reducing bioburden, and it may facilitate the remodeling processes. It was reported that larvae and their secretions have antibacterial effects, reduce inflammation and neo-angiogenesis, and improve wound healing (Bexfield et al., 2010; Horobin, Shakesheff, & Pritchard, 2005; van der Plas et al., 2007). Although the debridement and wound-healing efficacy of MDT has been proven in clinical studies, no mechanism has been proposed recently (Dumville et al., 2009; Opletalova et al., 2012).

In this study, we investigated the neo-angiogenic factors in wounds before and after MDT, and used human umbilical vein endothelial cells (HUVECs) as a model system (Park et al., 2006) to determine these changes.

2. Materials and methods

2.1. Patients

Patients in this study were randomly chosen. Informed consent was obtained before MDT. The study was allowed by Junxie Hospital Ethical Committee (which is the other name of 454th hospital). The inclusion criteria are patients who were range from 2 to 3 with Wagner Classification, which has no lower limb ischemia.

2.2. Application of maggots

Sterile larvae (Lucilia sericata) obtained from our lab were provided free of charge to the patients participating in the study. The young larvae were prepared individually for each patient and applied at a density of one maggot per 50–80/cm² of wound area. The maggots were applied for approximately 72 or 120 hours, depending on the patient’s tolerance and
medical evaluation of the wound. The criteria to stop treatment may include 1) severe pain, which patients strongly reject the procedure; 2) the maggot is too big to escape from wound.

2.3. Pathology

Tissues from patient ulcers were fixed in formalin. Five-micrometer-thick paraffin-embedded sections were used for staining. The sections were briefly immersed in xylene, hydrated through graded ethanol solutions, and incubated in 3% hydrogen peroxide for 5 minutes to eliminate intrinsic peroxidase activity. The sections were incubated overnight at 4 °C with anti-CD34 and anti-CD68 antibodies (1:100), and incubated for 30 minutes in goat anti-goat horseradish peroxidase (HRP)-antisera, and then for 1 hour in species-specific peroxidase antiperoxidase complex. 3,3-Diaminobenzidine (DAB) was used as the chromogen with sections developed in 0.75 mg/mL DAB with 0.015% hydrogen peroxide in Tris buffer.

2.4. Cell culture

HUVECs were grown in 100 mm tissue culture dishes (Falcon; BD Bioscience Discovery Labware, Bedford, MA, USA), containing standard

Fig. 1. Effect of MDT on wound healing in diabetes. (a) Sterile larvae (L. sericata) obtained from our lab were prepared individually for each patient with one maggot per 50–80/cm² wound area. The maggots were applied every 72 or 120 hours, and photographs were taken to determine changes. (b) Before and after MDT, tissues from patients’ ulcers were fixed in formalin and stained with anti-CD34 and anti-CD68; expression levels were observed by light microscope. (c) Quantitative real-time PCR was used to evaluate mRNA expression of CD34 (left) and CD68 (right) from patient tissue before and after MDT.
cell culture medium comprised of Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MI, USA). Cells were incubated at 37 °C in a 5% (volume/volume) CO₂ humidified atmosphere.

2.5. Excretion/secretion (ES) collection

*L. sericata* larvae that had been hatched and kept in sterile conditions were used for MDT. The ES were collected from the third-instar larvae. Briefly, 20 larvae were washed in 10 mL of sterile phosphate-buffered saline solution (PBS, pH 7.3; Life Technologies) for 48 hours at 37 °C to recover ES products. Protein concentrations were determined using a protein colorimetric assay (Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer’s instructions.

2.6. HUVEC stimulation assay (excretion/secretion of maggots)

Approximately $1 \times 10^4$ HUVECs were seeded in 96-well plates, and incubated for 24 hours at 37 °C in a 5% (volume/volume) CO₂ humidified atmosphere. After incubation, each well was washed with phosphate buffered saline (PBS). Fresh medium containing ES, DMEM, 0.5% fetal bovine serum (FBS), and penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin; Life Technologies) was added. ES was diluted with DMEM to reach the appropriate protein concentration and an equivalent volume of PBS was added instead of ES for the control.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, after incubating with ES, 20 μL of 5 mg/mL MTT solution was added to each well and the cells were incubated for...
an additional 4 hours. Next, 150 μL of DMSO was added to each well, and absorbance was read at 490 nm on a microplate reader.

2.7. Quantitative real-time PCR

RNA was extracted from the samples in the wells using the phenol-chloroform method. For reverse transcription, an ImProm-II reverse transcription system (Promega, Madison, WI, USA) was used. Next, quantitative real-time PCR was performed to detect CD34, CD68, and β-actin. PCR conditions were the following: denaturation at 95 °C for 10 seconds with subsequent annealing at 54 °C for 30 seconds and extension at 72 °C for 30 seconds. Using SYBR Green 1 fluorescence, amplification was conducted (LightCycler480; Roche Diagnostics, Tokyo, Japan) according to a standard protocol. Vascular endothelial growth factor (VEGF), CD34, and CD68 mRNA expression was normalized to β-actin.

2.8. Endothelial cell tube formation

Matrigel was thawed at 4 °C overnight and each well of pre-chilled 24-well plates was coated with 100 μL of Matrigel and incubated at 37 °C for 45 minutes. HUVECs (4 × 10⁴ cells) were added to 1 mL of DMEM with various concentrations of ES. After incubation for 12 hours at 37 °C and 5% CO₂, endothelial cell tube formation was assessed after imaging with a Nikon digital camera inverted microscope. Tubular structures were quantified by manually counting low-power fields (50×), and the inhibition percentage was expressed as a fraction of control (untreated wells = 100%).

2.9. Statistical analysis

For the in vivo studies, results are expressed as means ± standard error (SE). The Student’s t-test was used to compare results and differences were considered significant when P < 0.05.

3. Results

3.1. MDT promotes diabetic wound healing

To determine the role of MDT on diabetic wound healing, we collected tissue from patients before and after MDT. Our results showed clinical improvement, such as a reduction in necrotic tissue and the formation of healthy granulation tissue (Fig. 1a). Side effects of MDT were not observed except for analgesic-controllable pain (unpublished data). Moreover, we observed significant elevation of CD34 and CD68 levels after MDT by immunohistochemistry (Fig. 1b) and by PCR (Fig. 1c).

3.2. ES increases cell proliferation and angiogenesis in HUVECs

To identify the mechanism by which MDT affects wound healing, we assessed the effects of ES, which is the main active ingredient produced by maggots, on HUVECs. We also evaluated proliferation (MTT) and apoptosis by annexin V/propidium iodine (PI) staining in HUVECs treated with ES. Compared with the untreated group, ES treated cells had significantly increased in HUVEC proliferation (Fig. 2a), and the apoptosis significantly decreased (Fig. 2b). Meanwhile, ES also stimulated histomorphological tube formation (Fig. 2c). These results suggest that ES might trigger endothelial cell activity.

3.3. ES promotes VEGFR-2 and CD34 expression in HUVECs

We further investigated tested the expression of CD34 and VEGF-R2 in HUVECs by PCR. As shown in Fig. 3, VEGF-R2 and CD34 expression increased in the ES treated group, which indicates that VEGF-R2 and the CD34 may regulate endothelial cell activity following ES treatment.

4. Discussion

Foot problems in diabetes continue to be a challenge for clinicians. The healing process is often disturbed as a result of vascular ischemia and various metabolic abnormalities that occur in diabetic patients (Falanga, 2005). Therefore, re-establishment of a functional vascular network is a critical component of successful wound repair (Wietecha & Dipietro, 2013). Angiogenesis induces endothelial cell proliferation, differentiation, migration, and organization into a branched tubular network (Ribatti, Nico, & Crivellato, 2009). It has been reported that advanced glycation end-products can induce endothelial cell dysfunction in diabetes (Liu et al., 2013). We hypothesize that strategies that promote endothelial cell activity can contribute to diabetic wound healing.

MDT is an accepted method of biosurgical debridement (Cickova, Cambal, Kozanek, & Takac, 2013), which was approved by The United States Food and Drug Administration for the treatment of non-healing wounds. Furthermore, a recent meta-analysis showed that MDT significantly improved wound healing (Wilasrusmee et al., 2013). In addition to directly ingesting necrotic tissue, the wound healing properties of MDT have been demonstrated in many studies (Honda et al., 2011; Sun et al., 2014). Our clinical results showed that wounds improved and CD34 and CD68 levels increased after MDT, which are indicative of increased angiogenesis. This demonstrates that MDT might have a potential role in inducing angiogenesis during wound healing. However, the precise molecular mechanisms underlying this process remain unclear.

Early research has shown that the molecules that provide the beneficial effects of maggots were contained in ES (Bexfield, Nigam, X. Sun et al. / Journal of Diabetes and Its Complications 30 (2016) 318–322
Thomas, & Ratcliffe, 2004; Pavillard & Wright, 1957; van der Plas et al., 2007). Further study of ES has revealed that it might contribute to the debridement, disinfection, and wound healing action of maggot therapy (Bexfield et al., 2010). Our previous study demonstrated the antibacterial activity of ES (Jiang et al., 2012). In the present study, we observed that healthy granulation tissue was predominant in the wound after MDT. Therefore, ES was used to verify the mechanism of MDT in wound healing. As expected, there was increased tube formation following ES treatment. It is worthwhile to mention that ES may work in concert to achieve angiogenesis, which is ultimately beneficial for wound healing, and this process might involve VEGF-VEGFR signaling. Further studies on this signal pathway, including the factors that regulate angiogenic signaling, are ongoing.

In conclusion, we observed that MDT could promote wound healing by increasing endothelial proliferation, triggering angiogenesis, and maggot excretion/secretion might facilitate this process. Further identification of the specific molecule that enhances angiogenesis in MDT could potentially lead to drug discovery for intractable wound healing therapy.

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References


