Journal of Diabetes and Its Complications 28 (2014) 588–595

Contents lists available at ScienceDirect

Journal of Diabetes and Its Complications

journal homepage: WWW.JDCJOURNAL.COM

Mesenchymal stem cells ameliorate impaired wound healing through enhancing keratinocyte functions in diabetic foot ulcerations on the plantar skin of rats

Jiro Kato [a], Hideki Kamiya [b,*,1], Tatsuhito Himeno [a], Taiga Shibata [a], Masaki Kondo [a,1], Tetsuji Okawa [a], Atsushi Fujiya [a], Ayako Fukami [a], Eita Uenishi [a], Yusuke Seino [a,c], Shin Tsunekawa [a], Yoji Hamada [a,c], Keiko Naruse [d], Yutaka Oiso [a], Jiro Nakamura [a,1]

[a] Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan
[b] Department of CKD Initiatives, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan
[c] Department of Metabolic Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan
[d] Department of Internal Medicine, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, 464-8650, Japan

ARTICLE INFO

Article history:
Received 31 January 2014
received in revised form 29 April 2014
accepted 14 May 2014
Available online 22 May 2014

Keywords:
Diabetic complication
Cell therapy
Mesenchymal stem cell
Diabetic foot ulcer
Wound healing

ABSTRACT

Aims/hypothesis: Although the initial healing stage involves a re-epithelialization in humans, diabetic foot ulceration (DFU) has been investigated using rodent models with wounds on the thigh skin, in which a wound contraction is initiated. In this study, we established a rodent model of DFU on the plantar skin and evaluated the therapeutic efficacy of bone-marrow-derived mesenchymal stem cells (BM-MSCs) in this model.
Methods: The wounds made on the hind paws or thighs of streptozotocin induced diabetic or control rats were treated with BM-MSCs. Expression levels of phosphorylated focal adhesion kinase (pFAK), matrix metaroprotease (MMP)-2, EGF, and IGF-1, were evaluated in human keratinocytes, which were cultured in conditioned media of BM-MSCs (MSC-CM) with high glucose levels.
Results: Re-epithelialization initiated the healing process on the plantar, but not on the thigh, skin. The therapy utilizing BM-MSCs ameliorated the delayed healing in diabetic rats. In the keratinocytes cultured with MSC-CM, the decreased pFAK levels in the high glucose condition were restored, and the MMP2, EGF, and IGF-1 levels increased.
Conclusions/interpretation: Our study established a novel rat DFU model. The impaired healing process in diabetic rats was ameliorated by transplantation of BM-MSCs. This amelioration might be accounted for by the modification of keratinocyte functions.

1. Introduction

Among the populations of developed and developing countries, an enormous number of people, about 350 million adults in recent estimation (Danaei, Finucane, Lu, et al., 2011), struggle against diabetes. Diabetes is associated with the development of various complications: stroke, ischemic heart disease, polyneuropathy, nephropathy, retinopathy, and diabetic foot. Diabetic foot, which is predominantly characterized by foot ulcers (Boulton, Vinik, Arezzo, et al., 2005), is one of the critical underlying diseases resulting in non-traumatic lower extremity amputation (anon, 2000; Moxey, Gogalniceanu, Hinchliffe, et al., 2011), which substantially impairs quality of life and contributes to high mortality rate (Brownrigg, Davey, Holt, et al., 2012; Schofield et al., 2006). Therefore, it is important to elucidate the mechanisms of diabetic foot ulcers. A combined etiology consisting of chronic peripheral vasculopathy, peripheral neuropathy, and increased susceptibility to infection has been proposed to be a major mechanism of the ulcer and a cause of delay of wound healing in diabetic patients (anon, 2000). However, the mechanisms of impaired wound healing in diabetic patients have not yet been detailed. Furthermore, there is no appropriate animal model to elucidate the mechanism of foot ulcer healing in diabetic patients.

Although numerous studies aiming to investigate the mechanisms of wound healing have utilized skin ulcers performed on the rodent dorsal region (Xu, Wu, Zhang, et al., 2012; Yan, Chen, Lin, et al., 2010), the healing procedure differs from that in human feet. The rodent dermis is bounded by panniculus carnosus, which accelerates wound contraction immediately after incisions (Wong, Sorkin, Glotzbach, Longaker, & Gurtner, 2011), whereas in humans, wound contraction

Duality of interest: The authors declare that there is no duality of interest associated with this manuscript.

* Corresponding author at: 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan. Tel.: +81 561 63 1683; fax: +81 561 63 1276.
E-mail address: hkamiya@aichi-med-u.ac.jp (H. Kamiya).
[1] Current affiliation: Division of Diabetes, Department of Internal Medicine, Aichi Medical University School of Medicine, 21 Karimata, Yazako, Nagakute, Aichi 480-1195, Japan.

http://dx.doi.org/10.1016/j.jdiacomp.2014.05.003
1056-8727/

occurs after the formulation of a collagen-rich matrix which is produced by proliferated and migrated fibroblasts (Sonnemann & Bement, 2011). In other words, the dominant processes in the early stage of wound healing are re-epithelialization and granulation in humans, but contraction in rodents. Therefore, some researchers have tried to compensate for the difference by utilizing ring-shaped devices which can expand the excisional wounds and reduce the contraction (Wong et al., 2011).

However, as mentioned above, in diabetic foot, many etiological influences, especially neuropathy and vascular disease, are involved. A stricter model, which replicates the circumstance and process of wound healing in diabetic foot ulcers, is needed.

Bone-marrow derived mesenchymal stem cells (BM-MSCs) are regarded as one of the more easily-available stem cells. Numerous researchers have performed cell therapies utilizing the BM-MSCs due to the fact that the cells are relatively safe, differentiate several types of cells, and secrete various kinds of cytokines that promote cell survival and proliferation. In the field of wound healing, cell therapies using BM-MSCs have been reported to be effective through modulating inflammation, extracellular matrix production, migration of keratinocytes, and angiogenesis (Jackson, Nesti, & Tuan, 2012; O'Loughlin, Kulkarni, Creane, et al., 2013; Wu, Zhao, & Tredget, 2010).

In this paper, we attempt to establish a more comprehensive model of diabetic foot ulcers. Furthermore, we evaluate the therapeutic effects of transplantation of BM-MSCs on impaired foot ulcer healing using the revised ulcer model.

2. Methods

2.1. Animals and induction of diabetes

Five-week-old male Sprague–Dawley rats (Chubu Kagakushizai, Nagoya, Japan) with an initial body weight of 170–180 g were allowed to adapt to an experimental animal facility for 7 days. They were housed in an aseptic animal room at a temperature of 20–24°C and a humidity of 40–70%, with a 12-hour light cycle and fresh air changes per hour, and were allowed free access to rat diet and water. Diabetes was induced by intra-peritoneal injection of streptozotocin (STZ) (60 mg/kg; Sigma Chemical, St. Louis, MO). Control rats received an equal volume of citric acid buffer. One week after STZ administration, rats with plasma glucose concentrations of > 16 mmol/l were selected as the diabetic group (D). The Nagoya University Institutional Animal Care and Use Committee approved the protocols of this experiment.

2.2. Skin wounding models on the foot or the thigh

Eight weeks after the induction of diabetes, ulcers were created. The paw was rinsed with 70% ethanol. A 4 mm diameter full thickness wound was created on the paw of both hind limbs using Biopsy Punch (Kai-medical). A skin on the thigh was shaved with an electric clipper, depilated using a depilatory cream, then sterilized and wounded following the same procedure as the paws. Two wounds were created on the left and right sides of the mid-spinal line.

Digital photographs were taken on the day of surgery (day 1) and every 2 or 3 days thereafter. Wound size was defined as the area that was not re-epithelialized. Time to closure was defined as the time at which the wound bed was completely filled in with new epithelium. Wound area was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA) and as a percentage of the maximum wound area from day 1.

On the 3rd, 7th, and 14th days after wounding, the animals were euthanized, and the wounds were harvested and fixed in 4% paraformaldehyde and frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) after cryo-protection.

2.3. Isolation and expansion of the BM-MSCs

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old male Sprague–Dawley rats with PBS. Mononuclear cells were isolated from the collected marrow using the Histopaque-density centrifugation method (Shibata, Naruse, Kamiya, et al., 2008). Total mononuclear cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 5.5 mmol/L D-glucose, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS (Sigma-Aldrich) on plastic dishes at 37°C in 5% humidified CO_2. Non-adherent cells were washed off, and adherent cells were expanded. When adherent cells were confluent (defined as passage 0), they were continuously cultured as BM-MSCs until passage 3.

2.4. BM-MSC-conditioned media (MSC-CM)

The confluent BM-MSC cells were rinsed with serum free DMEM once and were maintained in serum free DMEM. After a 24-hour culture, the culture media were collected, centrifuged to remove floating cells, concentrated 10 times using 10 kD centrifugal filters (Amicom Ultra-15, Millipore, Billerica, MA, USA), and frozen at −80°C until use. We defined this media as "MSC-CM".

2.5. Characterization of the BM-MSCs

The cells were incubated with FITC-conjugated mouse monoclonal anti-rat CD90 antibody (Becton Dickinson, Franklin Lakes, NJ), FITC-conjugated hamster monoclonal anti-rat CD29 antibody (Becton Dickinson), phycoerythrin (PE)-conjugated mouse monoclonal anti-rat CD34 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and PE-conjugated mouse monoclonal anti-rat CD45 antibody (Becton Dickinson), followed by characterization utilizing FACS (FACS Canto; Becton Dickinson). Isotype-identical antibodies served as controls.

2.6. Transplantation of the BM-MSCs

The BM-MSCs were labeled with red fluorescent dye PKH26 (Sigma Chemical) according to the manufacturer's protocol. Briefly, 1×10^6 BM-MSCs were labeled by 2×10^{-6} mol/l PKH26 for 3 min at room temperature, and this reaction was terminated by the addition of 2 ml FBS. Then, the cells were washed twice with DMEM and continuously cultured in DMEM.

On the next day, 1×10^6 cells of the BM-MSCs in 50 μl saline were injected around the wound on each right paw of normal or diabetic rats using Hamilton syringe. The wounds on the opposite paws were treated with saline.

2.7. Tissue collection and histological analysis

The samples underwent routine histological processing with hematoxylin and eosin, or counterstaining with DAPI. Images were captured by a charge-coupled device camera (DP70; Olympus Optical) using a fluorescence microscope (BX51; Olympus Optical).

2.8. Culture of human keratinocytes (HKCs)

HKCs were obtained from Lonza Japan (Tokyo, Japan). For RT-PCR and Western blotting, HKCs were seeded onto 6 well plates pre-coated with type I collagen (5 μg/cm^2) at a density of 1.0×10^5 cells per well and cultured in KGM-GOLD Bullet kit (Lonza Japan). After 24 hours of culturing, the medium was changed to KBM-2 medium without any supplements. Cells were treated with 6, 12, or 25 mmol/l glucose with or without MSC-CM for 72 hours. For measurement of cell viability, HKCs were seeded onto 96 well plates at a density of 1.0×10^4 cells per well and cultured as described above.