Effect of osteopontin in regulating bone marrow mesenchymal stem cell treatment of skin wounds in diabetic mice

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Abstract

Background We aimed to investigate the role of osteopontin in regulating mesenchymal stem cells transplanted to promote wound healing in diabetic mice.

Methods The mesenchymal stem cells of osteopontin knock-out (KO) and wild-type (WT) mice were isolated separately for in vitro culture and characterization. A skin wound on the back of mice was established by skin punching. In 27 osteopontin KO male mice, induced diabetes mellitus was via intraperitoneal injection of streptozotocin. 9 normal mice were used as controls. The mice were divided into four groups and injected with Dulbecco’s modified Eagle’s medium (DMEM) or mesenchymal stem cells via the tail vein: A (diabetic mice injected with DMEM), B (diabetic mice injected with osteopontin KO mesenchymal stem cells), C (diabetic mice injected with WT mesenchymal stem cells), D (normal mice injected with DMEM). The healing times and closure rates of skin wounds were recorded. The microvessel density of healing wounds was measured, and the localized expression of osteopontin was identified by western blotting and immunohistochemistry. The migration of mesenchymal stem cells was observed on normal mice with skin wound injected with mesenchymal stem cells of C57BL6-GFP transgenic mice, which show green fluorescent under UV light.

Results Compared with normal mice, the healing time of wounds in the mice with diabetes and osteopontin KO was significantly prolonged (p < 0.01). After transplanting osteopontin KO mesenchymal stem cells, the healing time was slightly shorter. Meanwhile, the healing time was significantly shorter after transplanted with WT mesenchymal stem cells and more significant neovascularization at healing wounds (p < 0.05). The expression of osteopontin in local healing wounds after transplantation of WT mesenchymal stem cells was demonstrated with western blotting and immunohistochemistry. After 4 days, the green fluoresces were noted on the wounds of mice injected with mesenchymal stem cells of fluorescent mice.

Conclusions Mesenchymal stem cells can migrate to wound sites, and osteopontin plays a regulatory role in mesenchymal stem cells promoting the healing of diabetic skin wounds. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords osteopontin; mesenchymal stem cells; skin wound; diabetes mellitus

Introduction

Wound repair and regeneration of tissues is one of the most fundamental biological processes in animals and humans. However, as a result of its unknown
mechanism, clinical treatment to promote the healing of diabetic skin wounds is still a challenge [1]. The roles of various types of stem cells in wound healing have been widely studied, and mesenchymal stem cells (MSCs) in particular have a good effect on wound healing. MSCs, which have a large reservoir in vivo and are convenient to obtain, have multilineage differentiation potential to osteoblasts, neurocytes, adipocytes, myocytes, epithelial cells and vascular endothelial cells and thus are an important cell source for wound healing [2]. MSCs in wound tissues are closely related to high localized expression of osteopontin (OPN), and there is also a correlation between MSCs and OPN with regard to localization in vivo and in vitro [3].

Osteopontin is a secretory phosphorylated glycoprotein and participates in the physiological and pathological processes of skin wound healing [4], and it can induce monocytes to secrete inflammatory factors such as interleukin (IL)-1β, which will activate endothelial cells in the dermis to express selectin [5,6]. Selectin is a chemical inducer of lymphocytes and causes lymphocytes to exit vessels and travel to wounds [7,8]. Besides, OPN regulates myofibroblasts and transforming growth factor-β expression to form granulation tissues [9,10]. Therefore, OPN may be a potential target for influencing the healing speed of skin wounds. Here, we explore the role of OPN in MSCs promoting the healing of diabetic skin wounds.

Methods

Mice

Two pairs of OPN knock-out (KO) and one pair of enhanced green fluorescent protein (EGFP) transgenic mice [11] of clean grade were purchased from Cyagen Biosciences (Guangzhou). The OPN KO mice were backcrossed for six generations to C57BL/6. They were bred to mate for offspring in the Center of Experimental Animals, Daping Hospital, Third Military Medical University. Twelve wild-type (WT) and 30 OPN KO male mice of the fifth generation were selected for the experiment. All experimental procedures were approved by the Animal Ethics Committee of the Third Military Medical University.

Isolation, culture and characterization of MSCs

Pregnant WT and OPN KO female mice were anaesthetized by ether inhalation on gestational day 20 then soaked for 1 min in 75% alcohol for disinfection and thereafter placed on a super-clean bench. The abdomen was opened with ophthalmological scissors to expose the uterus, and then the uterine wall was opened to remove the fetal mice. The fetal mice were placed into the sterile culture dishes, and then their femurs were sheared off and placed into Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Gibco) supplemented with 10% fetal calf serum (Gibco). Thereafter, the femurs were sheared into pieces with ophthalmological scissors, filtered three times and transferred into culture flasks then cultured in an incubator under the culture condition of 37°C, 5% CO2, and 95% saturated humidity. After 24 h, the medium was changed, the cells and debris suspended, and impurities removed. After six passages, 1 × 10^6 adherent MSCs were harvested and washed twice with phosphate-buffered saline then resuspended in 100 μL FACS buffer, and 10 μL CD34-FLTC and 10 μL CD29-FLTC were added. Thereafter, they were incubated away from light at 4°C and mixed well once in every 10 min, and finally detected with flow cytometry after 30 min.

Establishment of a diabetes mellitus model

Osteopontin knock-out mice were subjected to intraperitoneal injection of streptozotocin (40 mg/kg) continuously for 5 days. At 1 week after the last injection, 3 mm mouse tails was sheared off with ophthalmologic scissors, the blood was slowly extruded from the base to the end of the tails and dropped onto blood glucose test strips, and then the blood glucose level was measured with a Roche glucometer. If the mice had a blood glucose level >11.1 mmol/L, the diabetes mellitus model had been successfully established, otherwise, continuous induction was performed with 3 days of continuous injection of streptozotocin. On the day of injection and at week 4 after injection, the weights and blood glucose levels of the mice were measured and recorded.

Establishment of a diabetic skin wound model

The hairs on the backs of diabetic mice were removed with cotton swabs wetted with 8% sodium sulfide, avoiding the sodium sulfide to enter the mouths of the mice. After removal of the hairs, the backs of the mice were cleaned with cotton swabs wetted with warm water and then wiped dry. At day 2, after removal of the hairs, the mice were anaesthetized with ether, then an 0.8 cm round wound deep to the muscular fasciae was made on the back using a puncher and the wound edge was trimmed with ophthalmological scissors under haemostasis by compression with cotton swabs. Thereafter, the mice were housed separately in single cages. From the
day of the invasive operation, the wound healing was observed daily until the wounds were completely closed, and the wound healing time was recorded.

**Grouping and MSCs transplantation**

Twenty-seven OPN KO diabetic mice were randomly divided into three groups (A, B and C). Group A only received an injection of 200 μL serum-free DMEM, group B an injection of 200 μL OPN KO MSCs and group C an injection of 200 μL WT MSCs. Nine normal mice with skin wound were injected with 200 μL serum-free DMEM as normal control group D. Before injection, two types of sixth-generation cultured MSCs were digested and centrifuged and then added into the serum-free DMEM to give cell suspensions of 2 × 10⁴/μL, and the cell suspensions were collected into 10 mL centrifuge tubes. The mouse tails were soaked in a 40-°C water bath for 5 min, and after the tail veins were engorged, 200 μL cell suspensions (4 × 10⁶ MSCs) was injected via the tail veins, using 1-mL sterile syringes.

**Observation of healing time and closure rate of wounds**

After the operation and the injection of MSCs via the tail veins, the animals were housed individually. The wound area was covered with a semiocclusive polyurethane dressing (Tegaderm). The dressing was changed twice a week; during that time, the animals were anaesthetized, and the wounds photographed from a standard height. The wound area was analysed using Image J software, and the wound closure rate was calculated using the following formula: wound closure rate = (initial wound area/C0 wound area on the day of observation)/initial wound area × 100%.

**Tissue samples and pathological sections**

On days 7 and 14, three animals from each group were euthanized, and the remaining animals were kept for the measurement of wound closure rate. The entire wound was harvested, including the surrounding skin area of 0.5 cm, and cut through the middle. One piece of tissue was treated with protein lysis buffer freshly prepared to extract total protein; the other piece was fixed in 10% neutral-buffered formalin solution for 24 h and stored in 70% alcohol at 4 °C until it was embedded in paraffin; then, the tissue samples were cut to 6-μm-thick sections.

**Histology and immunohistochemistry of healing wounds**

Some of the sections were stained with haematoxylin and eosin for structural evaluation. To test the microvessel density using immunohistochemistry, the sections were rehydrated, and antigen retrieval was performed; then, the sections were incubated with rabbit anti-CD34 (1 : 400; Santa Cruz) or rabbit anti-OPN (1 : 500; Santa Cruz) overnight at 4 °C. The secondary antibody used was conjugated with horseradish peroxidase (1 : 500; Milipore) at room temperature for 1 h. After development, the sections were rinsed with water, counterstained with diaminobenzidine and mounted. Images were taken with a light microscope (Eclipse 80i, Nikon); ImageJ was used for image analysis of the entire tissue sections. The microvessels in the selected region were counted at ×200 magnification, and then, the microvessel density (the number of microvessels in 1 mm²) was calculated.

**Western blotting**

Briefly, tissue proteins were extracted in a lysis buffer containing 1% Triton X-100 and proteinase inhibitors (Sigma). Equivalent amounts of each treatment group were run on 10% sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred electrically (12 V, 30 min) to a polyvinylidene fluoride membrane and incubated with a rabbit anti-mouse monoclonal antibody anti-OPN (1 : 400; Santa Cruz) for 2 h at 37 °C. After three washes with TBST, the membranes were incubated with fluorophore-conjugated secondary antibodies (either 680 or 800 nm emission). Detection and quantification of the bands was performed using the Odyssey Infrared Imaging System (Li-Cor). β-actin was used as the loading control.

**Watching the migration of MSCs with living imaging**

We isolate the MSCs of EGFP transgenic mice according to the previously mentioned methods and inject into the subcutaneous tissue of normal mice with skin wound. There is a 5-cm distance from the injecting site to the wound. The mice were watched, and images were taken with a fluorescence camera.

**Statistical analysis**

Data were expressed as means ± standard error. Statistical differences among the mean values of multiple groups were determined using analysis of variance followed by Student’s t-test; p < 0.05 was considered statistically significant.
Results

**In vitro culture of WT and OPN KO MSCs**

Hind femoral MSCs of WT and OPN KO fetal mice were extracted and isolated for culture. After 24 h of culture, the cells began to adhere to the wall and demonstrated a round or polygonal shape. After 3 days of culture, the adherent cells had a significantly increased size and started to divide; they gradually demonstrated a spindle-shaped or fusiform morphology and fibroblast-like growth and formed clusters in different sizes. Nonadherent cells were removed by changing the medium, and the remaining haematopoietic stem cells and vascular en-

![Figure 1](image1.png)

**Figure 1.** WT and OPN KO MSCs were separately cultured in complete DMEM. Bar = 50 μm

![Figure 2](image2.png)

**Figure 2.** Testing the expression of CD34 and CD29 on MSCs with a flow cytometry instrument: expression of (A) CD34 in WT MSCs, (B) CD29 in WT MSCs, (C) CD34 in OPN KO MSCs and (D) CD29 in OPN KO MSCs
dothelial cells did not survive for a long time, because of the limitations of the culture environment and thus died or disintegrated. Adherent MSCs gradually showed a ridge-shaped, fish swarm-shaped, swirl-shaped, and reticulate or radiate arrangement; the cell division growth was very evident, and there was plenty of cytoplasm and large nuclei (Figure 1).

**Characterization with flow cytometry**

The cultured sixth-generation MSCs were digested and harvested then incubated with fluorescein isothiocyanate-labelled mouse anti-human CD34 antibody and phycoerythrin-labelled mouse anti-human CD29 antibody, separately, in a ratio of 1:3 × 10^5 cells, and finally detected and analysed with flow cytometry. CD34 is a highly glycosylated type I transmembrane glycoprotein, selectively expressed on the surface of human and other mammal haematopoietic stem/progenitor cells, and is reduced with the maturation of cells until it vanishes. CD29 is a β1 integrin, and β1 integrins are the receptors of several extracellular matrix proteins. There are various kinds of β1 integrins with different functions, participating in such activities as cell proliferation, differentiation and apoptosis, and their positive expression is seen in MSCs. The detection results are shown in Figure 2A–2D, positive expression of CD29 in WT and OPN KO MSCs and negative expression of CD34 in the MSCs.

**Wound healing of each group**

A round skin wound on the back of mice was established. After grouped, different types of MSCs were injected via the tail veins; and thereafter, wound healing was observed. The complete healing time of wounds in the four groups was 19.90 ± 0.55 days (A), 18.52 ± 0.42 days (B), 13.70 ± 0.28 days (C) and 5.54 ± 0.21 days (D) (Table 1). The closure rate in group C was accelerated markedly after 5 days and was significantly faster than group A and B (p < 0.05) (Table 2). The wound healing condition from 3–14 days after making the wounds was shown in Figure 3. Haematoxylin and eosin staining of the four groups was carried out at 7 and 14 days to watch tissue repairing status. Diabetes mellitus and OPN KO significantly delayed the repairing of wound, and transplanting MSCs improved the repairing process. There was more faster and better quality repairing in the mice transplanted with WT MSCs than KO MSCs (Figure 4).

**Microvascular density**

The expression of CD34 in the wounding area was checked with immunohistochemistry at 14 days. CD34 is mainly expressed in vascular endothelial cells; therefore, CD34 immunohistochemical staining was used to indicate microvascular density. The number of microvascular was counted in 10 random fields of each group and compared among the four groups. Compared with group A, group B had increased microvascular density. Group C had more significantly expressed CD34 than group B (p < 0.05), even if it was less than group D (Figure 5).

**OPN expression in healing wound tissues**

In order to clarify the expression of OPN in the healing wound tissues, dermal tissues of healing wounds in each group were collected. Parts of these tissues were homogenized to extract total protein for western blotting, whereas the remaining tissues were sectioned and detected with immunohistochemistry. There was no OPN expression in groups A and B. However, a significant OPN expression was seen in group C; however, it was lower than normal control group. A similar phenomenon was showed with immunohistochemistry (Figure 6).
Migration of MSCs to wound with living imaging

In order to watch the migration of MSCs from transplanting site to the wound, the MSCs of EGFP transgenic mice was isolated and injected into the subcutaneous tissue of normal mice with skin wound. There is a 5-cm distance from the injecting site to the wound. After 4 days, we can find the green fluorescence in the wound area under the fluorescence camera (Figure 7).

Figure 3. Changes in wound area during 3 to 14 days after the wound model was set up. Upper: range of wound models; A: changes in wound area of a group A mouse; B: changes in wound area of a group B mouse; C: changes in wound area of a group C mouse; D: changes in wound area of a group D mouse
Discussion

In this study, we established a diabetic skin wound model with OPN KO mice, and normal mice served as controls. Then, we compared the effects of OPN KO MSCs versus WT MSCs, injected via the tail veins, on wound healing. The results showed that after OPN KO MSCs transplantation into OPN KO mice, the wound growth rate was accelerated compared with the diabetes group, but the difference was not statistically significant; the pathology of healing wound tissues found no OPN expression. However, after WT MSCs were transplanted into OPN KO mice, the wound growth rate was markedly accelerated, the wound closure rate was significantly increased and the total healing time was evidently shortened; the pathology of healing wound tissues showed high expression.

Figure 4. Haematoxylin and eosin staining of wound area from four groups of mice at 7 and 14 days. (A) Granulation tissue is present, with scattered distribution of fibroblasts and inflammatory cell infiltration at 7 days. There are still many inflammatory cells and fibroblasts that can be seen in the wound, but there is no intact epidermal structure at 14 days. B: Fibroblasts are visible and many inflammatory cells can be seen at 7 days. Angiogenesis is visible, but there are inflammatory cells, and the skin is not intact at 14 days. C: Fibroblasts and inflammatory cells are visible in the granulation tissue at 7 days. The wound is completely epithelialized, and the skin is intact at 14 days. D: The wound skin tissue is completely normal at 7 and 14 days. Bar = 100 μm
of OPN and significant neovascularization. These findings suggest that OPN expression in MSCs promotes local neovascularization and improves local microcirculation, thus accelerating localized wound healing. The OPN expressing in the wound tissues came from the WT MSCs, and we verified the migration of MSCs from

Figure 5. Detecting microvascular density with immunohistochemistry. After the 14-day wound model was set up, the expression of CD34 in each group was tested by immunohistochemistry in wound tissues, and the average number of microvessels was deduced. Compared with A group, other groups have significantly increased CD34 expression (*p < 0.05), and transplanting with WT MSCs shows more addition of CD34 expression than transplanting with KO MSCs (**p < 0.05). Bar = 100 μm
transplanting site to the wound with EGFP transgenic mice. It is worth noting that compared with normal mice, the diabetic KO mice transplanted with WT MSCs still had slower healing speed, which means that diabetes mellitus treatment is also important in the wound healing.

Diabetic skin wounds are usually long-standing and difficult to cure, mainly because fibroblast proliferation and collagen synthesis surrounding the wounds are reduced and there is an inflammatory over-response [12,13]. The process of skin wound healing can be divided into three contiguous periods: the inflammation period, the deposition of extracellular matrix period and the tissue remodelling period. This process involves a variety of cytokines, various types of cells and extracellular matrix [14]. In the inflammation period, the involvement of neutrophils and macrophages is dominant, whereas in the extracellular matrix deposition period closely following it, the proliferation of epithelial cells, vascular endothelial cells and fibroblasts are predominant, and in the tissue remodelling period, the epithelium and mesenchyme permanently regulate the integrity and dynamic balance of the skin to complete the wound healing process. If the regulation of these cytokines is disordered, it may interfere with the normal process of tissue repair and healing, prolong healing time or even construct a scar [15]. MSCs play a definite role in promoting neovascularization and have the capability to encourage the neovascularization of receptors.MSCs also have multilineage differentiation potential and enable the healing and dermal remodelling of refractory wounds [16].

The mechanism of regulating MSCs transplanted to promote wound healing and the mechanism of MSCs migrating to wounds are as yet both unknown. OPN is coincident with MSCs migration. When the binding of OPN with its receptor CD44 is interrupted, MSCs migration becomes markedly slow [17]. The detection of
cultured MSCs found positive expression of both CD44 and OPN in MSCs, and the immunofluorescence staining of wound tissue sections also showed OPN expression in CD44-positive cells. It is thereby inferred that over-expressed OPN mobilizes MSCs and promotes them to migrate to the wounds, and thus induces them to differentiate into various types of cells participating in wound repair. Recent studies have shown that CD44 has a new function of regulating the homing of MSCs, and a fixed glycosyl group in the molecular structure of CD44 induces the homing of MSCs to bone tissues by interaction with E-selectin [18]. Once cell surface molecules are highly expressed in specific organs of cells and tissues and thus lead to the interaction between MSCs and endothelial cells, the MSCs homing signature makes the targets of MSCS more accurate, which guides the MSCs to arrive at the wounds and participate in wound repair. CD44 is the receptor of OPN, whereas OPN is capable to mobilize bone marrow cells and induce the migration and differentiation of human embryonic kidney 293 cells [19], thus OPN can regulate MSCs homing via the CD44/E-selectin pathway and promote MSCs migration to the wound site and thus induce MSCs differentiation. This may be the potential molecular mechanism.

In conclusion, MSCs transplantation can promote the healing of diabetic skin wounds, whereas OPN may be an important target to regulate MSCs migrating to wounds and differentiating into other cells near to wounds, and plays a regulatory role in MSCS promoting the healing of diabetic skin wounds.

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Conflicts of interest
The authors have no conflicts of interest.

References


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