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

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Abstract

**Background** We aimed to investigate the role of osteopontin (OPN) in regulating MSCs transplanted to promote wound healing in diabetic mice.

**Methods** The MSCs of OPN 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. 27 OPN KO male mice were induced diabetic mellitus via intraperitoneal injection of streptozotocin (STZ) and 9 normal mice were as control. The mice were divided into 4 groups and injected DMEM medium or MSCs via the tail vein: A (diabetes injected with DMEM), B (diabetes injected with OPN KO MSCs), C (diabetes injected with WT MSCs), D(normal 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 OPN was identified by western blotting and immunohistochemistry. The migration of MSCs was observed on normal mice with skin wound injected MSCs of fluorescent mice.

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

**Conclusion** MSCs can migrate to wound site and OPN plays a regulatory role in MSCs promoting the healing of diabetic skin wounds.

**Keywords** osteopontin; mesenchymal stem cells; skin wound; diabetes mellitus
**A: 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].

OPN 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 regulate myofibroblasts and transforming growth factor-β (TGFβ) 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.

**B: Methods**

**+B:Mice**

Two pairs of OPN KO and one pair of 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. 12 WT and 30 OPN KO male mice of the fifth generation were selected for experiment. All experimental procedures were approved by the Animal Ethics Committee of the Third Military Medical University.

**B: Isolation, culture and characterization of MSCs**

Pregnant WT and OPN KO female mice were anaesthetized by ether inhalation, 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 (FCS; 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, 95% saturated humidity. After 24 h, the medium was changed and the cells and debris suspended and impurities removed. After six passages, 1 × 10⁶ adherent MSCs were harvested and washed twice with phosphate-buffered saline (PBS), 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.

**B: Establishment of a diabetes mellitus model**

OPN KO mice were subjected to intraperitoneal injection of streptozotocin (STZ, 40 mg/kg) continuously for 5 days. At 1 week after the last injection, 3 mm mouse tails were 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 STZ. On the day of injection and at week 4 after injection, the weights and blood glucose levels of the mice were measured and recorded.

+B: Establishment of a diabetic skin wound model

The hairs on the backs of diabetic mice were removed with cotton swabs wetted with 8% sodium sulphide, avoiding allowing the sodium sulphide 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.

+B: 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. 9 normal mice with skin wound were injected 200μL 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 $2 \times 10^4$/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 ($4 \times 10^6$ MSCs) were 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 semi-occlusive polyurethane dressing (Tegaderm). The dressing was changed twice a week, during which 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:

\[
\text{wound closure rate} = \frac{\text{initial wound area} - \text{wound area on the day of observation}}{\text{initial wound area}} \times 100\%.
\]

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 one 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 (H&E) for strutural 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 DAB (Sigma) 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 x200 magnification and then the microvessel density (the number of microvessels in 1 mm²) was calculated.

**+B: 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% SDS polyacrylamide gels. Proteins were transferred electrically (12 V, 30 min) to a PVDF 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.

**+B: Watching the migration of MSCs with living imaging**

Isolate the MSCs of EGFP transgenic mice according to above-mentioned methods, inject into the subcutaneous tissue of normal mice with skin wound. There are 5cm distant from the injecting site to the wound. The mice were watched and Images were taken with a fluorescence camera.

**+B: Statistical analysis**

Data were expressed as means ± standard error (SE). 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.
**A: Results**

**B: 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 d 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. Non-adherent cells were removed by changing the medium, and the remaining haematopoietic stem cells and vascular endothelial cells did not survive for a long time, due to the limitations of the culture environment, and thus died or disintegrated. Adherent MSCs gradually showed a ridge-shaped, fish swarm-shaped, swirl-shaped, reticulate or radiate arrangement; the cell division growth was very evident, and there was plenty of cytoplasm and large nuclei (Figure 1).

**B: Characterization with flow cytometry**

The cultured sixth-generation MSCs were digested and harvested, then incubated with FITC-labelled mouse anti-human CD34 antibody and PE-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–D; positive expression of CD29 in WT and OPN KO MSCs and negative expression of CD34 in the MSCs.
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 4 groups was 19.90 ± 0.55 d (A), 18.52 ± 0.42 d (B), 13.70 ± 0.28 d (C) and 5.54± 0.21d (D) (Table 1). The closure rate in group C was accelerated markedly after 5d, and was significantly faster than group A and B (p < 0.05) (Table 2). The wound healing condition from 3d–14d after making the wounds was shown at Figure 3. H&E staining of the 4 groups was carried out at 7d and 14d to watch tissues repairing status. Diabetes mellitus and OPN knockout significantly delayed the repairing of wound, and transplanting MSCs improved the repairing process. There were more faster and better quality repairing in the mice transplanted with WT MSCs than KO MSCs(Figure 4).

**+B: Microvascular density**

The expression of CD34 in the wounding area were checked with immunohistochemistry at 14d. 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 4 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).

**+B: 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, while the remaining tissues were sectioned and detected with
immunohistochemistry. There was no OPN expression in group 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).

**+B: 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 inject into the subcutaneous tissue of normal mice with skin wound. There are 5cm distant from the injecting site to the wound. After 4 days we can find green fluorescence in the wound area under the fluorescence camera (Figure 7).

**+A: 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 to 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 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 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.

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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, while in the extracellular matrix deposition period closely following it, the proliferation of epithelial cells, vascular endothelial cells and fibroblasts is 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 cells of organs 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, while OPN is capable to mobilize bone marrow cells and induce the migration and differentiation of human embryonic kidney (HEK) 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, while 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.

+B: Acknowledgement

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+A: Conflict of interest

The authors have no conflicts of interest.

+A: References


Table 1. Wound-healing time of each group

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>Healing time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>19.90 ± 0.55</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>18.52 ± 0.42</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>13.70 ± 0.28*#</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>5.54 ± 0.21**##</td>
</tr>
</tbody>
</table>

Compared with group A, * (p < 0.05), **(p < 0.01);
Compared with group B, * (p < 0.05), ##(p < 0.01).
Table 2. Wound healing rate of each group of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>8.51 ± 1.06</td>
<td>33.09 ± 3.78</td>
<td>56.21 ± 5.39</td>
<td>81.52 ± 6.54</td>
<td>99</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>8.62 ± 2.10</td>
<td>34.85 ± 2.51</td>
<td>59.54 ± 1.58</td>
<td>85.14 ± 4.21</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>13.12 ± 2.60</td>
<td>39.15 ± 5.01*</td>
<td>67.82 ± 8.13*#</td>
<td>99.57 ± 2.51*#</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>46.54 ± 5.36*#</td>
<td>69.78 ± 8.98**##</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Compared with group A, * (p < 0.05), ** (p < 0.01);
Compared with group B, * (p < 0.05), ** (p < 0.01).
Figure 1. WT and OPN KO MSCs were separately cultured in complete DMEM. bar=50μm
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; (D) CD29 in OPN KO MSCs.
Figure 3. Changes in wound area during 3 days 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.
Figure 4. H&E staining of wound area from 4 groups mice at 7 days 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 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 complete normal at 7 days and 14 days. bar = 100 µm
Figure 5. Detecting microvascular density with immunohistochemistry. After the 14 days 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=100μm
Figure 6. Expression of OPN is different in each group. A: Observing the expression of OPN with western blot, and β-actin was used as the loading control. There were not expressed of OPN in group A and B, but a significant expression of OPN was found in group C, even if the expressional level was lower than normal group D (*p < 0.05). B: Observing the expression of OPN with immunohistochemistry. The paraffin sections of wound tissues after the 14 days wound model were immunolabelled with rabbit anti-OPN. OPN (yellow) was seen in group C and D. Representative images are shown from three independent experiments. scale bars = 50 µm
Figure 7. Migration of MSCs from transplanting site to the wound with living imaging. The MSCs of EGFP transgenic mice were isolated and injected into the subcutaneous tissue of normal mice with skin wound. There are 5cm distant from the injecting site to the wound. Every day watched the migration of MSCs under the fluorescence camera and taken picture, and at 4days green fluorescence was founded in the wound area.