Comparison of Bone Marrow-derived Mononuclear Cells vs. Mesenchymal Stem Cells in Protecting Random-pattern Skin Flap Survival in Rats

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Abstract
Both cultured Bone Marrow Mesenchymal Stem Cells (BMMSCs) and non-cultured Bone Marrow Mononuclear Cells (BM-MNCs) can improve random-pattern skin flap survival. However, whether prior culture expansion of bone marrow cells is beneficial for this therapy remains unclear. In the current study, the protective effects of BM-MNCs and BMMSCs derived from identical bone marrow aspirates were compared in a random-pattern skin flap rat model. The mean skin flap survival rates were 71.8 ± 8.4% in the BM-MNC-treated group and 66.2 ± 3.1% in the BMMSC-treated group, both of which were significantly higher than the control group (55.3 ± 3.4%). The protective effects were confirmed by blood perfusion analysis and vessel density assay. However, no significant difference was observed between the cell transplanted groups. These results indicate that the current method for pre-culture of BMMSCs does not bring therapeutic benefits in skin flap protection. Therefore, BM-MNCs without pre-culture might be more practicable in the clinical setting.

Keywords: Random-pattern skin flap; Cell therapy; Bone marrow mononuclear cells; Bone marrow mesenchymal stem cells

Introduction
The random-pattern skin flap is one of the most widely used flaps in the repair of skin defects. However, partial necrosis, especially in the distal part, is a major problem because of lack of blood supply and severe ischemia [1]. This problem therefore limits the length-width ratio of random-pattern skin flaps. Previous researchers have found that sympathetic drugs, vasodilators and calcium channel blockers, could improve ischemia of skin flaps [2,3]. However, applying high doses of these drugs can lead to many side-effects. Various growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF) and transforming growth factor-β (TGF-β), have been proven to be beneficial for the survival of skin flaps, although they are also restricted by their short half-life and poor long-lasting effects [4-6].

In recent years, cell therapy has become a new strategy for tissue repair and regeneration. Both Bone Marrow Mononuclear Cells (BM-MNCs) and Bone Marrow Mesenchymal Stem Cells (BMMSCs) derived from bone marrow cell culture have been proven to improve skin flap survival [7,8]. However, it is unclear which cell population is optimal for treatment. Ideally, if a small number of BM-MNCs could be expanded to achieve a large number of BMMSCs without losing their protective function, the amount of bone marrow aspirate could be greatly reduced to meet current clinical requirements. On the contrary, if cells lost their protective function during cultivation, cell expansion might not be favorable for clinical application. To date, no study has compared the protective potential of cultured and non-cultured bone marrow cells in skin flap survival. To address this, we measured the phenotypic changes of bone marrow cells before and after culture, and compared the protective effects of non-cultured BM-MNCs and expanded BMMSCs derived from the same bone marrow aspirates in a random-pattern skin flap rat model.

Materials and Methods
Male Wistar rats (3-6 weeks old) were purchased from Shanghai Chuansha Experimental Animal Raising Farm (Shanghai, China). Animal procedures were approved by The Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine. All animals were maintained in a pathogen-free environment. Surgical procedures were performed under aseptic conditions.

Isolation of BM-MNCs
Isolation of BM-MNCs was performed according to standard protocol [8]. Briefly, male Wistar rats (3 weeks old) were sacrificed by cervical dislocation. Bilateral femurs and tibias were dissected and the bone marrow was harvested and suspended in low glucose Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). BM-MNCs were isolated by density gradient centrifugation of bone marrow cells on Ficoll-paque (Sigma, St. Louis, MO). Cells were resuspended in phosphate buffered saline (PBS) before transplantation.

Expansion of BMMSCs
Bone marrow was harvested and cultured to expand BMMSCs as previously described [9]. Briefly, whole bone marrow cells were plated onto tissue culture dishes in DMEM with 10% FBS and maintained at 37°C in 5% CO2. After 24 hours, the plates were washed with PBS to remove non-adherent cells. The resulting adherent cells were grown...
Multilineage differentiation of BMSCs

The adipogenic, osteogenic and chondrogenic differentiation of BMSCs (passage 4) were performed respectively as previously described [10,11]. After 3 weeks of induction, adipogenic differentiation was measured by Oil red O staining; osteogenic differentiation was detected by Alizarin red staining; and chondrogenic differentiation was measured by Alcian blue staining. All the stainings were performed according to the standard protocols [10,11].

Flow cytometric analysis of BM-MNCs and BMSCs

Freshly isolated BM-MNCs or cultured BMSCs (P4) were collected and 5×10⁶ cells were resuspended in 200 μl of wash buffer (PBS containing 4% FBS). Freshly diluted fluorescein isothiocyanate (FITC)-conjugated anti-CD29 (1:200), CD90, phycoerythrin (PE)-conjugated anti-CD45 (all from BD Pharmingen, San Diego, CA), CD14, CD133, CD144, KDR, CD31 and CD34 (all from Santa Cruz Biototechnology Inc; Santa Cruz, CA) monoclonal antibodies were added individually into respective tubes and incubated on ice for 30 minutes. After three washes, cells were resuspended in wash buffer for analysis. FITC or PE-conjugated isotype-matched immunoglobulins were used to determine nonspecific staining. Cells were analyzed on a FACs Caliber (Becton-Dickinson, San Jose, CA) and data were analyzed with Cell Quest software (Becton-Dickinson).

Skin flap model and cell transplantation

The skin flap model and cell transplantation procedure are shown in Figure 1. In brief, 6-week-old Wistar rats were anesthetized with chloral hydrate (0.4 ml/100 g) and the dorsal skins were shaved using electric clippers. Two days before operation (day -2), cells were subcutaneously injected in 7 points on the dorsal skin evenly (cell numbers for transplantation are described below). On day 0, an 8-cm×2-cm random-pattern skin flap was raised and sutured back in situ using a 3-0 silk suture [12]. The survival area of the skin flaps was measured on day 7.

To identify an optimal cell dose for therapy, either 1×10⁶ or 5×10⁶ of freshly isolated BM-MNCs were injected into rats (n=6/group). The same volume of PBS was injected as a control (n=6). The survival of the skin flaps was measured on day 7 and an optimal cell dose of 5×10⁶ cells per rat was chosen for further experimentation.

To compare the therapeutic effects of BM-MNCs and BMSCs, bone marrow aspirates collected from 4 rats were divided equally into halves. One half was used to isolate BM-MNCs directly and the other half was used to expand BMSCs in culture as described above. Step 1: the total cell number of BM-MNCs was counted and the total number of animals that could be treated with the BM-MNCs was calculated by (total BM-MNC number)/(5×10⁶). Step 2: the total cell number of BMSCs at passage 4 was counted and an equivalent number of cells to be injected into each animal was calculated by (total BMSC number)/(total number of animals that could be treated by BM-MNCs). Step 3: 5×10⁶ of BM-MNCs or equivalent number of BMSCs were suspended in 700 μl of PBS and subcutaneously injected in each animal (7 injection points in each animal with 100 μl in each point, n=6/group). Control animals received the same volume of PBS (n=6).

Survival of skin flaps

On day 7, rats were anesthetized and images of each flap were taken with a digital camera. The survival area of each flap was grossly determined based on its appearance, color and texture. The survival area was quantified by Image-Pro Plus software (Version 6.0, Media Cybernetics, Rockville, MD). Results were expressed as percentage of survival in relation to the total surface area of the skin flap.

Blood perfusion measurement

On day 7, rats were anesthetized and blood perfusions of the skin flaps were detected with a Full-Field Laser Perfusion Imager (FLPI, Moor Instruments Ltd, Axminster, UK). Results were presented as the laser intensity of the flaps.

Histology

Rats were sacrificed with an overdose of anesthesia on day 7. Specimens were taken from the middle part of each flap. The specimens were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned as 6-μm slices. Immunohistochemical staining of anti-CD31 antibody (Dako, Glostrup, Denmark) was carried out using standard protocol, followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Dako) and colorized with diaminobenzidine tetrahydrochloride (DAB, Dako). Five randomly selected fields were captured by a light microscope. The number of blood vessels was calculated by Image-Pro Plus software (Version 6.0, Media Cybernetics). Three slices from each group were analyzed.

Statistical analysis

All data were expressed as mean ± SD. Comparisons of flap survival...
rate and capillary number between groups were performed using the Student–Newman–Keuls q test. A value of P<0.05 was considered significant.

Results

Characterization of BM-MNCs and BMSCs

BMSCs (P) exhibited a typical fibroblast-like morphology with strong proliferation ability in vitro (Figure 2A). These cells were able to differentiate into adipocytes, osteoblasts and chondrocytes in the presence of certain factors (Figures 2B-2D). The cell surface marker expression profiles of BMSCs and non-cultured BM-MNCs were analyzed by flow cytometry. Compared to BM-MNCs, BMSCs expressed higher levels of mesenchymal cell markers CD29 and CD90, but lower levels of endothelial cell marker CD31 and hematopoietic cell marker CD45 (Figure 3).

Optimal cell dose for transplantation

To determine the optimal cell dose for therapy, 1×10^6 or 5×10^6 of freshly isolated BM-MNCs were injected into rats. On day 7, the boundaries between survival and necrotic areas were observed in each skin flap (Figure 4A). The mean survival rates of skin flaps were 64.0 ± 6.1% in the 1×10^6 cell-treated group and 70.8 ± 5.3% in the 5×10^6 cell-treated group, which were significantly higher than in the PBS group (54.3 ± 2.5%, P<0.05). However, no significant difference was observed between the cell transplanted groups (P>0.05; Figure 4B). An optimal dose of 5×10^6 cells per rat was chosen for subsequent experiments.

Flap survival improved by transplantation of BM-MNCs and BMSCs

Gross views of skin flaps on day 7 of treatment are shown in Figure 5A. Quantitative analyses of skin flap survival rate showed that 66.2 ± 3.1% of flaps survived in the BMSC-treated group and 71.6 ± 8.4% in the BM-MNC-treated group, and both groups were significantly higher than the PBS group (55.9 ± 3.4%, P<0.05). However, no significant difference was observed between the cell transplanted groups (P>0.05) (Figure 5B). FFLPI revealed that blood perfusions in the cell transplanted groups were better than those in the PBS group (Figure 5C).

Capillary density in flaps

To measure capillary density after cell transplantation, immunohistochemical staining of CD31 was performed in the skin flaps collected from identical anatomical positions. As shown in Figure 6, capillary numbers were 42.7 ± 5.1 in the BMSC-treated group and 58.2 ± 6.8 in the BM-MNC-treated group, which were significantly higher than the PBS group (22.8 ± 3.1, P<0.05). In addition, a significant difference was observed between the BMSC-treated and BM-MNC-treated groups (P<0.05).

Discussion

The pro-angiogenic potentials of BM-MNCs and BMSCs have been demonstrated not only in skin flap survival model but also in the other ischemic diseases [7,8,13-17]. However, which is the optimal cell source for clinical application is not clear. The expansion of cells in culture could reduce the initial amount of bone marrow aspirates, but would limit the treatment in the facilities with qualified cell culture unit and raise the safety concerns about cells after long-term culture. It is important to clarify whether cell expansion could bring great benefit to the therapy.

In order to obtain a certain amount of cells for transplantation, we expanded BMSCs for 4 passages in culture. The multipotency of expanded BMSCs was proved by their adipogenic, osteogenic and chondrogenic differentiation capacity (Figure 2). In addition, flow cytometric analysis confirmed that cells expressed typical MSC surface markers (Figure 3), indicating that these are functional BMSCs. Our preliminary study found that flap raising could induce tissue inflammatory reaction which may not be good for the survival of transplanted cells. Therefore, we adopt the strategy from Yang et al. that BMSCs and BM-MNCs were injected two days pre-operation [8]. We demonstrated that both freshly isolated BM-MNCs and cultured BMSCs (P4) were able to improve random-pattern skin flap survival in a rat model. More importantly, injection of equivalent numbers of BM-MNCs and BMSCs, derived from identical bone marrow aspirates, displayed comparable effects without significant difference, indicating that current cell expansion procedures may not bring therapeutic benefit.

It is well known that neovascularization is crucial for the survival of skin flaps following ischemic injury. However, flap survival may not completely rely on blood supply in such an acute ischemic model. Other protective factors may also play a role in the flap survival. We found that capillary density was significantly higher in BM-MNCs group than that in BMSCs group (Figure 6). This accords with others finding that...
BM-MNCs could differentiate into endothelial cells to participate in the neovascularization [8,18], while BMSCs barely differentiate into endothelial cells [19-22]. Interestingly, the flap survival rate was similar between BM-MNCs group and BMSCs group (Figure 5). BMSCs have been used in the treatment of many ischemic disorders [23-26]. It has been reported that BMSCs barely participate in neovascularization directly but through paracrine secretion of factors to protect resident cells, promote cell proliferation and neovascularization, and regulate inflammatory responses [19-22]. This may explain why the flap survival rate was similar in both groups. Since cells were not labeled before injection, the exact fate of transplanted cells is not clear. It is worth to be investigated in future.

Although BMSCs area relatively pure population that might be good for reducing cell-related side-effects, other pro-angiogenic components, such as endothelial progenitor cells and hematopoietic cells [27], are lost during expansion (Figure 3). This might be one reason why no further improvement were observed following BMSC treatment compared with BM-MNC treatment. Yang et al. demonstrated that bFGF and VEGF were highly expressed in the flaps receiving BM-MNC transplantation [8]. In addition, they proved that the pro-angiogenic effect of BM-MNCs was comparable with adipose derive stem cells (ADSCs), which have been also used to enhance flap survive [28]. Compare to BMSCs and ADSCs, BM-MNCs apparently have distinct advantages that could be used without complicate and time-consuming culture process.

To achieve large amount of functional cells in culture, maintaining the “stemness” of cells during expansion is still a big challenge. The current standard protocol for BMSC culture is widely used but not ideal [27,29]. Cells are reported to lose their multipotent differentiation potential in later passages [27]. Cells at P4, which still possessed multipotent differentiation potential (Figure 2), were used for transplantation in this study. Compared with similar studies, the number of cells injected in this study was higher than others [8,30-33]. Despite this, cells expanded at passage 6 could increase the number of cells for injection, but did not improve the protective effects in skin flap survival (data not shown). Clearly, the current culture protocol...
Figure 6: Capillary density in skin flaps measured by immunohistological staining of CD31. *P<0.05. Scale bars: 100 μm.

is not suitable for expansion of bone marrow cells for this particular therapeutic purpose. An enhanced cell culture system needs to be developed to meet this requirement.

Conclusion

This study demonstrates that both BM-MNCs and BMSCs are comparable in improving the survival of random-pattern skin flaps. However, non-cultured BM-MNCs would be more practicable for clinical application.

Conflict of Interest

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this manuscript.

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