Development of Prevascularized Cell Sheets Using Single Bone Marrow Mesenchymal Stem Cell Source for Tissue Regeneration

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Abstract

Developing a vascular network for tissue engineering constructs is still a challenge. Cell-sheet technology has already brought promising potential in vascularization. However, endothelial cell source for vascularization is limited due to the difficulties in obtaining endothelial cells from peripheral blood or bone marrow. In this study, we differentiated rabbit bone marrow mesenchymal stem cells (rBMSCs) into endothelial like cells (ECs) by adding vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) into the culture medium of BMSCs. Then, the induced ECs were seeded on rBMSCs sheets to form vascularized ECs/BMSCs cell sheets. Results show that ECs on the undifferentiated BMSCs sheets formed networks in vitro and in vivo. In vivo results demonstrated that the quantity of cells in the non-prevascularized constructs was lower than that of prevascularized constructs. Furthermore, the prevascularized rBMSCs sheet constructs induced functional anastomosis with the host vasculature. These results suggested that using BMSCs as an endothelial cell source and cell sheet technology to create prevascularized cell sheet brings a promising potential for tissue regeneration.

Keywords: Bone marrow mesenchymal stem cells; Cell sheet; Prevascularization; Endothelial like cells

Introduction

Insufficient vascularization in tissue-engineered implants results in the failure of implantation [1,2], especially in the thick constructs [3-6]. The lack of nutrient and oxygen causes low cell survival rate and slow growth of new vessels [7]. A number of strategies have been developed to engineer blood vessels by either combining endothelial cells with scaffold materials or loading angiogenic growth factors on scaffolds [8-10]. However, these technologies have not fully shown the success in forming functional blood vessels for sufficient blood supply and nutrient transportation after post-implantation. Cell sheet technology has brought promising potential in tissue regeneration and been widely used in tissue engineering for cartilage, cornea, myocardium, periodontal tissue, blood vessels and other soft tissues regeneration [11-15].

To develop a vascularized cell sheet, endothelial cells (ECs) are a crucial element to form blood vessels in a 3D cell sheet microenvironment. However, it is difficult to obtain endothelial cells from the source of the terminally differentiated blood vessel or microvascular cell by the direct isolation method [16]. In addition, studies have showed that the proliferation ability of the terminally differentiated blood vessel or microvascular endothelial cells was limited and matured endothelial cells are easy to become aging in the process of expansion [17,18]. Therefore, seeking a better method to acquire ECs became very important in vascularization strategies [19]. Studies also showed that the ECs induced from bone marrow derived mesenchymel stem cell (BMSCs) had a stronger ability of differentiation compared with that of differentiated ECs [15-20].

In tissue engineering, BMSCs is an important source for tissue regeneration [21]. Studies have shown that BMSCs can be used as vascular pericyte cells. Co-culturing the BMSCs and endothelial cells at a certain proportion contributes to form ripe vascular system in tissue engineering bone [22,23]. It has been reported that BMSCs have the ability of differentiating into endothelial cells under the appropriate induced condition [24-26].

Therefore, the objective of this study is to investigate the ability of BMSCs differentiating into ECs and the vascularization ability of induced ECs under specific inductive conditions. We hypothesize that autologous stem cells can differentiate to endothelial-like cells and stem cell-derived endothelial cells can prevascularize stem cell sheets. To test these hypotheses, in this study, we isolated and cultured rabbit BMSCs and then induced the BMSCs differentiation into endothelial like cell (ECs). Afterwards, the induced ECs were seeded on undifferentiated BMSCs cell sheets to form prevascularized tissue-engineered sheet constructs. We investigated cell migration and network formation in vitro, and functional vascularization in the subcutaneous tissues of nude rats in vivo by histological examinations.

Our main purpose in this study is to investigate the vascularization ability of ECs/BMSCs cell sheet construct using BMSCs as a single cell source. The in vivo implantation results suggested the formation of functional blood vessels in the prevascularized constructs.

Materials and Methods

Cell isolation and culture

The animal procedures were approved by the University Research Ethics Committee of Lanzhou University. Primary rabbit BMSCs were isolated and cultured according to a previously described method [27]. Briefly, the Japan big white rabbit (3 weeks old and about 500
g) were euthanized with overdose of anesthetic, 3% Chloral hydrate 3 ml/kg (fanke, Shanghai). Then the hind limbs were aseptically removed and the femurs were dissected free of soft tissues. No. 16 puncture needle punctured to the bone marrow cavity in both sides of the greater trochanter of the femurs 2 to 3 ml bone marrow was extracted from the femoral bone with injector treated by heparin (600 U/ml). Centrifugation was performed at 1500 r/min, and 0.5 ml concentrated bone marrow was obtained. Whole bone marrow adherent method was used to separate and purify the bone marrow mesenchymal stem cells. In our previous studies, the cultured BMSCs were differentiated into osteoblasts, cartilage and fat, which confirmed that the cells could be BMSCs, and BMSCs had multiple differentiation potential [28]. The obtained cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO CA, USA) containing 10% fetal bovine serum (Hyclone, USA), 0.29 g/L L-glutamine (Sigma, USA) and 2% antibiotics (200 mg/mL penicillin and 200 mg/mL streptomycin). The cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 or 3 days. The adherent cells were cultured until confluence and then passaged BMSCs after digestion with 0.25% trypsin / 1 mL EDTA. Second generation of the cells was used for all experiments.

Cell induced differentiation and identification

Subsequently, the second generation BMSCs was utilized for inducing differentiation into endothelial cells. The BMSCs were seeded on the cell culture dish at a density of 1×10⁶ cells/cm² and cultured in the DMEM medium. After 2 days, BMSCs reached confluence, and the DMEM medium was changed to endothelial cell medium consisting of DMEM medium plus vascular endothelial growth factor (VEGF 10 μg/L), basic fibroblast growth factor(b-FGF 10 μg/L). At the same time, the cells were cultured in the DMEM medium as a control. The cells were also cultured in a humidified atmosphere of 5% CO₂ at 37°C. Media were changed every 2 or 3 days. An inverted microscope was used to observe the morphological changes of experimental group cells at 3,7,14 days after induction. Immuno-fluorescent staining of the von Willebrand factor (vWF) and platelet endothelial cell adhesion molecule (PECAM-1 or CD31) was carried out to identify the induced ECs. After 7 and 14 days, the cell climbing pieces were separately made up of the two groups at a density of 1×10⁶ cells/cm². When the cells reached confluence at forty or fifty percent, the sample was washed by using PBS for 3 times, each time for 5 minutes. The sample cells were fixed in 4% paraformaldehyde for twenty minutes and then washed using PBS for 3 times for 5 minutes each time. After washing the cells three times in PBS, the samples were blocked in a 5% goat serum-PBS buffer solution for 30 minutes at room temperature and then washed using PBS for 3 times. A primary antibody rabbit anti-rabbit vWF (bs-0586R, Bioss, dilution1:500) and human anti-rabbit CD31 (ab9498, Abcam, dilution1:200) in 1% BSA-PBS were respectively added into the sample and incubated overnight at 4°C. Then the corresponding secondary antibody goat anti-rabbit (ZDR-5209, 0.5 ml, ZSGB-BIO) and goat anti-human (ZDR-5201, 0.5 ml, ZSGB-BIO) in 1% BSA-PBS were added and incubated in the dark for 2 hour at room temperature. A DAPI (0.5 μg/mL) solution was added to counterstain the cell nuclei for 5 minutes and then the samples were washed using PBS. Ultimately, mounted the slide with anti-fluorescence quenching agent. The fluorescent staining was recorded by an inverted fluorescence microscope (Olympus).

Flow cytometry analysis of ECs

To further assess the endothelial phenotype of induced ECs, flow cytometry analysis was done for identifying EC specific marker PECAM-1(CD31). After 14 days, cells were trypsinized, and then added to a tube at a concentration of 1×10⁶ cells per tube, fixed using 4% paraformaldehyde for 10 minutes. After washing the cells in PBS one time, the samples were blocked in a 2% BSA-PBS buffer solution for 10 minutes at room temperature. The monoclonal antibodies, human anti-rabbit (ab9498, Abcam, dilution1:20) for CD31, were added into the samples and then the samples were incubated at 4°C for 30 min. In contrast, an equal amount of PBS was added into the cells as a control. Then the samples were washed by PBS a time and the fluorescently-labeled rat anti-human secondary antibody (1:200; Vector Laboratories) were used. Afterwards, the samples were incubated at 4°C for 30 min in dark. After washing the specimen in PBS two times, the cells were resuspended in 500 microliters PBS and mixed in dark. Flow cytometry detection was performed on a FACS Aria Flow Cytometry System (BD Biosciences, San Jose, CA, USA).

Preparation of prevascularized cell sheet

To produce a prevascularized cell sheet in vitro, BMSCs were seeded on a cell culture dish at a cell density of 9×10⁴ cells/cm² and cultured in DMEM undifferentiated medium. After cells reached confluence, changed the DMEM medium containing 50 μg/ml ascorbic acid, which promotes the production of extracellular matrix. After 14 days of culture, undifferentiated BMSCs can form a thick cell sheet layer. Then, the induced ECs at 14 days were passaged. After passing, the ECs were cultured and amplified, and the amplified ECs were seeded onto the surface of the BMSC cell sheet at a cell density of 5×10⁴ cells/cm². Afterwards, the endothelial cell culture medium, DMEM medium plus VEGF (10 μg/L), b-FGF (10 μg/L) was added to the growing cells for 7 days. Medium was changed every 3 days. At the same time, the simple undifferentiated BMSCs cell sheet and ECs were separately seeded on a cell culture plate as a control. A fluorescent microscope was used to observe the ECs cell migration on the BMSCs cell sheet. After 3, 7 and 14 days, the samples of 3 groups were fixed in 4% paraformaldehyde for 15 minutes, then washed using PBS for three times. Immunofluorescence staining for CD31 was performed the same as staining the ECs. The frozen sections were produced of the two groups, the prevascularized cell sheet and the simple undifferentiated BMSCs cell sheet for HE staining to observe the samples after culturing for 3,7 and 14 days.

Production of three-dimensional prevascularized ECs/BMSC constructs

Cell sheet folding technique was used to engineer a 3D prevascularized ECs/BMSCs structure in our study [29]. BMSCs and ECs were co-cultured for 7 days in a diameter of 10 cm round tissue culture plate, when the ECs/BMSCs cell sheet was formed, a cell scraper was used to gently scratch the edge of the thick cell sheet and detach it. The harvested cell sheet was clipped into approximately a square. The manucured ECs/BMSCs cell sheet was then folded 3 times to form 8 layers EC/BMSCs constructs with a point forces (Figure 1). The width of the construct was approximately 8 mm and length was around 8 mm. The EC/BMSCs construct was further cultured for 24 hours to integrate the 8 layer cell sheets into one unit.

In Vivo subcutaneous implantation

To investigate whether the eight-layer ECs/BMSCs cell sheet constructs have an ability of neovascularization in vivo, ECs/BMSCs sheets were subcutaneously implanted into mice. BMSCs cell sheet constructs without ECs were produced as a control and the constructs were prepared with the same method as the ECs/BMSCs constructs.
Then, the 3D pre vascularized cell sheet constructs were incubated for 1 day in an endothelial cell medium before implantation, and the control groups were incubated for 1 day in DMEM medium. Afterwards, the cell sheet constructs were implanted into the subcutaneous dorsal tissues of nude mice. Five male BALB/c immunodeficient nude mice (6-week-old, 20–25 g body weight, Vital River Laboratories, Beijing, China) for each group at each time point were implanted. After implantation, one mouse per cage was housed. The nude mice were euthanized with overdose of anesthetic, 3% Chloral hydrate 3 ml/kg (fanke, Shanghai), to remove the constructs after 1 and 2 weeks, then the constructs were detected for histological analysis.

**Histological and immunohistochemical analyses**

The implants were fixed in 10% formalin buffer solution for 24 h, dehydrated in serial degraded ethanol, embedded in paraffin, and then sectioned. The specimens were sectioned into 5 μm thickness for hematoxylin & eosin (H&E) staining to observe the formation of luminal blood vessel structures containing red blood cells. For the immunohistochemistry staining of CD31, after deparaffinized and retrieved by an antigen retrieval solution at 95–100ºC for 20 minutes, specimens were then washed by PBS three times and blocked by blocking serum (5%) for 30 minutes. Human anti-rabbit CD31 (ab9498, Abcam, dilution1:10) was used. Then rat anti-human biotinylated secondary antibody (1: 500; Vector Laboratories) and DAB substrate kit (Vector Laboratories) were used followed by hematoxylin counterstaining and permanent mounting. Eight view fields (under 20×magnification) from each specimen of H&E or CD31 staining slides were randomly selected using the Image-Pro plus 6.0 software to quantify the density of micro vessels formed in the constructs in vivo. The lumen of the structures containing erythrocytes was observed in the slides, which were defined as micro vessels. The quantitative assay of rabbit lumens containing murine erythrocytes was also performed with the same method.

**Statistical analysis**

All data were expressed as mean ± standard deviation. The one way ANOVA and Tukey post hoc tests were performed to analyze the results using the SPSS 17.0 software (SPSS). A p value less than 0.05 was considered to be statistically significant (Figure 1).

**Results**

**Induced differentiation of BMSCs into ECs and immunofluorescence staining**

The isolated and primary cultured BMSCs presented long spindle observed by the inverted microscope. Figure 2a shows the cellular morphology of the BMSCs, which shows long spindle. The experimental BMSCs got shortened slightly when induced 3 days (Figure 2b) were. The morphology of the cells changed significantly which showed oval after 7 days (Figure 2c). Cells presented “slabs tone” structure and swirling growth at 14 days (Figure 2d). Immuno-fluorescent staining result shows that vWF and CD31 of the experimental group expressed positively. On the contrary, vWF and CD31 expressed negatively in the control group (Figure 3).

**Flow cytometry detection of induced ECs for CD31**

After inducing in endothelial cell medium for 14 days, the cells showed a significantly higher number of cells with positive expression of CD31 (49.40 ± 3%) when compared to the control group (3.90 ± 2%) (Figure 4).

The inserted ECs were distributed in the BMSCs cell sheet construct. Hematoxylin and eosin (H&E) staining were used to observe the microstructure of the BMSC sheets. After 3 days of culture, the inserted ECs rearranged and shaped cavitional structure (Figure 5a). Then the network structures formed by the ECs on the BMSCs sheet were inspected at 7 days (Figure 5b). The network structures performed even more obviously after culturing ECs on the BMSCs sheet for 14 days (Figure 5c).

**Immunofluorescent staining for CD31**

Immunofluorescent staining for CD31 was performed to detect the network formation of ECs on the BMSCs sheet. Immunofluorescent staining for CD31 result reveals that after seeding the ECs on the BMSCs cell sheet for 3 days, the ECs migrated fused and formed the vacuoles structure (Figure 6a). The aggregation of vacuoles structure appeared at 7 days (Figure 6b). Finally, lumens of the micrangium formed after culturing the pre-vascularized cell sheet for 14 days (Figure 6c).
result of immune-fluorescent staining for CD31 shows that a large quantity of networks formed in the multiple layer cell sheets.

To confirm whether the multiple layer BMSCs cell sheet structures with endothelial network have the ability of neovascularization and whether the vascular networks can anastomose with host vasculature in vivo, the BMSCs cell sheets with or without ECs were surgically implanted on the dorsal subcutaneous tissue of nude rat. H&E staining results show that a number of micro vessels were observed in the ECs-positive transplanted grafts (Figures 7a and 7b). The blood vessels were
defined as lumen containing red blood cells. The magnified image from Figure 7c expresses the blood vessels containing erythrocytes. The control group without ECs, few blood vessels was observed at 7 and 14 days (Figures 7b and 7d). Form the quantification, the number of the micro vessel density showed a statistically significant difference between the pre-vascularized ECs/BMSCs sheet structures and BMSCs sheet structures (Figure 7e). The result of the quantification revealed that the quantity of the micro vessel in pre-vascularized sheet structures is larger than that in non-pre vascularized sheet structures at 7 and 14 days (p<0.05, n=5) (Figure 7f). Between 7 and 14 days, there was not a statistically significant difference in density of blood vessels of these two groups.

**Immunohistochemistry staining of functional vessels**

To further investigate whether the vessels formed in vitro have the ability to anastomose with host vasculature in vivo, immunohistochemistry staining for CD31 was performed on the paraffin sections. There were many blood vessels stained positive for CD31 in the ECs/BMSCs sheet constructs at two time points (black arrows, Figures 8a and 8b). A magnified image from Figure 8c shows that positive rabbit CD31 lumen contains murine blood cells, which confirmed that murine erythrocytes invaded into the lumens, and functional blood vessels were formed. Meanwhile, some murine blood vessels invading into the constructs were seen (green arrows, Figures 8a and 8c). In contrast, few rabbit blood vessels were discovered in the
BMSCs sheet constructs without ECs at day 7 or 14 (Figures 8b and 8d). The result of the quantification revealed that there is no significant difference in the density of rabbit lumens in the ECs/BMSC sheets between days 7 and 14 (Figures 8e and 8f).

**Discussion**

Rapid and sufficient vascularization plays an important role in tissue-engineered constructs for optimal cell survival and implants integration [7]. ECs are a prerequisite in the process of constructing vascularized structures. Due to the restriction of donor area, the previous method of obtaining terminally differentiated cells from vascular or microvascular by direct separation is limited [17,18]. And further studies found that terminally differentiated ECs from vascular or micro vascular have limited ability in proliferation, and are easy to become aging in proliferation process in vitro [16]. Besides, they have the loss of function and the presence of biological activity is not strong and other issues [16]. Therefore, seeking better access to obtain ECs seed cells becomes a hot topic in the study of vascularization [19]. Studies have shown that BMSCs can be induced to differentiate into ECs [25-30]. And the induced ECs had an ability of differentiation than terminally differentiated ECs.

In this study we adopted VEGF and b-FGF to induce the rabbit BMSCs differentiation into ECs [30]. Our *in vitro* results indicated that the morphology of BMSCs changed at day 3, which presented shrink of cytoplasm and the large nucleus. BMSCs changed from long fusiform to cobblestone-like morphology after inducing 7 and 14 days. Immuno-fluorescent staining results implied that the expressions of CD31 and vWF were positive in induced ECs, suggesting that BMSCs had the ability to differentiate into ECs under experiment condition.

To further study the induction rate of endothelial cells from BMSCs, we conducted flow cytometry analysis for identifying EC specific marker PECAM-1(CD31). Our result indicated that the induced ECs showed a significantly higher number of cells with positive expression of CD31. Similar results can be found in Divya Pankajakshan’s study, which reported the ability of porcine MSCs to differentiate into ECs under *in vitro* inducing conditions [24]. These findings suggest that we can obtain ECs by inducing the BMSCs to differentiate. BMSCs have multiple differentiations potential [17,31]. The ease of isolation, rapid growth in vitro allowing for extensive culture expansion, maintenance of differentiation potential, immune-modulatory effects, and their ability to home to the sites of injury and subsequent wound healing, makes BMSCs attractive candidates for regenerative medicine [32]. In this study, we seeded the passaged ECs onto the BMSCs cell sheets, fabricating prevascularized cell sheets using single bone marrow mesenchymal stem cell source for tissue regeneration, which had higher induction efficiency and the method was easy. The results showed that vascular network structures could be formed in the pre vascularized cell sheets, which prompted that ECs obtained by induction *in vitro* could
form capillary network. Therefore, we effectively solved the problem of the source of endothelial cells.

Effective construction strategy is crucial in fabricating vascularized tissue. In this study we used the cell sheet technology based on Okano’s cell sheet stacking method [11]. We seeded the induced ECs onto the surface of the cell sheet at a cell density of 5x10^5 cells/cm² then the ECs fused and connected to each other and the capillary-like structures were gradually formed. Similar results were also found in our previous study, which reported that seeding HUVECs on hMSC sheet could form a pre vascularized cell sheet and 3D pre vascularized constructs was further fabricated. The pre vascularized constructs showed remarkable vascularization ability both in vitro and in vivo [29]. It is known that the formation of a stable and functional vessel not only requires migration of ECs but also depends on cooperation of ECs with pericytes. Recently, it has been proven that BMSCs can support in vitro vasculogenesis for at least several reasons, acting as perivascular cells [33] and releasing angiogenic factors [34]. Therefore, combination of ECs and the undifferentiated BMSC sheets showed rich networks in vitro in our current study, suggesting MSCs play key roles for vascularization. Although the pre vascularized networks formed in vitro are assumed to anastomose with the host vascular system in vivo after implantation and to be functional, there are different mechanisms of angiogenesis and anastomoses in such type of graft. For example, in a similar mouse model for engineering vascularized grafts, Tsikou O [22-34] reported that the new vessel generated by the implanted cells was gradually replaced by host vasculature. To reveal this, vWF staining was marked as marker of functional blood vessels accompanied by cell-labeling may be an effective and helpful way.

Our main purpose in this study is to investigate a new approach of obtaining endothelial cells by inducing BMSCs, and then develop pre vascularized cell sheets using the induced ECs and BMSCs cell sheet. Subcutaneous implantation in vitro results showed the formation of functional blood vessels in the constructs. In addition, it has been reported that BMSCs in vitro can support new vessel formation as pericytes [33] and can stimulate endothelial cell migration by releasing some angiogenic factors [34]. Our results confirmed the robust vascularization ability, but the transplantation experiments in situ need to be carried out to investigate reconstruction abilities of BMSCs cell sheet construct for specific tissues, such as for bone regeneration.

Conclusion
In the present study we obtained ECs by inducing rabbit BMSCs and examined angiogenesis abilities of the induced ECs and fabricated a pre vascularized cell sheet construct that had the potential to form blood vessels in vitro and in vivo. Meanwhile, the formed vessels functionally anastomosed with the host vascular network system. Our results demonstrate that using rabbit BMSCs as an endothelial cell source to form a pre vascularized ECs/BMSCs cell sheet construct is feasible, which provides a promising potential for structuring pre vascularized tissues and contributing to regenerative medicine.

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References


