Local Transplant of Human Umbilical Cord Matrix Stem Cells Improves Skin Flap Survival in a Mouse Model

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A skin flap is a piece of skin that has its own blood supply, which is useful to repair large skin defects and deep wounds in plastic surgery. However, partial skin flap necrosis usually occurred. Bone marrow mesenchymal stem cells (BM MSCs) are effective in improving the ischemic flap survival, but their clinical application is restricted by their limited source. Human umbilical cord matrix stem (HUCMS) cells are easily isolated in a large number, compared to BM MSCs. In this study, we evaluated the therapeutic potential of HUCMS to improve the survival of ischemic skin flap. HUCMS cells were characterized with surface markers, and were labeled with 5-acetylene base-2 ‘deoxidizing uracil nucleoside (EdU) in vitro. Twenty male immunodeficient BALB/c mice with an epigastric flap were randomly divided into two groups. HUCMS cells or Dulbecco’s Modified Eagle Medium (DMEM) were injected into the subcutaneous flap tissues. On the 7th postoperative day, flap survival, capillary density, levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and EdU-positive cells in the skin flap were examined. Results showed that flap survival rate was higher in the HUCMS cell group ($P < 0.05$). Capillary density, VEGF level, and bFGF level were higher in the HUCMS cell group ($P < 0.05$). EdU-labeled HUCMS cells were mainly distributed in the subcutaneous flap tissues. These findings suggest that HUCMS cells can improve the survival of ischemic skin flap by promoting vascularization, which may be attributed to the increased expression of VEGF and bFGF.

**Keywords:** 5-acetylene base-2 ‘deoxidizing uracil nucleoside; growth factor; human umbilical cord matrix stem cells; skin flap; vascularization


The skin flap is one of the most important surgical techniques in reconstructive plastic surgery that can repair large skin defects and deep wounds formed from injuries, surgeries, ulcers, or congenital defects (Pearl et al. 1983). However, the partial necrosis of the flap is a major problem for skin flap transfer in clinical applications. Ischemia-reperfusion injury caused directly by vascular endothelial cell damage has been identified as an important cause of skin flap necrosis, which is the key obstacle in flap survival (Schmidt et al. 2012).

In the past decades, extensive research has been performed on enhancing skin flap survival through pharmacological manipulation, including vasodilators, calcium channel blockers, and low-dose radiation. These manipulations have been either inconsistent in the prevention of flap necrosis or associated with potential side effects (Akamatsu et al. 2000; Karacal et al. 2005; Kim et al. 2009). Recently, application of growth factors such as vascular endothelial growth factor (VEGF) (Zhang et al. 2003) and basic fibroblast growth factor (bFGF) (Haws et al. 2001) has been demonstrated to be a promising therapeutic method to improve the survival of ischemic flaps, but it is restricted by their short half-lives.

Stem cells have multiplex differentiation potentials, which have led to them becoming an important seed cell in tissue engineering (Caplan 2007). Recent studies have demonstrated that bone marrow mesenchymal stem cells (BM MSCs) transplantation can improve ischemic flap survival by promoting neovascularization (Uysal et al. 2010; Wang et al. 2011). BM MSCs could promote the secretion of a variety of angiogenesis growth factors, such as VEGF and bFGF, which are critical for their neovascularization capacity (Uysal et al. 2010; Ding et al. 2011). However, the low number of BM MSCs in bone marrow (BM) restricted its clinical application.

Human umbilical cord matrix stem (HUCMS) cells are

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another type of all-around stem cells, which are derived from human umbilical cord Wharton’s jelly. Compared with BM MSCs, HUCMS cells have some advantages. First, the isolation of HUCMS cells is noninvasive, whereas the procedure to isolate BM MSCs is invasive, painful, and raises the possibility of bleeding and infection (Petsa et al. 2009). Second, HUCMS cells are much closer to embryonic stem cells and have greater capabilities of proliferation, differentiation and plasticity compared to BM MSCs (Taghizadeh et al. 2011). Third, HUCMS cells have low immunogenicity compared to BM MSCs. They can be isolated in large numbers and grow robustly (Mitchell et al. 2003). However, their therapeutic effect on flap ischemia has remained unclear. In this study, the effect of HUCMS cell transplantation on the ischemic flap survival was examined and the potential mechanism was also explored.

Materials and Methods

Animals and surgical procedures

Twenty BALB/c severe combined immunodeficiency (SCID) mice (4-week-old; male) were purchased from Lu Kang Company (Shandong, China) and maintained in a pathogen-free environment. All of the animal procedures in this study were performed under aseptic conditions, which were approved by the Institutional Animal Care and Use Committee of Qingdao University Medical School Affiliated Hospital.

Expansion and characterization of HUCMS cells

HUCMS cells of the P1 generation were obtained from the Stem Cell and Regenerative Medicine Center in Qingdao University Medical School Affiliated Hospital. Informed consent was obtained from the mother before parturition in accordance with the ethical committee of The Affiliated Hospital of Medical College Qingdao University. HUCMS cells were prepared according to previously described methods (Mitchell et al. 2003). HUCMS cells of the P1 generation were grown in complete Dulbecco’s Modified Eagle Medium (DMEM) culture medium (low-sugar DMEM medium supplemented with 10% fetal bovine serum, Hyclone, Logan, UT, USA) and maintained in a humidified incubator at 37°C (95% humidity, 5% CO2). The cell medium was changed every 3 days until the cells were approximately 80% confluent. Then 0.25% of trypsin (Hyclone, Logan, UT, USA) was used to digest the cells. The cells were subcultured as the P2 generation at 1 : 2 ratios after digestion.

Mesenchymal stem cells (MSCs) have characteristic surface molecules expression profile, which must be positive for cluster of differentiation (CD) 90, CD105, and CD146 and negative for hematopoietic associated markers including CD34, CD45, and human leukocyte antigen (HLA)-DR. HUCMS cells of the P3 generation were incubated with a series of antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) including CD90, CD105, CD146, CD34, CD45, and HLA-DR, and detected with flow cytometry (FACS Calibur, BD Company, USA) according to previously reported methods (Weiss et al. 2006).

EdU labeling HUCMS cells

HUCMS cells of the P3 generation were subcultured at 1 : 2 ratios in complete DMEM medium. Two days later, the culture medium was changed to complete DMEM medium containing 10 μmol/l 5-acytylene base-2 ’deoxidizing uracil nucleoside (EdU) (Ruibo biotechnology, Guangzhou, Guangdong, China) and cells were cultured for 24 hours. Then, cells of the P4 generation were collected with digestion and washed with PBS. The cell density of the HUCMS cells was adjusted to 4 × 10^5/ml. To observe the efficiency of EdU labeling, HUCMS cells were fixed for fluorescent staining as previously reported (Huang and Qin 2010) and imaged through fluorescence microscopy (DMI 4000B, Leica Company, Germany).

Epigastric skin flap model and cell transplantation

Twenty BALB/c SCID mice were randomly divided into two groups, each consisting of 10 mice. Epigastric flaps were established in mice as previously reported (Miyamoto et al. 2008). The mice were fasted one day pre-operation. Then the mice were anesthetized with hydrated chlorine aldehyde in a supine position, and their abdominal hairs were removed. An axial skin flap pattern measuring 3 × 6 cm^2 was designed at the pedicled superficial inferior epigastric vessels bundled in the right lower quadrant of the abdomen. Then a flap edge was cut in the deep fascia layer along the design marker, the skin flap was lifted from its remote end, the vascular pedicle was clamped with a tiny vascular clamp to block the blood supply of the flap, and the flap was sutured in situ. Six hours later, the flap was opened, and the vascular clamp was removed to allow the blood supply recanalization, which resulted in ischemia reperfusion in the skin flap. The flaps were sutured in situ, and flap ischemia-reperfusion injury models were established.

In the HUCMS cell transplantation group, 4 × 10^5 HUCMS cells suspended in 1.0 ml DMEM were injected into the subcutaneous tissue of the flap at 10 evenly distributed points along the axis of the flap from the base to the distant end, with 4 × 10^5 HUCMS cells suspended in 0.1 ml DMEM being injected at each point. The injection points were 1 cm apart. For the DMEM control group, equivalent injection points were injected with 0.1 ml of DMEM for each point (Fig. 1).

Flap assessment

After surgery, the mice were returned to their individual cages. After 7 days, they were re-anesthetized as previously described. The...
survival area of each flap was assessed in a blinded fashion by two experienced specialists, with respect to the gross appearance, color, texture, thickness, hair growth, necrosis, and presence or absence of bleeding when cut with a scalpel. A viable flap was characterized by warm, pink, hair bearing skin, and bleeding when cut with a scalpel. Nonviable flaps were characterized by a dry, hard, hairless eschar, and no bleeding when cut with a scalpel. The total and survival areas of the flap were portrayed on semitransparent papers, and then analyzed by Image Pro Plus Software (version 6.0, Media Cybernetics). The flap survival rate was calculated using the following formula: flap survival rate = (flap survival area/flap total area) × 100%.

Vascularization detection

The neovascularization level of skin flaps was detected by capillary density 7 days after surgery. Six tissue specimens (1 × 1 cm²) were collected from each group 1 cm from the demarcation of survival, fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, sectioned to 5 μm continuous slices, and prepared for H&E staining to detect capillary. A capillary was defined as a ring or cylindrical structure with an internal endothelial cell lining (a lumen) in which red blood cells could be observed. Neovascularization was assessed by averaging the number of capillaries in subcutaneous tissues in 10 randomly chosen fields (100 × magnification; measurements were performed by two blind reviewers).

Expression of VEGF and bFGF in flap tissues

For detection of growth factors, enzyme-linked immune sorbent assay (ELISA) was applied. Tissues (0.5 g each) in the above area from each group were collected (n = 6 for each group). The tissues were homogenized in 500 μl tissue protein extraction reagent (CWBO, Beijing, China) and 5 μl PMSF (Sigma-Aldrich). After centrifuged at 10,000 rpm for 10 min, the supernatant was collected for the assay of VEGF and bFGF using ELISA kit according to the manufacturer’s instructions (R&D, Minneapolis, USA). Optical density (OD) values were examined on a microplate reader (DENLEY DRAGON, Lab systems) at 450 nm. The VEGF and bFGF levels were calculated using standard curves obtained with recombinant VEGF (from 15.6 to 1,000 pg/ml) and with recombinant bFGF (from 15.6 to 1,000 pg/ml).

Tracking of transplanted HUCMS cells in vivo

Slices from the same part were subjected to H&E staining and EdU staining to track EdU-positive cells according to a previously established method (Yu et al. 2010). EdU-positive cells were observed under a fluorescence inverted microscope (DMI4000B, Leica Company, Germany).

Statistical analysis

The results are expressed as the mean ± s.d. (Standard Deviation).
Results

Cell growth, EdU labeling, and the validation of HUCMS cells

The cells grew steadily after passage, and the cell morphology showed the following characteristics: single cells presenting a spindle or fiber appearance, clumpy distribution, closely spaced, stuck to the wall, large, and mononuclear (Fig. 2A). EdU-labeled HUCMS cells were stained and photographed under a fluorescence microscope (Fig. 2B). Flow cytometry assays showed that CD34, CD45, and HLA-DR were barely expressed on HUCMS cell surface, with positive rates of 0.10 ± 0.02%, 0.09 ± 0.01%, and 0.99 ± 0.00%, respectively (Fig. 2C-E). In addition, CD90, CD105, and CD146 were highly expressed on HUCMS cell surface, with positive rates of 99.78 ± 0.10%, 99.90 ± 0.08%, and 46.63 ± 0.15%, respectively (Fig. 2F-H). These data meet the requirements of the International Cell Transplant Society for mesenchymal stem cells (Dominici et al. 2006).

Flap survival

In the epigastric flap model, mice were injected with DMEM or HUCMS cells (Fig. 1). On the 7th postoperative day, for the DMEM control group, the flap edge and flap itself showed different sizes of dark crusts, the skin texture was shallow, and no bleeding occurred when the crusty areas were cut with a scalpel (Fig. 3A).

On the 7th postoperative day, for the HUCMS cell treatment group, the flap edge was completely healed, new hair grew, the flap tissue was thicker than that before operation, it bled when cut with a scalpel at a pink region, and the skin texture was normal (Fig. 3B). Small (0.1 - 0.3 cm) dark crusts appeared distal of the skin flap in two mice, large (0.4 - 0.6 cm) dark crusts appeared distal of the skin flap in another two mice, and no bleeding occurred when dark regions were cut with a scalpel. The remaining six mice flaps survived completely (Fig. 3B).

On the 7th postoperative day, the regions of survival and necrosis were clearly demarcated in each flap. All of the mice were sacrificed. The mean survival percentage of the flaps was 97.58 ± 3.41% in the HUCMS cell group (Fig. 3C), which was significantly higher than the DMEM control group (54.37 ± 8.78%).

Fig. 3. Flap survival status on the 7th postoperative day in mice.

The survival status of the flaps in the DMEM (A) and HUCMS cell (B) groups on the 7th postoperative day. Partial sutures were demolished. C. The mean survival percentage of flaps in HUCMS cell group was significantly higher than that of control group (n = 10 for each group; *p < 0.05).
Neovascularization of skin flaps

Improvement of angiogenesis in flaps was observed in HUCMS cell group, as evidenced by the high levels of capillaries distributed in sections excised from surviving regions close to the necrosis-survival margin (Fig. 4A). However, only a small number of new capillaries were observed in the flap tissue of the control group (Fig. 4B).

The capillary density was significantly higher in the HUCMS cell group (8.2 ± 0.9) compared with the DMEM control group (3.7 ± 0.4) (Fig. 4C).

Protein levels of VEGF and bFGF in skin flaps

On the 7th day after surgery, the levels of VEGF and bFGF proteins in the flap tissues were measured using ELISA. The expression of VEGF in the flaps of HUCMS cell group was higher than that in the control group (Fig. 5A). The bFGF protein level in the control group was statistically lower than that of HUCMS cell group (Fig. 5B).
ELISA. The VEGF protein level was found much higher in HUCMS cell treated flap tissues (292.88 ± 10.68 pg) than that in the control group (89.42 ± 9.59 pg) (Fig. 5A). Similarly, bFGF in HUCMS cell group (62.03 ± 4.25 pg) was significantly higher than that in the control group (30.25 ± 2.32 pg) (Fig. 5B).

The tracking of transplanted cells in vivo

EdU-labeled HUCMS cells released specific red fluorescence. On the 7th day after surgery, for the HUCMS cell group, EdU-labeled HUCMS cells were continuously distributed in the subcutaneous flap tissues (Fig. 6A, B). However, for the DMEM group, no red fluorescence was observed in the flap tissues (data not shown).

Discussion

Skin flap necrosis caused by a lack of blood supply and severe ischemia, especially in the distal region of flaps, is a common problem in the clinic. Experimental studies have shown that neovascularization in the skin layer is the most important factor for the survival of skin flaps. If early angiogenesis could be initiated, the survival of the flap could be improved (Asahara et al. 1999; Li et al. 2000).

Mesenchymal stem cells, such as BM MSCs (Zheng et al. 2008) and adipose-derived stem cells (ADSC) (Uysal et al. 2009), were proved to be effective at increasing the viability of ischemic flaps by promoting the formation of new blood vessels and improving the blood flow perfusion of ischemic flaps. Capillary density is one of the main histologic markers when evaluating angiogenesis (Park et al. 2004). In our study, using a standard mouse epigastric skin flap model, we demonstrated for the first time that local injection of HUCMS cells could improve the skin flap survival by promoting the vascularization by virtue of increasing capillary density.

The neovascularization of the skin flap is a complex process that involves the proliferation of endothelial cells and cooperation among various growth factors (Tucci et al. 2001; Zhang et al. 2001). The steps between mesenchymal stem cell application and neovascularization remain unclear, but experimental studies have shown that BM MSCs and ADSCs can promote angiogenesis through promoting the release of growth factors (Uysal et al. 2010; Ding et al. 2011). Among them, VEGF and bFGF have been proved to be the important growth factors in promoting angiogenesis of skin flaps in various animal models (Padubidri et al. 1996; Tucci et al. 2001; Zhang et al. 2001). In the complex neovascularization cascade, VEGF is thought to be the main controller, with a positive feedback control exerted by endothelial cells (Murohara et al. 1998; Taub et al. 2000). Our results demonstrated that both VEGF and bFGF were elevated in the skin flap on the 7th day after local HUCMS cell transplantation. These data suggest that HUCMS cells can promote the formation of new blood vessels via increasing the secretion of VEGF and bFGF.

In this study, HUCMS cells were labeled with EdU to track their fate in vivo. EdU is a new type of tracer marker developed in recent years, which can be incorporated into the newly synthesized DNA of replicating cells. The terminal alkyne group of EdU allows for chemical detection using a fluorescent azide that covalently binds to the alkyne group. This detection method is fast and specific and does not require DNA denaturation (Lin et al. 2009). In our experiment, EdU-labeled HUCMS cells were found to be mainly distributed in the subcutaneous flap tissues on the 7th day after transplantation, which indicated that HUCMS cells were able to survive all through the neovascularization process.

Conclusion

This study demonstrated that increased ischemic skin flap survival could be achieved by local application of HUCMS cells via increasing the secretion of angiogenesis factors such as VEGF and bFGF. With wider source and without morbidity at the donor site, HUCMS cell transplantation may be a promising therapy and deserves further investigation.
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Conflict of Interest
The authors report no conflict of interest.

References


