Therapeutic angiogenesis promotes efficacy of human umbilical cord matrix stem cell transplantation in cardiac repair

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ABSTRACT

Objective(s): Although previous studies have confirmed the beneficial effects of human umbilical cord matrix stem cell (hUCM) transplantation post myocardial infarction (MI), but this stem cell resource has no potential to induce angiogenesis. In order to achieve the process of angiogenesis and cardiomyocyte regeneration, two required factors for cardiac repair agents were examined namely; hUCM and VEGF on an infarcted heart. The main objective of this research is to investigate the combinatorial effect of dhUCM and VEGF transplantation on an infarcted heart.

Materials and Methods: 45 min of ligating the left anterior descending coronary artery, the MI-induced animals received 50 μl PBS, 5 μg VEGF, 5×10^6 hUCM cells alone, combined with 5 μg VEGF and 5×10^6 differentiated hUCM cells alone or combined with 5 μg VEGF through intramyocardial injection. MI group, without hUCM and VEGF served as the control group. Left ventricular function and angiogenesis were also evaluated.

Results: After eight weeks post MI, there were significant rise in left ventricular ejection fraction in dhUCM+VEGF group compared to the other treated and non-treated groups (P<0.05). Fibrosis tissue was markedly lower in the dhUCM+VEGF and hUCM+VEGF groups compared to the other treated and non-treated groups (P<0.05). Despite these benefits, vascular density in dhUCM+VEGF group was not markedly different compared to VEGF and hUCM+VEGF groups. The transplanted hUCM and dhUCM cells survived and migrated to the infarcted area.

Conclusion: Our findings demonstrated that the dhUCM cells transplantation combined with VEGF were more efficient on an infarcted heart.

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Introduction

Despite many advances in medical sciences, coronary disease which may lead to myocardial infarction (MI) is a complex one and still remained the leading cause of mortality in developed countries (1) Previous studies have proven that human umbilical cord matrix stem cell (hUCM) injected intravenously improved the functional recovery of a traumatic brain Injuries in rats (2). Angiogenic and stem cell therapies have generated much excitement as novel therapeutic approaches that might provide additive benefits on conventional treatments to restore left ventricular dysfunction after MI (3-6). Angiogenesis and replacement of lost cardiomyocytes with new, live cardiomyocytes are considered as key factors in cardiac repair (7). Apart from the use of stem cells and angiogenic therapies, the use of antioxidants to protect

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that have examined the effects of transplantation of hUCM into infarcted heart are rare and therefore its application requires more assessment. Study have demonstrated the beneficial effects of hUCM cells on improvement of cardiac function 4 weeks post MI, however angiogenesis was never observed (18). We assume that of beneficial synergic effects are existed between stem cells combined with VEGF transplantation which may cover the limitations related to stem cells or VEGF alone transplantation. Here we have described the effects of myocardial injection of hUCM and hUCM-derived cardiomyocytes either alone or in combination with VEGF into infarcted heart on cardiac function, 8 weeks post MI.

**Materials and Methods**

**Isolation and culture of hUCM cells**

Umbilical cords were obtained after birth and collected in Hank’s balanced salt solution (HBSS) (Gibco, USA), with informed consent of the parents. Umbilical cord blood vessels were cleared off and the remaining matrices were divided into fragments and then cultured in culture plates containing a complete medium DMEM-F12 (Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 15% FBS (Gibco, USA), 100 U/ml penicillin, 60 μg/ml streptomycin (Gibco, USA) in a humidified 37 °C incubator with 5% CO2. After expansion of fibroblast shape cells, the fragments were discarded and non-adherent cells were removed by changing the media every three days. Cells at 80-90% confluence were trypsinized and sub-cultured in new medium.

**Characterization of hUCM cells**

A) Expression of cell surface molecules

After trypsinization, the detached cells were washed and suspended in PBS. 1×10⁶ cells/ml hUCM cells were incubated with primary antibodies against CD73, CD29, CD34, CD44, CD45, CD90, CD105, HLA-ABC (HLA-1) and HLA-DR (all ebiosciences, USA) as described in previous studies (16, 17) at room temperature followed by fluorescent secondary antibodies. Control hUCM cells were stained with a secondary antibody alone. The cells were assessed in a FAC Scan (Fluorescence Associated Cell Sorter Scan) machine (Becton, Dickinson).

B) In vitro differential potential

hUCM cells at passage 4 were plated into adipogenic differentiation media containing complete medium supplemented with 10% FBS and 100 nM dexamethasone [Sigma, St. Louis] and into osteogenic differentiation media containing complete medium, 80 μg/ml ascorbic acid, 10 nM dexamethasone, and 10 nM β-glycerophosphate [Sigma, St. Louis].

Three weeks later, the cells were fixed by 4% paraformaldehyde in PBS and underwent both oil red and Alizarin red staining for detection of adipogenic and osteogenic differentiation, respectively.

**Induction of cardiac differentiation**

hUCM cells at passage 4 were incubated with cardiogenic differentiation medium containing complete medium and 10 nM Oxytocin (Wako chemical, Germany) for 72 hr. Then the differentiation medium was replaced with complete medium and was regularly refreshed every three days, for three weeks.

**Immunochemical analysis**

Oxytocin- treated cells (dhUCM) and hUCM cells served as the control and were fixed in 4% formaldehyde (Sigma, USA), permeabilized with 0.1% triton X-100 (Sigma, USA) in PBS for 15 mins and then incubated overnight at 4 °C with primary antibodies against cardiac troponin T (cTnT, abcam, UK), sarcomeric actin (sA, abcam, UK), cardiac actin (cA, sigma, USA) followed by fluorescent secondary antibodies (FITC and Alexa Flou) for 2 hr at 37 °C (19).

**Cell preparation**

hUCM and dhUCM cells were labeled by Cell tracker CM-Dil according to manufacturer’s instruction (Invitrogen, USA), before cell transplantation.

**Induction of MI model and cell transplantation**

Eighty white New Zealand male rabbits (weighing 3-3.5 kg) were randomly divided into eight groups of 10 animals each: (a) intact group (non-MI); (b) MI group consisting of MI model; (c) PBS group consisting of MI model and PBS; (d) VEGF group; (e) hUCM group; (f) dhUCM group; (g) hUCM + VEGF group; (h) dhUCM + VEGF group. Rabbits were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally), intubated and ventilated using a ventilator. After the left thoracotomy, a 0.6 mm silk ligature was placed and tied closely around the proximal end of the left anterior descending coronary artery (LAD). 45 min later, 5x10⁶ cells with or without 5 μg VEGF in normal saline were injected into the margin of MI region of the VEGF recipient groups (d, e, f, g, h). The injection was administered by 28 gauge needle attached to an insulin syringe. Cyclosporine A (10 mg/kg, subcutaneously) was administered 24 hr before the cell therapy, once daily. This investigation was approved by the Ethical Committee at Tehran University of Medical Science, Iran.

**Echocardiography**

Left ventricular ejection fraction (LVEF) was measured using a trans-thoracic echocardiographic device (Vivid3; General Electric) equipped with a 5-MHz linear transducer, five days and eight weeks...
Umbilical cord matrix stem cells improve left ventricular function.

**Histological and immunohistochemical analysis**

Eight weeks post MI, animals were sacrificed and their hearts were then removed, washed in PBS and fixed in 10% formaldehyde. Then, 5 µm thick sections were prepared from infarcted areas and stained with hematoxylin and eosin (H&E), as well as trichrome masson. Some selected sections were incubated with primary antibody against CD31 (Bioscience, USA), followed by peroxidase conjugated secondary antibody, each one for 1 hr at room temperature. CD31-positive cells with a vascular structure were counted as a vessel (capillary or artery). Vascular density and fibrosis tissue of myocardium have been quantified as described in previous studies (17, 18). For immunohistochemical studies, the prepared sections from cell-treated groups were incubated overnight 4 °C with primary antibody against cardiac α-actinin (cAn, Sigma, USA) followed by Fluorescein isothiocyanate (FITC) and Alexa Fluor for one hour.

**Statistical analysis**

Statistical analyses were performed by ANOVA test using SPSS 19.0 software. A P-value of <0.05 was considered significant. Data is expressed as means±SD.

**Results**

Culture and Characterization of hUCM cells

The hUCM cells have expressed mesenchymal specific markers [CD29 (93%), CD44 (89%), CD73 (97%), CD90 (92%)], but did not expressed CD34 and CD45 hematopoietic specific markers. Moreover, the hUCM cells were positive for HLA-ABC (95%) and negative for HLA-DR (Figure 1). The primary isolates appeared in fibroblast shape cells possessing cytoplasmic processes which remained in similar morphology after several passages (Figure 2c). Differential potential was confirmed following their differentiation into adipogenic and osteogenic lineages (Figure 2a, 2b).

Cardiac differentiation of hUCM cells

Three weeks after induction of cardiogenic differentiation, the Oxytocin-treated cells appeared as flat shape cells with short cytoplasmic and striped cytoplasm as well as spontaneous beating of these cells was observed under microscope (Figure 2d). Also, dhUCM cells expressed some contractile proteins including sA, cA and cTnT, indicating fibrillar and contractile structure of the dhUCM cells (about 65% of oxytocin-treated cells). The hUCM cells served as the control group and were negative for contractile proteins (Figure 3).

Figure 1. Flowcytometric analysis of hUCM cells. The hUCM cells were positive for mesenchymal specific markers (CD29, CD44, CD73 and CD90) but negative for (CD34, CD45). hUCM cells were positive for HLA-1 but negative for HLA-DR, which indicated the low risk of recipient inflammatory reaction. The represented data obtained after 3 experiments.

Figure 2. Adipogenic and osteogenic differentiation of hUCM stem cells were verified by (a) Oil-Red and (b) Alizarin Red Staining, respectively. After treating hUCM cells with oxytocin, fibroblast shape hUCM cells (c) were turned into cardiomyocytes with short cytoplasmic extensions and fibrillar structure (d).
Evaluation of myocardial function

Five days post MI, LVEF decreased significantly in all MI-induced groups compared to the control group (P<0.01) but there was no significant difference among the MI groups. Eight weeks post MI, LVEF improved significantly (P<0.05) in all treated groups compared to the MI and PBS ones. The dhUCM+VEGF group exhibited significant increase in LVEF (P<0.05) level when compared to other treated groups. Increase in LVEF was significantly higher (P<0.05) in dhUCM+VEGF group compared to VEGF, hUCM, hUCM and hUCM+VEGF groups (Figure 4).

Histological and immunohistochemical analysis

Vascular densities were significantly higher in VEGF, hUCM+VEGF and dhUCM+VEGF groups than other treated and non-treated groups (P<0.05). There were no significant differences in vascular density among VEGF, hUCM+VEGF and dhUCM+VEGF groups (Table 1). The amount of fibrosis tissue of myocardium was determined by trichrome Masson staining. The fibrosis of myocardium in all treated groups were significantly less than to other non-treated groups (P<0.05). The hUCM+VEGF and dhUCM+VEGF groups showed significantly the lowest level of fibrosis compared to other treated groups (P<0.05) (Table 1). No significant difference was observed between hUCM+VEGF and dhUCM+VEGF groups. The transplanted hUCM and dhUCM cells were observed in infarcted myocardium and in a remote distance from the injection sites (Figure 5a, 5b, 5e, 5f). The α-actinin stained sections confirmed that the transplanted dhUCM cells expressed the α-actinin, but transplanted hUCM cells did not express α-actinin in cardiac microenvironment (Figure 5c, 5d, 5g, 5h).
Table 1. Vascular density and fibrosis tissue of the myocardium

|                | Intact | MI       | PBS     | VEGF   | hUCM    | hUCM + VEGF | dhUCM   | dHUCM + VEGF |
|----------------|--------|----------|---------|--------|---------|-------------|---------|--------------|
| CD32+ vessels  | 108±3.6| 51.8±4.2 | 54.8±4.6| 83.6±2.8| 59.3±5.1 | 89.6±3.6   | 57.3±47 | 86.3±3.7     |
| Fibrosis area  | 0.4±0.1| 34.5±1.8 | 35.1±2.1| 17.6±2.1| 14.5±1.6 | 9.3±1.7    | 13.4±16 | 8.8±1.7     |

Values are means±SD. *P<0.05 compared to myocardial infarction and myocardial infarction Phosphate-buffered saline. # P<0.05 compared to vascular endothelial growth factor, human umbilical cord matrix and differentiated human umbilical cord matrix (dHUCM)

Discussion

Our achievement confirmed that transplantation of hUCM and dhUCM cells either alone or in combination with VEGF via direct intramyocardial injection improve cardiac functions and attenuate fibrosis tissue formation in infarcted myocardium eight weeks after MI. Although previous studies had confirmed the beneficial effects of various stem cell resources on restoration of ischemic heart function but practical application of these sources is limited because of some associated problems (13, 19-21). The hUCM stem cells were obtained from the umbilical cord that have been routinely discarded at parturition, thus eliminating some of the problems associated with other stem cell resources (15, 18,22). Echocardiographic measurements revealed that the LVEF increased markedly in all treated groups compared to MI and PBS groups at eight weeks post MI (P<0.05). The dhUCM+VEGF group shows significant increase in level among the treated groups (P<0.05) (Figure 4). The dhUCM+VEGF transplantation was more efficient when compared to VEGF, hUCM and dhUCM and hUCM+VEGF transplantation, one possible reason is because of the synergic effects of dhUCM cells and VEGF.

Scientists have rarely investigated synergic effects of stem cells and pro-angiogenic factors administration on MI. In Yang's study BM-MSCs transplantation combined with HGF (proangiogenic factor) improved cardiac function and angiogenesis, but were not efficient when compared to only BM-MSCs transplantation (23). Our study confirmed that the combination of dhUCM cells and VEGF have more beneficial effects than the administration of dhUCM cells or VEGF on infarcted myocardium. The proposed potential synergistic effect between dhUCM cells and VEGF is explained as thus. Although, cardiac microenvironment potentially induces stem cells to cardiomyocyte differentiation, some studies have shown that less than 5% of cardiomyocytes are derived from stem cells in the recipient cardiac tissue (24, 25). About one billion cardiomyocytes are lost during post MI, therefore the number of transplanted stem cell-derived cardiomyocytes in vivo, may not be enough to influence the contractility of the infarcted myocardium (27).
induced the hUCM cells to undergo cardiogenic differentiation with high quality prior to the in vivo administration by oxytocin, which has been confirmed using immunocytochemical analysis (Figure 3). On one hand, the contractile functional cardiomyocytes have been delivered into the infarcted heart and the loss or mal-differentiation of hUCM cells in cardiac microenvironment has been prevented. So on the other hand, VEGF by itself diminished cardiomyocyte apoptosis, stimulated cell proliferation and mediated the migration of cardiac stem cells to infarcted myocardium post MI (28-29). According to the above statements, we delivered and protected the enough cardiomyocytes into ischemic hearts which hypothetically participated in cardiac regeneration but the exact mechanism by which they act still remain unclear. There was an insignificant increase in LVEF’s level in hUCM+VEGF group than in VEGF, hUCM and dhUCM groups. The transplanted hUCM cells did not express α-actinin in recipient cardiac tissue (Figure 5 (g, h)).

Poor synergetic effect between hUCM cells and VEGF could be because of the lack of cardiogenic differentiation of the transplanted hUCM cells in recipient cardiac microenvironment. Immunohistochemical study has revealed that vascular density was significantly higher in the VEGF, hUCM+VEGF and dhUCM+VEGF groups than in other treated and non-treated groups (P<0.05). Vascular density was insignificantly different among the VEGF, hUCM+VEGF and dhUCM+VEGF groups (Table 1). Our results have suggested that the transplanted hUCM and dhUCM cells cannot stimulate angiogenesis, in accordance with Latifpour’s results (18). BM-MSCs potentially induce angiogenesis in infarcted hearts (30) but the hUCM cells do not possess this potential. Lack of the ability to induce angiogenesis is considered as a defect for dhUCM cells but this defect could be eliminated by combination with VEGF (18). VEGF induces angiogenesis through pathways which lead to induction of growth, proliferation and migration of endothelial cells to infarcted myocardium. Many studies have reported the beneficial effects of VEGF administration into infarcted hearts, however, conflicting data about its efficacy has been reported which is primarily associated with an exposed dose of it (31-35). We have considered using 5 µg VEGF in normal saline as a sufficient dose (36). Lost and hibernated cardiomyocytes replaced by fibrosis tissue after MI. Transplanted stem cells have potential to activate signaling pathways that lead to reduction of the fibrosis tissue formation in myocardium. Fibrosis tissue in the treated groups was markedly lower when compared to the MI and PBS groups. Animals in the hUCM+VEGF and dhUCM+VEGF groups markedly have lower myocardium’s fibrosis tissue when compared to animals in the other treated groups (Table 1).

Immunohistochemical analysis confirmed that the hUCM and dhUCM cells survived and migrated to infarcted myocardium, therefore lost cardiomyocytes seems to be replaced by the transplanted cells (Figure 5). Previous studies have documented that VEGF administration into infarcted heart induces some beneficial effects such as: Endothelial, CSC and aortic smooth muscle cells migration to the infarcted myocardium, neovascularization and survival of blood vessels, migration and proliferation of cardiomyocytes as well as prevention of cardiomyocytes apoptosis (28, 29, 36-38). It can be concluded that the chances of fibroblasts to proliferate in the infarcted myocardium were reduced because of the administration of hUCM, dhUCM and VEGF.

Negative influence of the transplanted cells and VEGF fibrosis tissue formation in myocardium has been duplicated in hUCM+VEGF and dhUCM+VEGF groups and as a result, hUCM+VEGF and dhUCM+VEGF groups have significant reduction in fibrosis tissue when compared to the other treated and non-treated groups.

**Conclusion**

The dhUCM cells combined with VEGF transplantation can improve left ventricular dysfunction, induce angiogenesis and decrease fibrosis tissue formation in infarcted myocardium in eight weeks post MI owing to the synergetic effect between dhUCM cells and VEGF. The (dhUCM+VEGF) is more effective when compared to VEGF, hUCM, dhUCM and (hUCM+VEGF) administration on infarcted heart.

**Conflict of interest**

The authors confirm that this article content has no conflict of interest.

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