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The effects of dan-shen root on cardiomyogenic differentiation of human placenta-derived mesenchymal stem cells

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ABSTRACT

The aim of this study was to search for a good inducer agent using for cardiomyogenic differentiation of stem cells. Human placenta-derived mesenchymal stem cells (hPDMSCs) were isolated and incubated in enriched medium. Fourth passaged cells were treated with 10 mg/L dan-shen root for 20 days. Morphologic characteristics were analyzed by confocal and electron microscopy. Expression of α-sarcomeric actin was analyzed by immunohistochemistry. Expression of cardiac troponin-I (TnI) was analyzed by immunohistofluorescence. Atrial natriuretic factor (ANF) and beta-myocin heavy chain (β-MHC) were detected by reverse transcriptase polymerase chain reaction (RT-PCR). hPDMSCs treated with dan-shen root gradually formed a stick-like morphology and connected with adjoining cells. On the 20th day, most of the induced cells stained positive with α-sarcomeric actin and TnI antibody. ANF and β-MHC were also detected in the induced cells. Approximately 80% of the cells were successfully transdifferentiated into cardiomyocytes. In conclusion, dan-shen root is a good inducer agent used for cardiomyogenic differentiation of hPDMSCs.

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1. Introduction

Mammalian heart has been considered terminally differentiated with a static number of cardiomyocytes that are incapable of self-renewal. However, there is emerging evidence that there may be replicating cardiomyocytes and stem cells that are participating in the process of cellular maintenance and myocardial regeneration in patients with heart failure [1,2]. However, the numbers of cells involved in this process are believed to have insignificant effect on the repair of major injuries such as myocardial infarction. Although heart transplantation has been a therapy for these cases for several decades, its usage is much limited by the shortage of donors and the host immunorejective reaction to the grafts [3–6]. As a newly developed strategy, cellular cardiomyoplasty, which involves the injection of suspended cultured cells into myocardial scar tissue, is showing a bright future in this field.

Adult bone marrow may contain multipotent stem cells that are capable of participating in tissue regeneration and repair. Myocardial transplantation of bone marrow stem cells has been associated with improved cardiac performance in experimental models [7–9], and significant effects were observed. However, recent papers have shown that the number and function of the circulating stem cells were depressed in older patients and in patients with diabetes mellitus [10,11], suggesting that stem cells obtained from patients with coronary risk factors may not function well. This suggests limits to the utilization of autologous stem cells for the ischemic cardiomyopathy patient. Recently, investigators isolated a cell population from human placenta. Their morphology and immunophenotype were similar to those MSCs isolated from bone marrow, and they showed the ability of multi-directional differentiation [12].

Recovery of cardiac performance following cellular transplantation in experimental models has been partly attributed to transdifferentiation of stem cells leading to de novo formation of cardiomyocytes [13,14]. However, the types and characteristics of these stem cells remain poorly defined with widely different transdifferentiation efficiency reported [14,15]. Pre-differentiation of stem cells towards a defined cardiac lineage before transplantation may be more advantageous than transplanting uncommitted stem cells that may undergo unanticipated differentiation. MSCs have been reported to transdifferentiate into cardiomyocytes following co-culturing with rat cardiomyocytes [16,17] or 5-azacytidine (5-aza) treatment [18]. However, co-culturing technique and 5-aza treatment are not likely to be applicable clinically due to their low efficacy and potentially harmful effects of nonspecific demethylating activity of 5-azacytidine [19].

A closely regulated in vitro environment may be necessary for the occurrence of the transdifferentiation process that would utilize chemically defined culture media supplemented with recombinant cytokines and growth factors. Dan-shen root is a traditional Chinese drug and commonly used to treat ischemic diseases in clinic. This study was conducted to investigate the ability of

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hPDMSCs to differentiate into cardiomyocytes after treatment with dan-shen root in vitro.

2. Materials and methods

2.1. hPDMSCs isolation and culture

Term (38–40 weeks gestation, $n = 10$) placentas from healthy donor mothers were obtained with informed consent approved according to the procedures of the institutional review board. Briefly, the decidua of the maternal part was separated and discarded. Placental tissues from the fetal part were cut into pieces approximately 5 mm^3 in size. The pieces were washed in phosphate buffered solution (PBS) supplemented with 100 U/mL penicillin–streptomycin, until the supernatant was free of erythrocytes. Some pieces of chorionic plate were attached to the substratum in a 10-cm-diameter dish. Culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 mmol/L L-glutamine, 0.1 mmol/L, β-mercaptoethanol and 1% non-essential amino acids was added. The culture medium was changed every 3 days. When cells were more than 80% confluent, they were harvested with 0.25% trypsin–EDTA and subcultured at a 1:3 split ratio onto new 10-cm-diameter dishes.

2.2. Flow cytometry analysis

Placenta-derived cells at passage 4 were treated with 0.25% trypsin–EDTA, harvested, and washed twice with PBS. The cells were incubated on ice with labeled mouse anti-human antibodies for CD13, CD73, CD90, CD105, CD166, HLA-DR (PE-conjugated), CD14, CD29, CD31, CD44, CD45, CD105 HLA-ABC (FITC-conjugated). Control groups were incubated with FITC- and PE-conjugated antibodies against mouse IgG. The labeled cells were analyzed by flow cytometry.

2.3. Preparation of murine fetal cardiomyocytes

Fetal cardiomyocytes were obtained from the hearts of day 3 mouse fetuses. Hearts were minced with scissors and washed with PBS, and the minced hearts were incubated in 0.25% trypsin–EDTA for 15 min at 37 °C. After DMEM supplemented with 10% FBS was added, the cells were centrifuged at 1000 rpm for 5 min. The pellet was then resuspended in 10 mL of culture medium containing DMEM supplemented with 10% FBS, 1 mmol/L L-glutamine, 0.1 mmol/L, β-mercaptoethanol and 1% non-essential amino acids and incubated on 10-cm-diameter for 90 min to separate the cardiomyocytes from fibroblasts. The medium which was used to incubate fetal cardiomyocytes was collected everyday, we called it conditional medium.

2.4. Cardiomyogenic differentiation

hPDMSCs of the forth passage were resuspended after trypsin treatment in complete medium containing DMEM supplemented with 10% FBS, 10 mg/L dan-shen root, 1 mmol/L L-glutamine, 0.1 mmol/L, β-mercaptoethanol and 1% non-essential amino acids for induction of cardiomyogenic differentiation. Since in vitro simulation of the heart by the environment has been shown to be an efficient means of inducing the transdifferentiation of MSCs, we incubated hPDMSCs in conditional medium to induce cardiomyogenic differentiation for positive control. We also employed a culture system with uninduced cells for negative control. Morphologic characteristics of the cells were analyzed by confocal and electron microscopy everyday. Growth curves of the cells were measured by trypan blue dye exclusion test every 2 days. The experiment was terminated at the 20th day.

2.5. Immunocytochemistry analysis

Cells were fixed in PBS containing 4% paraformaldehyde (PFA) for 20 min, permeabilized in PBS containing 0.2% Triton X-100 for 10 min, and blocked in a serum-free blocking solution for 5 min at room temperature. Cells were then incubated with primary antibody α-sarcomeric actin diluted 1:100 overnight at 4 °C. After extensive washing with PBS, cells were incubated with biotinylated rabbit anti-human IgG as secondary antibody. Finally, nuclei were stained with hematoxylin.

2.6. Immunofluorescence analysis

Cells were washed with PBS and fixed in PBS containing 4% PFA for 30 min, washed extensively with PBS after fixing and permeabilized in PBS containing 0.2% Triton X-100 for 10 min, and washed extensively with PBS after permeabilizing and blocked in a serum-free blocking solution for 5 min at room temperature. Cells were then incubated with primary antibody cTnI diluted 1:50 overnight at 4 °C. After extensive washing with PBS, cells were incubated with the fluorescence isothiocyanate (FITC)-conjugated anti-rabbit IgG diluted 1:50 for 1 h at room temperature. After extensive washing with PBS, the cells were analyzed using fluorescence microscope. The cardiomyogenic induction rate was calculated as the fraction of cTnI-positive cells in the total cells which were in counterpart visual field. The rate was calculated as the average from more than 10 separate experiments.

2.7. RT-PCR analysis

Total RNA was extracted from cells of the three culture system using Trizol reagent according to the manufacturer's instructions. The total RNA was used for RT-PCR. We used Taka RNA PCR Kit (mv) Ver.3.0 for RT-PCR and performed RT-PCR according to the manufacturer's instructions. The PCR conditions included a denaturation step (94 °C for 2 min), amplification and quantification repeated 35 times: 94 °C for 30 s, (Anf: 54.7 °C, β-MHC: 62.5 °C, β-actin: 54.5 °C) for 30 s, and 72 °C for 45 s, with an additional 7 min at 72 °C after completion of the final cycle. The primers used for real-time PCR were as follows: β-actin (TCATGTTTGAGACCTTCAA, GTCTTTGCGGATGTCCACG, 512 bp), Anf (TCTGCCCTCCTA AAAAGCAA, ATCACAACTCCATGGCAACA, 406 bp), β-MHC (GATGGC CAGTCTTTGGGGCTGC, TGTAGAGCCA CCGCGGGCTCTCAT, 285 bp). The PCR products were analyzed by electrophoresis with 1.2% agarose gel, visualized with goldview staining, and photographed under UV light.

3. Results

3.1. Characterization of human placenta-derived cells

A small population of single cells was observed at 3–5 days after initial plating. Majority of the cells displayed spindle-like fibroblastic shape. These cells began to proliferate at about day 7, and gradually grew to form small colonies. The colonies reached confluency at 21–28 days after initial plating. After passaging with trypsin, the placenta-derived cells grew to form colonies again (Fig. 1A). The surface markers of placenta-derived cells were exactly the same as those of previously reported bone-marrow- and cord blood-derived mesodermal cells, i.e., positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166 and HLA-ABC, and negative for CD14, CD31, CD45 and HLA-DR (Fig. 1)B.

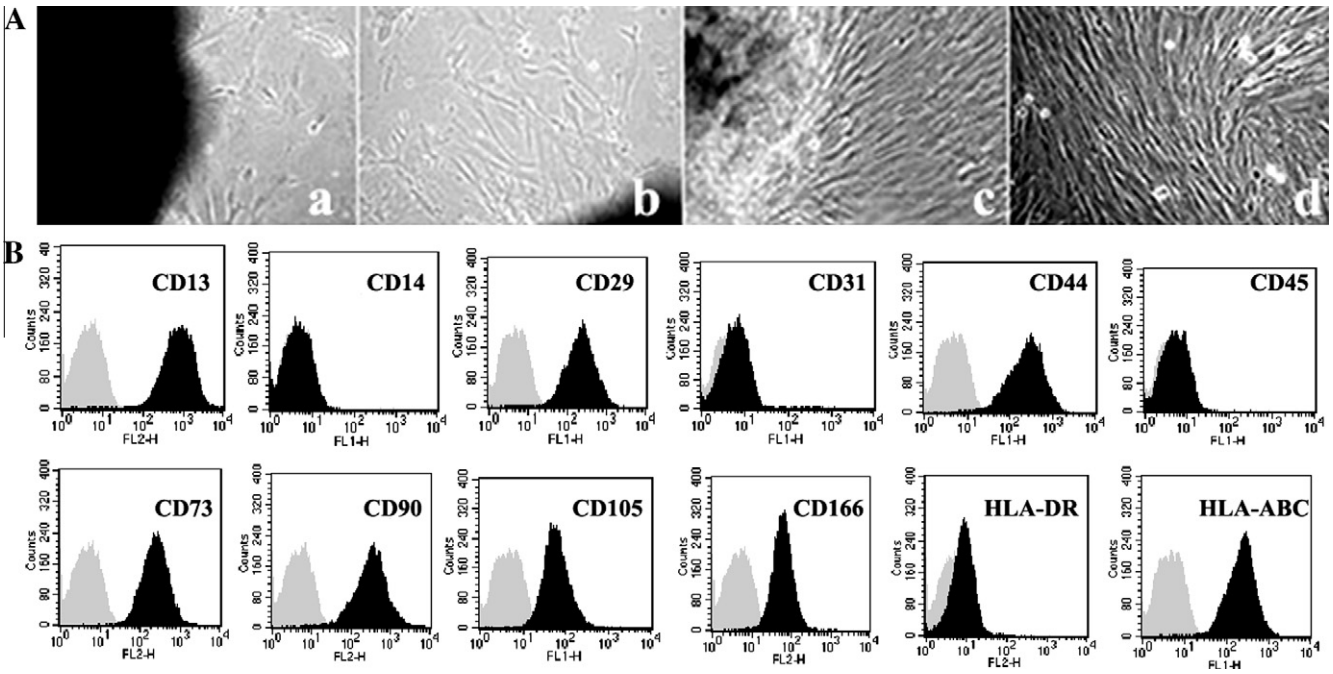

Fig. 1. Characterization of human placenta-derived cells. (A) The morphologic characteristic of human placenta-derived cells: (a) primary culture 3d; (b) primary culture 10d; (c) primary culture 3w; (d) passage 2. (B) The immunophenotyping of human placenta-derived cells: it is common to BMSCs.

3.2. Cell proliferation rate and morphological changes

Uninduced cells still maintained rapidder growth rate (Fig. 4A) and maintained fibroblast-like morphology (Fig. 2a). However, induced cells significantly slowed down the growth rate (Fig. 4A) and began to change their morphology. They gradually increased in size to form a stick-like appearance. At the 20th day, the cells became enlarged and showed a number of branches connecting with adjoining cells (Fig. 2b and c).

3.3. Expression of cardiac markers

Immunocytochemistry and immunofluorescence revealed that induced cells stained positive for α-sarcomeric actin and TnI. RT-PCR analysis showed that induced cells expressed ANF and β-MHC. All these cardiomyocyte-specific genes were not expressed in uninduced cells (Fig. 3).

3.4. Cardiomyogenic differentiation rate

We calculated the percentage of cTnI-positive cells to determine the cardiomyogenic differentiation rate of hPDMSCs. Uninduced hPDMSCs did not show any TnI expression, however, most of induced hPDMSCs became positive for TnI antibody. The cardiomyogenic induction rate of dan-shen root was slightly high compared to that of conditional medium (Fig. 4B). It is noted that dan-shen root could be used as an inducer agent to induce hPDMSCs to differentiate into cardiomyocytes.

4. Discussion

MSCs give origin to the marrow stromal environment that supports hematopoiesis. These cells present a wide range of differentiation potentials, they can be induced to differentiate into bone, adipose, cartilage, muscle, and endothelium if these cells are cultured under specific permissive conditions [20,21]. Therefore, MSCs may have a wide range of potential applications in clinical therapy. Currently, bone marrow represents the main source of MSCs for both experimental and clinical studies. However, there are only a small quantity of MSCs in bone marrow, and recent papers have shown that the number and function of the circulating stem cells were depressed in older patients and in patients [10,11], suggesting that stem cells obtained from patients may not function well. This suggests limits to the utilization of autologous stem cells for patients. On the other hand, in order to do allogenic stem cell transplantation, human leukocyte antigen (HLA)-type matching is very important for

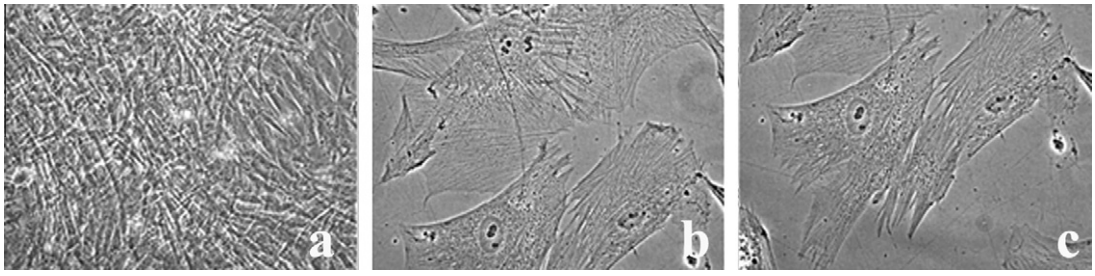

Fig. 2. The morphological changes of the cells. (a) Negative control: cells still maintained their fibroblast-like morphology. (b) Conditional medium inducing group and (c) dan-shen root inducing group: cells became enlarged and showed a number of branches connecting with adjoining cells.

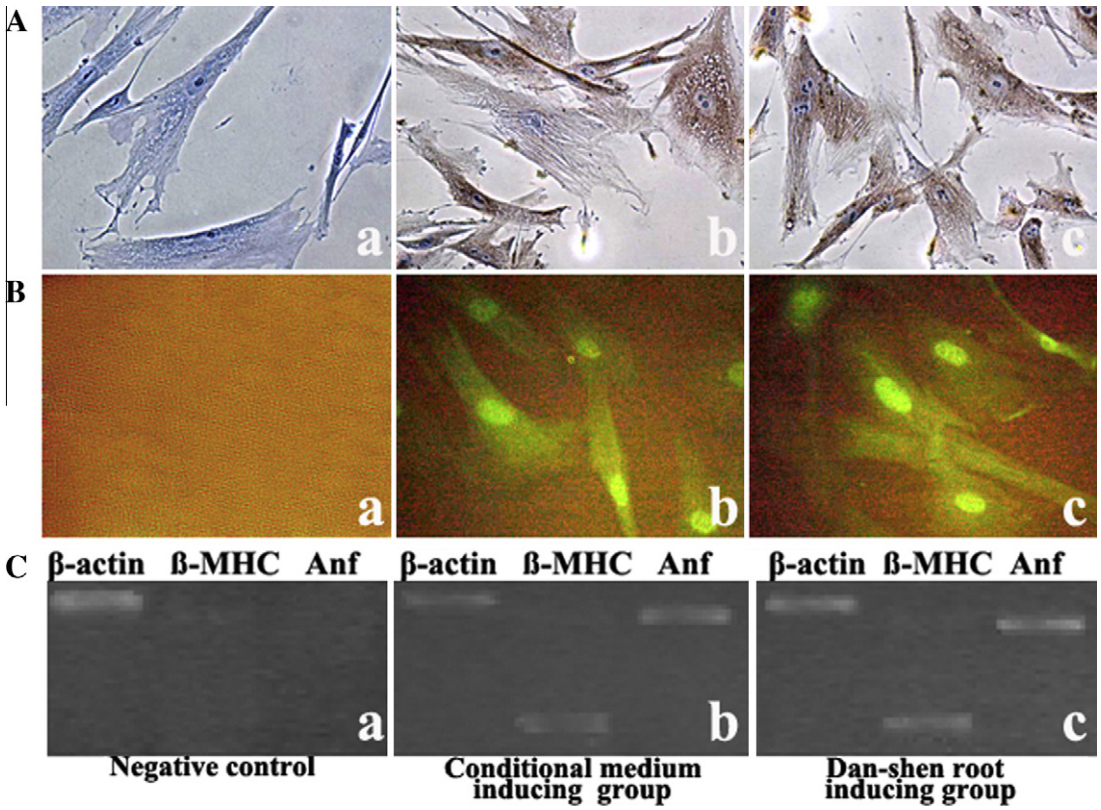

Fig. 3. Assessment of cardiomyogenic differentiation. (A) Expression of α-sarcomeric actin: (a) negative control: cells stained negative for α-sarcomeric actin; (b) conditional medium inducing group and (c) dan-shen root inducing group: cells stained positive for α-sarcomeric actin. (B) Expression of TnI: (a) negative control: cells stained negative for TnI; (b) conditional medium inducing group and (c) dan-shen root inducing group: cells stained positive for TnI. (C) RT-PCR analysis of expression of β-MHC and ANF: (a) negative control: cells did not express β-MHC and ANF; (b) conditional medium inducing group and (c) dan-shen root inducing group: cells expressed β-MHC and ANF.

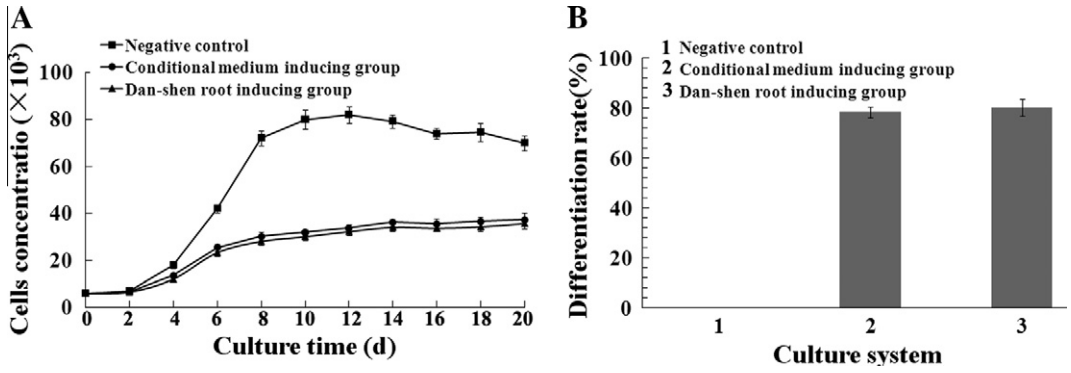

Fig. 4. Cell proliferation rate and cardiomyogenic differentiation rate. (A) Proliferation rate: uninduced cells still maintained rapidder growth rate, but induced cells significantly slowed down the growth rate. (B) Cardiomyogenic differentiation rate: the cardiomyogenic induction rate of dan-shen root was slightly high compared to that of conditional medium, but there was no significant difference.

the stable survival of grafts. Therefore, the sample, which can be noninvasively collected from many volunteers, is a desirable source of stem cells due to the ease of establishing cell banks that can store all HLA-types. Recently, a population of MSCs was isolated from human placenta. These cells possess morphologic, immunophenotypic, and functional characteristics similar to those MSCs isolated from bone marrow [12,22]. Since the placenta is usually discarded, it can be collected at usual delivery or cesarean section and can be banked or stored. Cells with almost all the HLA types can be collected after several generations. Therefore, hPDMCs may be an attractive, alternative source of progenitor or stem cells for basic and clinic researches.

Dan-shen root is a traditional Chinese drug and commonly used to treat ischemic diseases in clinic. It can increase the blood flow through improving the microcirculation of ischemic region, inhibit platelet aggregation, and decrease blood fat and delay development of arteriosclerosis. Studies also show that dan-shen root can inhibit the glycolysis, rectify acidosis, increase the production of ATP of the cardiomyocytes in ischemic region and inhibit the lipid peroxidation in the progress of ischemical reperfusion injury.

Therefore, dan-shen root is one of the contributing factors in the regeneration of the injured myocardium.

Our results indicated that hPDMSCs were easier to get in comparison with BMSCs. They could be induced to differentiate into cardiomyocytes without relying on cytotoxic agents or co-culture technique but with relying on dan-shen root. However, the mechanism by which dan-shen root promotes cardiomyogenic differentiation remains unclear. Pre-differentiation of stem cells into cardiomyocytes may potentially enhance their abilities to survive and engraft as cardiomyocytes following myocardial transplantation. Furthermore, transplantation of cells with pre-defined myocardial characteristics may circumvent the controversies revolving transdifferentiation and cell fusion in the generation of cardiomyocytes by undifferentiated stem cells in regenerating myocardium. Pre-treatment of these multipotent cells by dan-shen root can be used prior to transplantation studies to ensure that the differentiation process will be directed towards the cardiomyogenic lineage in the in vivo environment.

5. Conflict of interest statement

None declared.

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