Induced Differentiation of Human Cord Blood Mesenchymal Stem/Progenitor Cells into Cardiomyocyte-like Cells In Vitro

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Summary: The feasibility of using cord blood mesenchymal stem/progenitor cells (CB-MSPCs) to regenerate cardiomyocytes and the optimal inducing conditions were investigated. The CB mononuclear cells were cultured in low serum DMEM medium to produce an adherent layer. After expansion, the adherent cells were added into cardiomyocyte inducing medium supplemented with 5-azacytidine. Cardiogenic specific contractile protein troponin T staining was performed to identify the cardiomyocyte-like cells. The results showed that the frequency of CB-MSPCs clones in CB mononuclear cells was 0.5 x 10^-6 and about 1.3 x 10^3-fold expansion was achieved within 20 sub-cultivation. After cardiogenic induction, 70% CB-MSPCs was differentiated into cardiomyocyte-like cells. It was indicated that low serum culture could expand CB-MSPCs extensively and the expanded CB-MSPCs could be induced to differentiate into cardiomyocyte-like cells in high efficiency.

Key words: cord blood; mesenchymal stem/progenitor cell; differentiation; cardiomyocyte; 5-azacytidine

It has been confirmed that adult human bone marrow contained a rare population of mesenchymal stem/progenitor cells (MSPCs). Morphologically, MSPCs in their undifferentiated state are spindle shape and resemble fibroblasts. They can be extensively expanded in vitro and, when culture under specific permissive condition retain their ability to differentiate into multiple lineage including bone, cartilage, tendon, muscle, nerve and stromal cell1]. Recent studies revealed that MSPCs were also present at low frequency in term CB mononuclear cells (MNC). The CB-derived MSPCs had nearly the same proliferation and differentiation potential as those from bone marrow and might be one of the optimum seed cells for tissue regeneration without any biological and ethical problem2]. The purpose of this study was to investigate the possibility of using CB-derived MSPCs to regenerate cardiomyocyte and define the optimal inducing condition for cardiomyocyte differentiation.

1 MATERIALS AND METHODS

1.1 Cord Blood Harvest and Preparation of Adherent Cells

Cord blood harvests were obtained with the mothers' consent from the term deliveries of uncomplicated pregnancies at the time of babies birth in Xiehe Hospital of Tongji Medical College, Huazhong University of Science and Technology. The blood was set in a sterilized plastic bag containing ACD-A solution (4:1 v/v). All blood samples were processed immediately after harvest and CB mononuclear cells were obtained by gradient centrifugation using Ficoll-Hypaque (Sigma, USA). The CB MNC were washed and suspended in DMEM culture medium (Gibco, USA) containing 10% or 2% (v/v) fetal bovine serum (FBS Hyclone), with 100 U/ml penicillin G and 100 U/ml streptomycin. Then they were seeded on T-25 flask (NUNC) at a density of 1 x 10^4/ml at 37 °C in a humidified atmosphere containing 5% CO_2. After 72 h non-adherent cells were removed and same medium was replaced, at 80% confluence the cells were harvested with 0.25% trypsin (Gibco, USA) for 5 to 10 min. To expand the cells through successive passage, they were seeded at 10^4 cells/ml grown to near confluence and harvested with the same protocol. MSPCs frequency in CB MNC was assessed by the limiting dilutions described by Verfaillie and Reys3]. The purpose of this study was to investigate the possibility of using CB-derived MSPCs to regenerate cardiomyocyte and define the optimal inducing condition for cardiomyocyte differentiation.

1.2 Preparation of Human Cardiomyocyte Conditioned Medium

Human fetal heart tissue was come from a male infant whose mother was terminated pregnancy due to non-healthy reason, with the gestational age of 14+4 weeks. The tissue collection was approved by the research ethics committee of Tongji Medical College. The myocardial tissue from ventricle was sterilely taken, sheared, grinded into homogenate, washed with PBS and centrifuged. The supernatant
was retarded. The cells were adjusted to a density of 10^5/ml and suspended in DMEM supplemented with 10 % FBS, 100 U/ml penicillin G, and 100 U/ml streptomycin, and put into T-25 flask. 72 h later the culture medium was withdrawn gently and the same culture medium as above was added. When cells were confluent to 60 %, successive passage was needed. Every 3 days supernatant was collected and culture medium was replaced. The conditioned culture medium was collected till sixth passage.

1.3 Cardiomyocytic Differentiation

The inducing medium for cardiomyocytic differentiation was consisted of DMEM (50 %) and human fetal cardiomyocyte conditioned medium (40 %) supplemented with 10 % FBS, 100 U/ml penicillin G, 100 U/ml streptomycin. The inducing agent was 5-azacytidine (5-aza, Sigma, USA) at the concentration of 10 μmol/L. For inducing the cord blood derived adherent cells to differentiate to cardiomyocytic-like cells, two steps were used; (1) Inducing culture: Cord blood-derived adherent cells at their 5—10th passages were put in 6-well plates (NUNC) at a cell density of 2-5 × 10^4/ml adjusted with inducing culture medium. Some wells had been put with a sterile glass slide at the bottom. The culture plates were incubated at 37℃ in a humidified atmosphere containing 5 % CO₂. 24 h later, 5-aza was added at the final concentration of 10 μmol/L and the culture was maintained for another 24 h. (2) Culture after induction: 24 h after induction with 5-aza, the culture medium was replaced by inducing culture medium not containing 5-aza. The medium was changed twice a week for 2 weeks. Phase contrast microscopy was performed for morphological observation.

1.4 Immunohistochemical Identification of Specific Cardiac Contractile Protein in Induced Cord Blood Derived Adherent Cells

The un-induced adherent cells were used as negative controls and the adherent cells from the DMEM containing 10 % FBS as controls. These cells were immunofluorescently stained for human cardiac specific troponin T (spectral diagnostic) at day 14 after induction. The procedures were performed according to the manufacturer’s guideline. Briefly, the slides which lied on the well bottom in each well was taken out gently, washed with PBS and air dried at room temperature. The slides were fixed for 15—20 min in 95 % ethanol at room temperature, washed with PBS 3 times, and air dried. The slides were incubated with a monoclonal antibody against cardiac specific troponin T at 37℃ for 1 h. To remove the unbound antibody, the slide was gently shaken at room temperature and washed 3 times with PBS. A mouse antibody conjugated with FITC against human IgG was added to the slide, incubated and rinsed with PBS as described above for the first antibody. The slide was examined under a fluorescent microscope and photographed.

2 RESULTS

2.1 Morphological and Growth Characteristics of Cord Blood Derived Adherent Cells

Cord blood derived mononuclear cells were set in culture and onset of an adherent layer was monitored continuously. By the day of 14 in low serum DMEM, all 12 cord blood harvest produced adherent clones (>100 cells) and reached 80 % confluent at day 35—40. These adherent layers were formed by a homogeneous cell population which resemble closely to fibroblast. These cells were smaller (diameter 10—15 μm) and had scant cytoplasm with fewer vacuoles or granules. The frequency of cord blood mononuclear cells giving rise to these adherent culture was 0.5 × 10^-4. Their cells doubling time was 60—72 h. Within their 20th sub-cultivation, near 50 cell proliferative periods, these cells could maintained morphological feature constantly. This indicated that these cells had been extensively expanded about 1.3 × 10^7 fold. Cytochemical analysis showed that these cells were strongly positive for α-naphthyl acetate esterase and periodic acid Schiff but negative for peroxidase.

By the day of 14, all 12 cord blood derived mononuclear cells set in 10% FBS DMEM culture medium gave rise to an adherent colonies also and were confluent at day of 28—30. Out of 12 cord blood harvests, the adherent layer in 9 cases was formed by heterogeneous populations which mainly was osteoclast-like cell. Microscopic examination revealed these cells had an elongated or oval/round shape with smooth border. Their remarkable feature was the presence of multinucleated cells which congregated around a central area. Other 3 samples evolved a homogenous population of cells showing a fibroblastoid spindle shape morphologically, but was larger (20—25 μm in diameter) and often contained vacuoles or granules. The cells doubling time was about 48 h. Within 10th sub-cultivation their morphological features could maintained constantly. Their cytochemical staining reaction was same as that in low serum.

2.2 The Morphological Change of Adherent Cells after 5-aza Induction

Cord blood mononuclear derived adherent cells showed a fibroblast-like morphology before 5-aza treatment and this feature was retained through sub-culture under inducing medium conditions. After 5-aza treatment the morphology of the cells gradually changed, approximately 70 % of the adherent cells which come from low serum culture was gradually increased in size but decreased in cytoplasm, formed a ball like appearance, or lengthened in one direction and formed a stick-like morphology one week later. Other 30 % adherent cells remained unchanged morphologically. In contrast, only about 40 % of adherent cells evolved from 10 % FBS DMEM culture condition produced a differentiated morphological change.