Swine endothelial progenitor cell culture

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Abstract

Objective: This study is to culture swine endothelial progenitor cells (EPCs) in vitro for future local transplantation.

Materials and methods: EPCs were isolated from peripheral blood of a domestic swine. After isolated with density gradient centrifugation, EPCs were cultured with EGM-2 medium in a standard condition for 12 days and identified by fluorescence microscopy and immunocytochemistry tests.

Results: EPCs were successfully isolated, cultured and characterized. From day 1 to day 12 after culture, their morphology changed from round cells to cobblestone or spindle cells. On day 4, EPCs showed adhesion ability to the flask wall. The EPCs were characterized with phenotype expressions and specific functions.

Conclusion: It is feasible to culture swine EPC in vitro for future transplantation.

Background

Endothelial progenitor cells (EPCs) have the ability to repair the injury vessels and promote neovascularization of ischemia tissues, namely angiogenesis [1]. It is also well known that if rapid re-endothelialization, which provides an inherent nonthrombogenic potential and interrupts cytokine driven activation of SMCs in vascular medial tissues, is achieved, accelerated normal wound healing at diseased sites may be realized [2].

To promote rapid re-endothelialization and improve the patency of affected vessels, the authors cultured swine endothelial progenitor cells (EPCs) in vitro for future local transplantation.

Materials and methods

Isolation, culture and identification of endothelial progenitor cells

EPCs were isolated from newly drawn porcine peripheral blood by the density gradient centrifugation method [3]. 20 mL peripheral blood was extracted from a young adult healthy domestic swine (female, weight 25 kg) (Jingling Farm Center for Animal Experiments, Nanjing, China), through femoral artery puncture under sterilization with heparin anticoagulation (100 IU/mL, Heparin Sodium, Qianhong Inc., Jiangsu, China). The blood was diluted at 1:1 ratio by phosphate-buffered saline (PBS). EPCs were isolated from the diluted blood by density gradient centrifugation (Biofuge, Heraeus, Germany) at 2000 rpm 24-25°C with lymphocyte isolation medium Histo-Paque 1077 (Sigma-Aldrich, St. Louis, MO) for 30 minutes. A sedimented layer of peripheral blood mononuclear cells was collected, washed twice with PBS, two more centrifugations were performed at 1000 rpm for 10 minutes each. The precipitated cell pellet was counted by cytometer and resuspended in 2 mL of microvascular growth medium-2 (EGM-2 MV; Cambrex, Walkersville, MD) in a T25 culture flask which pre-coated with fibronectin (Chemicon) and incubated at 37°C, full humidity, 5% CO2 (CO2 Incubator, Heraeus, Germany). Each 500 mL EGM-2 medium contains FBS 25 mL, hydrocortisone 0.2 mL, hFGF-B 2 mL, VEGF 0.5 mL, R3-IGF-1 0.5 mL, ascorbic acid 0.5 mL, hEGF 0.5 mL, GA-1000 0.5 mL. After 4 days, the suspended cells were removed and the adhering cells were changed every 4 days with a fresh culture medium. On day 8-12, while the cells occupied the more than 75% of the microfield, the passage begun by digesting the cells with 0.25% trypsin (Trypsin, Sigma) and transferring the pellet to a larger glass culture flask (50 mL).

EPCs engulfed Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL, Biomedical Technologies Inc., Stoughton, MA) and binding fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (FITC-UEA-I, Vector Laboratories Inc., Burlingame, CA), were examined by inverted fluorescence microscopy. The adherent cells that stained positive with both FITC-UEA-I and Dil-Ac-LDL were indicated to be differentiating endothelial cells. The EPCs phenotype was identified by immunocytochemistry including CD31, CD34, vWF, and VEGFR-2 (flik-1, fetal liver kinase-1) (TBD Co.). For immunocytochemistry, first passage cells were plated on 24-well chamber slides and fixed with 4% paraformaldehyde for 30 minutes. Slides were blocked with 3% BSA in PBS-T for 1 hour and incubated overnight at 4°C in 1:100 dilution of goat polyclonal anti-vonwillbrand factor (DiaSorin), FLK-1, CD31, CD34 (SantaCruz Biotechnology). Horseradish peroxidase activity was visualized with DAB.

Statistical analysis

Continuous variables are expressed as mean ± SD. Descriptive analysis was applied by using SPSS 20.0 (Windows Version, SPSS, Inc., Chicago, IL, USA)

Results

EPCs were in round or multangular shapes in P0 stage on day 1.

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Putative EPCs were the cells attached to the T-25 flask wall after 24 hours culture. On 12th day, EPCs had cobble-stone or spindle-like appearance and cluster formation. After first passage, EPCs were harvested, counted by cytometry, and identified by immunocytochemistry technique. There were $(2.0 \pm 0.5) \times 10^6$ cells in T25 glass flask, and $(5.5 \pm 1.2) \times 10^6$ cells in one 50 ml culture flask. Progenitor endothelial cell phenotypes including CD34, VEGFR-2, and vWF were positive in majority (≥95%) of day 7-9 EPCs.

Inverted fluorescence microscopy demonstrated EPCs emitting red fluorescence by endocytosed Dil-Ac-LDL and radiated green fluorescence while binding FITC-UEA-1, overlay image showed brown color.

**Discussion**

There are many animal and clinical investigations on promotion of endothelialization in various vascular stent models such as Bhattacharya V et al. and Griese DP et al. [4,5]. Obviously, this technique only demonstrates an approach to promote the endothelialization but inapplicable clinically. Stem cells such as EPCs have many advantages for such purpose, because EPCs can be conveniently obtained from peripheral blood and be differentiated to endothelium in vivo.

Shirota T reported successful fabrication of endothelial progenitor cell (EPC)-seeded intravascular stent devices in vitro [6]. We adapt similar technique in our study [7]. Our in vitro results show that it is feasible to construct an EPC implanted metal stent.

Currently, there are mainly two approaches to obtain circulation EPCs, including immune magnetic beads selection and density gradient centrifugation methods. The later one is cheaper than the previous one which depends on fluorescence -activated cell sorting (FACS) analysis. In this study we adapted the later technique.

To the purpose of accelerating endothelialization after a stent placement in vessels, an intraluminal delivery of a sufficient number of endothelial cells (ECs) to diseased vessel sites has shown promising results [8]. However, it is not practical to use mature ECs for the cell transplantation due to the limitation of difficulty of harvesting ECs [8].Irrespective of whether an on-stent or catheter-infusion delivery system is used, however, difficulty of harvesting EC from patients has hampered clinical usage of these methods [9].

Ashahra et al. identified that circulating EPCs in adult peripheral blood which is capable of trafficking toward ischemic sites and differentiating into mature endothelial cells [1]. Recently, the existence of circulating endothelial progenitor cells (EPCs) has been identified as a key factor for re-endothelialization [10]. Harvesting circulating EPCs requires only peripheral blood collection. This minimally invasive harvesting procedure indicates that EPC is a candidate for a new source of cells for endothelialization. The early establishment of a functional endothelial layer after vascular injury has been shown to assist in the prevention of neointimal proliferation and thrombus formation [11,12].

**Conclusion**

Porcine endothelial progenitor cells can be isolated, cultured and identified from peripheral blood.

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