



A new method of culturing rat bone marrow endothelial progenitor cells *in vitro*

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Background: Endothelial progenitor cells (EPCs) play an important role in the re-endothelialization of ischemic cerebrovascular disease. However, the current acquisition method has some deficiencies. This study aimed to design a new and practical method for obtaining EPCs.

Methods: Bone marrow was obtained autologously from the right tibia of living rats. Briefly, the right tibia bone was carefully exposed and two holes (1 mm in diameter) were made in the tuberosity and lower one-third of the tibia, respectively. A PE-50 catheter and syringe (5 mL) were inserted through the holes to aspirate the bone marrow. Bone marrow mononuclear cells (BMMCs) were isolated by density-gradient centrifugation with Ficoll and counted. Adherent cell culture continued for 2 weeks, and the medium was replaced every 3 days.

Results: During the first days of culture, adherent cells formed a monolayer, consisting predominantly of small-sized cells. Single large cells with endothelial morphology were observed. On day 4, the nonadherent cells were removed, and the adherent cells were left for further culture. On day 6–7, a proliferating population of round cells formed clusters in the culture chamber, and morphological analysis revealed a homogeneous population of colony-forming units (CFUs). Large, flat cells with endothelial morphology sprouted from the CFUs, which had nearly disappeared by day 14 of culture. The adherent cells were positive for CD133 and vascular endothelial growth factor receptor 2 (VEGFR2), internalized acetylated low-density lipoprotein, and bound ulex europaeus-agglutinin-I, but were negative for CD45, which correlated with the endothelial morphology and ability to form capillaries of EPCs.

Conclusions: Our results are direct evidence that mononuclear cells (MCS) from living rat bone marrow can be used to culture EPCs *in vitro* under certain culture conditions, providing a new method for the further study of autologous EPC transplantation.

Keywords: Bone marrow; cell culture; endothelial progenitor cells (EPCs); rats

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Introduction

Cell therapy has opened up a new avenue of treatment for ischemic disease (1-3). Endothelial progenitor cells (EPCs) are among the most widely used stem cells, owing to their

powerful potential for proliferation and differentiation to vascular ECs (4). The origin of EPCs is presumed to be the same as that of hematopoietic stem cells (HSCs), the progenitor cells of which arise in the embryonic mesodermal blood island (5). Because of their special origin,

EPCs and HSCs share certain antigenic determinants, including CD133 (AC133), CD34, vascular endothelial growth factor receptor 2 (VEGFR2, also called Flk-1 and KDR in mice and humans, respectively), Tie-2, c-Kit, and Sca-1 (6). According to the previous study, CD133 is a highly conserved antigen expressed on some stem cells, and has been proved to be the main surface marker distinguishing EPCs from ECs (7-9). After the differentiation step (4), EPCs have the same markers as ECs, such as vascular endothelial cadherin, Tie-2, and VEGFR2, along with the EC functions of releasing nitric oxide (NO), forming capillaries, internalizing Ac-LDL, and binding lectins such as ulex europaeus-agglutinin-I (UEA-I). Thus, CD133+/VEGFR2+ cells with the same functions as ECs were most likely represent EPCs (10). Despite the uncertainty regarding their origin, phenotype, and therapeutic viability, great interest surrounds the harnessing of EPCs to promote vascular regeneration.

At the same time, there is great academic debate. Firstly, there is no standardization of cell surface markers or culture protocols for the production of EPCs for therapeutic intervention (11). Secondly, some experts have reported that EPCs originate from the bone marrow, mobilize in response to ischemia, and home in to sites of vascular injury. However, recent research discovered that endogenous neovascularization is driven by tissue-resident EPCs with no direct contribution from bone marrow cells (12,13).

The basis of the debate may be related to the different sources of stem cells used in interventions. Previous studies have shown that bone marrow EPCs are associated with more consistent benefits than circulating EPCs (12). However, the current methods of EPC culture mainly use umbilical peripheral venous blood, and many deficiencies exist. For example, the success rate with peripheral blood (PB) is low, the mortality rate is extremely high, and umbilical peripheral venous blood is difficult to obtain. Moreover, dead rats have traditionally served as the bone marrow source, which makes follow-up autotransplantation research challenging to complete. Currently, there is little direct evidence on whether EPCs can be induced and differentiated *in vitro* from living rat bone marrow.

In the present study, we used unmanipulated rat bone marrow mononuclear cells (BMMCs) as the starting material for inducing EPC culture *in vitro*. We identified EPCs by their functional and phenotypic characteristics, and explored the requirements for further study of autologous EPC transplantation for cerebral ischemia in rats.

We present the study in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/cdt-20-536>).

Methods

Animals

Twenty adult male Sprague Dawley (SD) rats, weighing between 250 and 280 g, were obtained from Wuhan University Experiment Animal Center and used in all experiments. All experimental procedures were approved by the Research Animal Resources and Care Committee of Hubei Province, China (No. 50,2005), in compliance with the Laboratory Animal regulation of China [2001] for the care and use of animals.

BMMC acquisition

Bone marrow was obtained autologously from the right tibia of each animal. First, the rats were anesthetized with 10% chloral hydrate intraperitoneal injection (0.035 mL/kg). Under aseptic conditions, the right tibia was fully exposed and prepared for drilling. Two holes (1 mm in diameter) were made in the tuberosity and lower one-third of the tibia, respectively. A PE-50 catheter was inserted through the hole in the tuberosity, and a syringe (5 mL) was inserted through the lower tibial hole to aspirate the bone marrow as follows. Phosphate Buffered Saline (PBS) was infused through the PE-50 catheter by one researcher, while a second researcher aspirated approximately 5 mL of bone marrow with the syringe. The incision was sutured and the rats were allowed to recover.

Mononuclear cells (MCS) were isolated from the bone marrow by density-gradient centrifugation (14,15) (Ficoll-Hypaque, LTS1083; TBD Science, Tianjin, China). The harvested cells were supplemented with M199 medium (Sigma-Aldrich, USA), containing 10 ng/mL VEGF (PeproTech Inc., Rocky Hill, CT, USA), 1 ng/mL basic fibroblast growth factor (b-FGF, PeproTech Inc.), 1 ng/mL epidermal growth factor (EGF, PeproTech Inc.), 20% fetal bovine serum (FBS, Invitrogen), heparin, penicillin, and streptomycin.

Induced culture of bone-marrow EPCs

Cell suspensions of each rat were plated on 24-well culture plates with culture wells pretreated with human fibroectin

(hFN, Chemicon International, Temecula, CA, USA). The suspended cells were plated at three different densities: 2×10^6 , 3×10^6 , and 4×10^6 cells/well; each density group comprised of 3 wells from 6 rats. The cells were incubated at 37 °C with 5% CO₂ and left undisturbed for 4 days (14,15). The medium was completely replaced every 3 days to remove nonadherent cells. The induced culture of adherent cells continued for 2 weeks. On days 7, 10, and 14 of culture, each well was observed under $\times 400$ magnification, 5 randomly chosen microscopic fields were photographed, and the number of adherent cells and CFUs in each high-power field were counted by photo software (HPIAS2000). The averages of five fields in each well were compared to select the optimal density.

After choosing the optimal density, another five rats were euthanized to harvest BMMCs, and the suspended cells were plated at the optimal density on a 24-well culture plate. Each well contained a previously placed coverslip that was coated with human fibrolectin (hFN). After 14 days of induced culture, the coverslips with adherent cells were used for the identification of EPCs.

Immunophenotyping

Immunocytochemistry was performed according to standard protocols (16) and the manufacturers' instructions. EPCs were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at 4 °C, and washed in PBS. The fixed EPCs were identified by their expression of CD133 and VEGFR2 using immunohistochemistry. We used primary rabbit polyclonal antibodies against rat CD133 (BioTech, Beijing, China) and rat VEGFR2 (Boster, Wuhan, China). In the preliminary step, endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at 37 °C, and the cells were rinsed in distilled water. Antigen was retrieved with 0.1% trypsin solution for 10 min, the cells were rinsed in PBS, 5% bovine serum albumin (Boster, Wuhan, China) was applied for blocking, and 15 min later the excess solution was tapped. The cells were incubated overnight with 1:10 ratio $\mu\text{g/mL}$ primary antibody in PBS at 4 °C, then rinsed in PBS. We then proceeded with the biotinylated second antibody (goat anti-rabbit IgG) and streptavidin biotin peroxidase complex (SABC) following the instructions of the rat SABC kit (SA1022, Boster). Following the manufacturer's directions 3,3'-diaminobenzidine (DAB) was used for color development (ZSGB-BIO, Beijing, China),

and the nuclei were counterstained with hematoxylin.

Internalization of Dil-Ac-LDL and binding of FITC-UEA-I

To assess Ac-LDL internalization and UEA-I binding, we used Dil-labeled Ac-LDL (Dil-Ac-LDL, Biomedical Technologies, Inc., Stoughton, MA, USA) and fluorescein isocyanate (FITC)-labeled UEA-I (FITC-UEA-I, Sigma-Aldrich), as previously described (17,18). The samples were observed using confocal laser scanning microscopy (Leica TCS SP2 AOBS MP, Germany). On day 14, live cells were incubated with 10 $\mu\text{g/mL}$ Dil-Ac-LDL in M199 media (without other additions) for 4 hours at 37 °C, then the medium containing Dil-Ac-LDL was removed and the cells were washed several times with PBS. Next, the cells were fixed with 4% PFA for 20 min at 4 °C, washed several times with PBS, and then incubated with 10 $\mu\text{g/mL}$ FITC-UEA-I in PBS for 1 hour at 37 °C. Finally, the cells were washed several times with PBS and examined using a confocal laser scanning microscopy system (LCS Lite). All steps of the procedure were performed in the dark.

Flow cytometry

Cells were cultured for 9 days, then digested and collected with 0.25% (mass concentration) pancreatin and washed twice with PBS. The cells were set up in single standard tubes No. 1, No. 2, and No. 3, to which was human CD34-PE, CD45-PE, and anti-CD133 was added, respectively, before incubation at 4 °C in the dark for 30 min. After twice washing with PBS, single standard tubes No. 1 and No. 2 were tested on the flow cytometer; single standard tube No. 3 had a second antibody added and FITC, and was incubated at 4 °C in the dark for 30 min. In the negative control group, PBS was used to replace the cells, while in the blank control group, PBS was used to replace the first antibody. The positive expression rates of CD34, CD13, CD45, and CD44 in EPCs were detected.

Statistical analysis

Data are presented as mean \pm standard deviation. Statistical evaluation of the data was by one-way analysis of variance (ANOVA) for intergroup comparison analysis, and bivariate correlation analysis for the relevant factors. All statistical

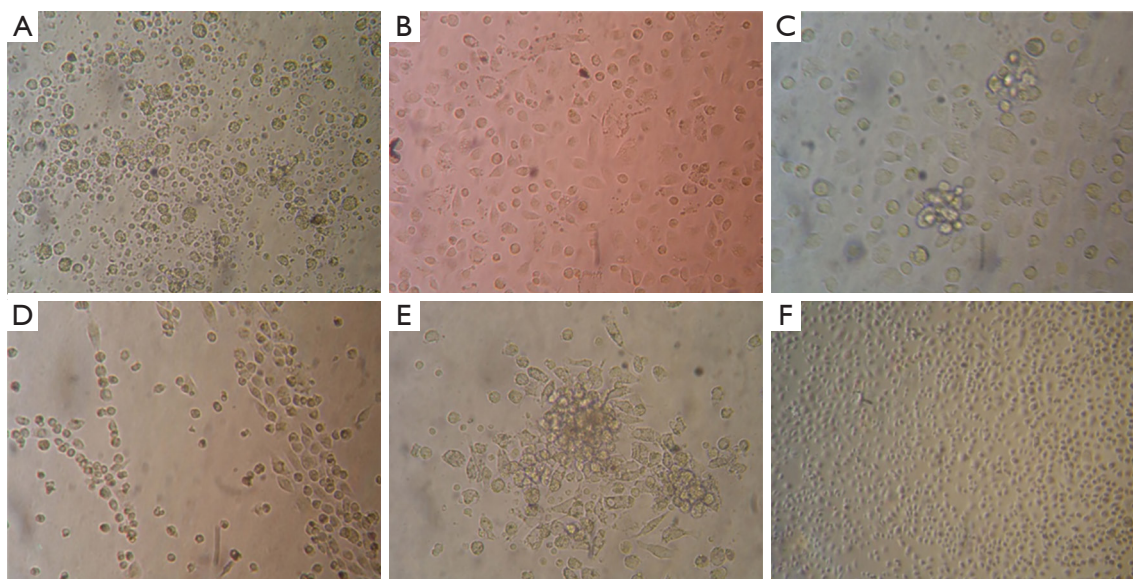


Figure 2 Morphological observation of cultured EPCs. Representative phase-contrast photomicrographs of EPCs. (A) On day 4 of culture, adherent cells have a round shape ($\times 400$). (B) On day 6 of culture, some adherent cells have extended into a spindle shape ($\times 400$). (C) On day 7 of culture, some adherent cells have proliferated into CFUs ($\times 500$). (D) On day 10 of culture, cells had heterogeneous shapes, and some of the spindle-shaped cells had linked together, forming a tube ($\times 400$). (E) On day 10 of culture, adherent cells had proliferated and differentiated, with some elongated cells sprouting from the central core of CFUs ($\times 400$). (F) On day 14 of culture, the CFUs had nearly disappeared, and the majority of the adherent cells had differentiated to spindle-shaped cells, with a “cobblestone” appearance ($\times 250$). CFU, colony-forming unit; EPC, endothelial progenitor cell.

3 testing time points, day 10 had the maximum number of CFUs, and by day 14, the CFUs in all density groups had disappeared (*Figure 3B*). These results indicated that 3×10^6 cells/well was the optimal plating density under our experimental conditions. Analysis of the correlation of adherent cells and CFUs of all samples on day 10 showed the two variables to have a satisfactory correlation ($r=0.829$, $P=0.000$, bivariate correlation analysis) (*Figure 3C*).

Immunocytochemical analysis of BMMNCs

After 12 days of primary culture, the MNCs had almost fused and were arranged in a typical cobblestone-like pattern. The number of cells could be expanded to approximately 106. The results of immunohistochemistry showed that CD34 ($86.36 \pm 5.12\%$), CD45 ($30.25 \pm 4.62\%$), and CD133 ($19.72 \pm 5.20\%$) stained positive, and the cytoplasm was brown (*Figure 4*). CD44 was negative, indicating that the cells detected were not bone marrow mesenchymal stem cells (BMMNCs), that CD44 had been labeled, and that cell purity was high.

Internalization of Dil-Ac-LDL and binding of FITC-UEA-I

On day 12 of induction culture *in vitro*, laser confocal microscopy showed that the cultured cells had the ability to phagocytize Dil-Ac-IDL and FITC-UEA-I: the cells combined with FITC-UEA-I showed green fluorescence, while the cells that had ingested Dil-Ac-Idl showed red fluorescence. Laser confocal image analysis was used for fluorescence superposition, and the results showed that the positive rate of double fluorescence was 65.01 ± 12.50 (*Figure 5*), indicating that cell activity was strong, which would meet the needs of future experiments.

Flow cytometric analysis of BMMNCs differentiating into EPCs

The presence of CD13 and CD45, and a subsequent loss of CD34, suggests that EPCs have differentiated into mature ECs. CD44 is a unique marker of BMMSCs, which are easily confused with EPCs. On day 12 of induction culture *in vitro*, the results of flow cytometry showed that the cells

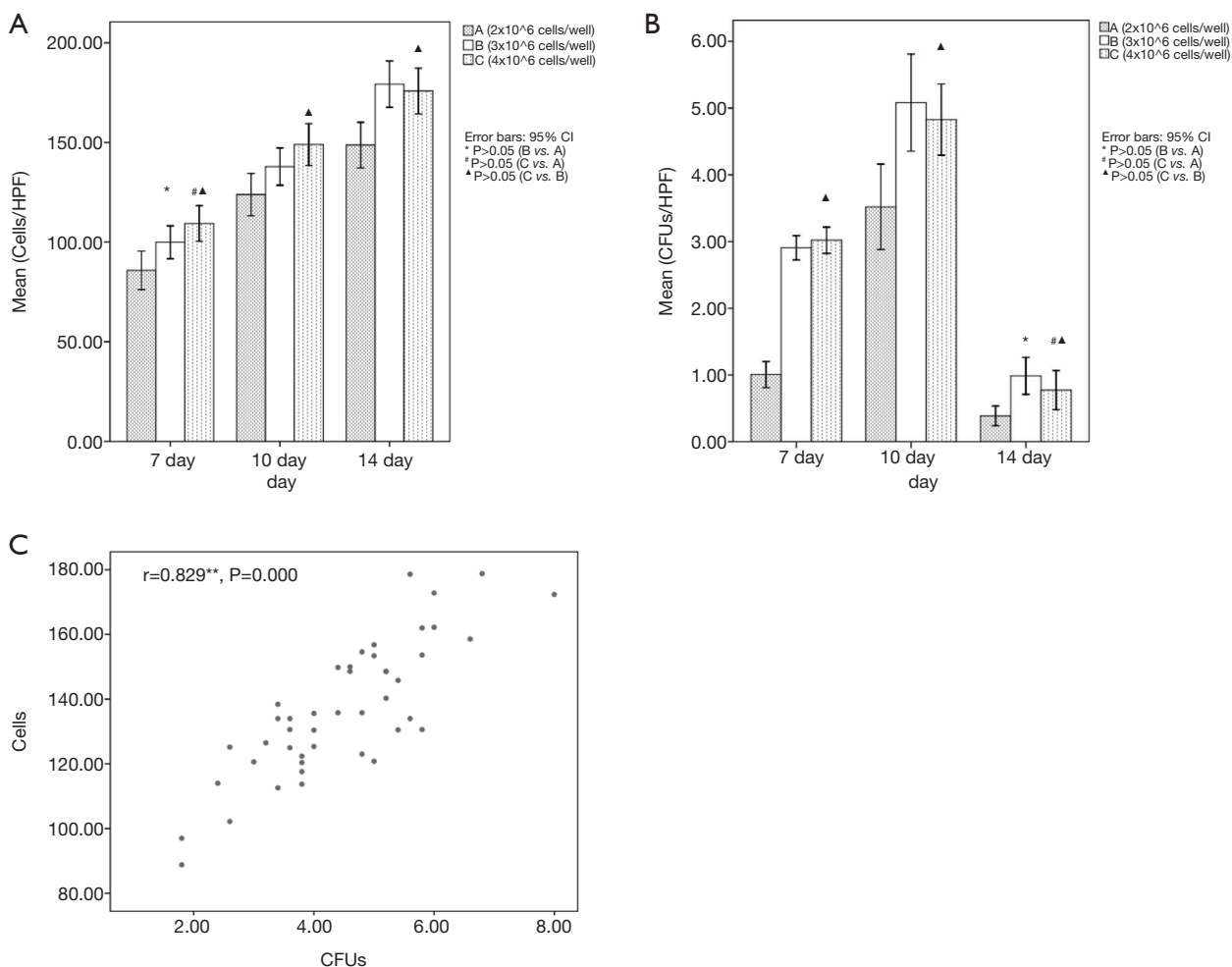


Figure 3 Adherent cells and CFU count at different plating densities. (A,B) The counts of adherent cells and CFUs in three groups at different plating densities. *, $P>0.05$ comparing group B with group A; #, $P>0.05$ comparing group C with group A; ▲, $P>0.05$ comparing group C with group B (one-way ANOVA). (C) Correlation of adherent cells and CFUs in total samples on day 10, $r=0.829$, $P=0.000$ (bivariate correlation analysis). CFU, colony-forming unit.

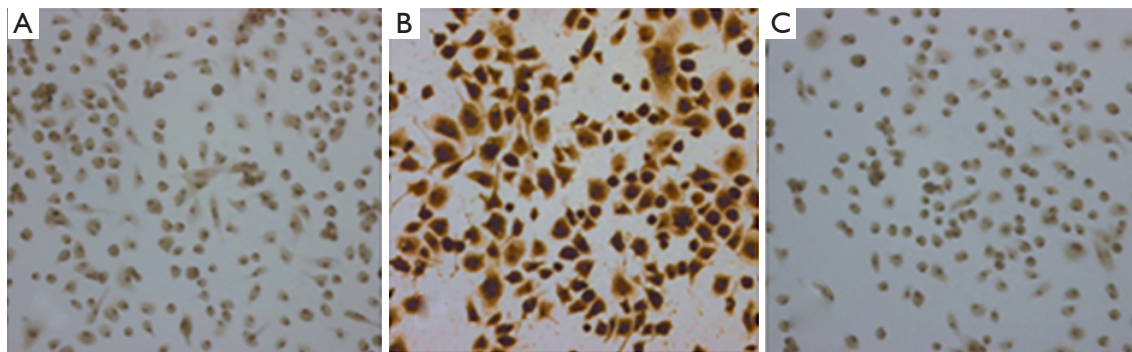


Figure 4 Immunocytochemical analysis of BMMNCs. After 14 days of culture, BMMNCs revealed expression of rat stem cell markers ($\times 400$). (A) CD34 is positive and the cytoplasm is brown (86.36 ± 5.12); (B) CD45 is positive (30.25 ± 4.62); (C) CD133 is positive (19.72 ± 5.20).

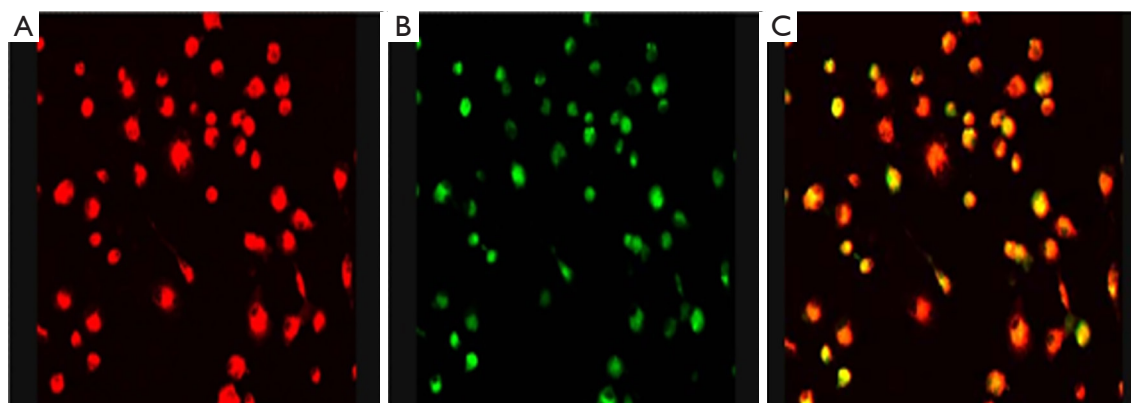


Figure 5 Internalization of Dil-Ac-LDL and binding of FITC-UEA-I. Imaging under $\times 400$ magnification. (A) Adherent cells have internalized Dil-Ac-LDL presenting red fluorescence ($\times 400$). (B) Adherent cells have bound FITC-UEA-I presenting green fluorescence ($\times 400$). (C) The majority of adherent cells are both Dil-Ac-LDL and lectin positive (yellow overlap, $\times 400$; $56.9\% \pm 4.2\%$, $n=8$ of 3 high-power fields per section). FITC, fluorescein isocyanate; UEA-I, ulex europaeus-agglutinin-I.

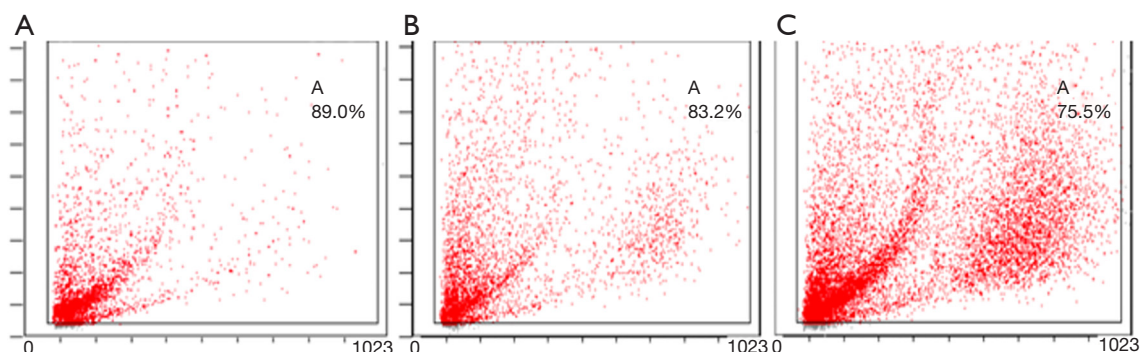


Figure 6 Flow cytometry analysis of BMMNCs differentiating into EPCs. After 12 days *in vitro*, the results of flow cytometry show that: (A) CD34 is positive (89.0%); (B) CD45 is positive (83.2%); (C) CD133 is positive (75.5%). BMMNCs, bone-marrow mononuclear cells; EPCs, endothelial progenitor cells.

were positive for CD34, CD45, and CD133, but negative for CD44. The results also discovered that the positive rate of double fluorescence in the cells were 89.0% for CD34, 83.2% for CD45 and 75.5% for CD133, respectively. *Figure 6* suggesting that cell activity was strong and could meet the needs of future experiments.

Discussion

EPCs can localize in the bone marrow and PB or can reside in the arterial wall (7,19,20). An earlier study showed that EPCs are a small subpopulation ($<0.05\%$) of the leukocyte fraction of bone marrow-derived mononuclear hematopoietic cells in adults humans (21). Experimental data support that EPCs can be induced and differentiated from rat bone marrow; therefore, we separated EPCs from

uncommitted MNCs utilizing the adherent properties of endothelial lineage cells. Being stimulated by some early-acting and endothelial lineage-restricted growth factors (i.e., VEGF, b-FGF, and EGF), the progenitor cells that were initially separated were induced to proliferate and trigger differentiation into EPCs.

EPCs are of great interest because of their potential in cell therapy for vascular diseases and ischemic conditions (22-24). Recent advances in stem cell technology have enabled researchers to separate EPCs from stem cells and other precursor populations. However, most of these studies mainly used PB EPCs (14). PB EPCs show high mortality and low successful culture rates, which has restricted research into autologous transplantation in rats. In the present study, we used rat bone marrow as the source of MNCs, and identified EPCs by their phenotypic

characteristics and function.

EPCs can be enriched by antigen-positive selection from among the MNCs of bone marrow or PB cells. CD133, VEGFR2, CD34 and other factors have been reported as antigens for EPC selection (7,19). We also explored EC functions such as releasing NO, forming capillaries, internalizing Ac-LDL, and binding lectins such as UEA-I (17,18). Thus, we determined that CD133+/VEGFR2+ cells with the same function as ECs most likely represent EPCs (10,17,18).

The number of positive cells, however, was very limited, and single-population culture is not always successful. For this reason, we used whole MNC culture to augment the differentiation and proliferation of EPCs under EC-specific culture conditions. As reported in previous studies, various differentiation techniques to induce EPC differentiation have been explored (1,18,19). Often, a combination of differentiation stimuli, including cytokines, extracellular matrix (ECM) coatings, seeding density, and mechanical stimulation improves the yield, quality, and differentiated state. Almost all studies have induced EPCs from adult stem or progenitor cells using VEGF alone or in combination with other growth factors, so VEGF is considered necessary. The effects of other growth factors in addition to VEGF have not been fully compared with those of VEGF alone (25-27). Different studies have used different VEGF concentrations, ranging from 5 to 100 ng/mL; therefore, the effect of a graded concentration has not been studied extensively. ECM is critical in all aspects of vascular biology, because it supports the adhesion, migration, proliferation, and survival of ECs, and gives cues for capillary morphogenesis and neovessel stability and maturation (28). Fibronectin (FN) is used in EPC-induced culture, because it has been proven to significantly enhance VEGF-induced differentiation of EPCs (29,30). As another culture parameter, the seeding density can influence the survival and proliferation of EPCs. A study of ESCs hypothesized that cell-cell contact and membrane-associated factors are important for the proliferation of stem cells, and that the appropriate microenvironment is essential (30). Our results suggest that VEGF used in combination with other growth factors (b-FGF and EGF) supports EPC growth and differentiation into ECs. From our observations, compared with culture conditions without growth factor, the combination of low-concentration growth factors can significantly enhance adherent cells' survival and differentiation. Pretreating culture wells with FN noticeably enhanced the adhesion and differentiation

of cells. Additionally, because the adherent cells were more likely to gather on the glass coverslips, we suggest that glass culture dishes might be most suitable.

We compared three different seeding densities of MNCs in this experiment, and discovered that 3×10^6 cells/well was the optimal plating density under our experimental conditions. EPCs cultured *in vitro* are adherent cells; their survival depends on the seeding density but they can be contact inhibited, which means a density that is too high or too low might decrease their survival and differentiation.

Because identification of EPCs remains controversial, the cells in different reports may not have the same phenotype. Each group has named the identified EPCs differently, according to their markers, adding to the confusion of EPC nomenclature. In our experiment, the adherent cells were positive for CD133 and VEGFR2, internalized Dil-Ac-LDL and bound FITC-UEA-I, which represented EC morphology and the ability to form capillaries, suggesting EPCs. Further research is needed to understand the mechanism of action of growth factors in EPC-induced culture.

Conclusions

This study provides strong evidence that EPCs can be induced from BMMCS using growth factor-supplemented medium under appropriate culture conditions. However, further study is needed to enable autologous bone marrow EPC transplantation in cerebral ischemic rats.

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Footnote

Reporting Checklist: The authors present the study in accordance with the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/cdt-20-536>

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Ethical Statement: The authors are accountable for all aspects of the work by ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were approved by the Research Animal Resources and Care Committee of Hubei Province, China (No. 50,2005), in compliance with the Laboratory Animal regulation of China [2001] for the care and use of animals.

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