Functional endothelial progenitor cells derived from adipose tissue show beneficial effect on cell therapy of traumatic brain injury

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\textbf{A B S T R A C T}

Endothelial progenitor cells (EPCs) are responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Adipose tissue (AT) is an abundant source of mesenchymal stem cells (MSCs), which have multipotent differentiation ability. We successfully derived EPCs from AT, which maintained a strong proliferative capacity and demonstrated the characteristic endothelial function of uptaking of acetylated low-density lipoprotein. They formed tube-like structures in vitro. Endothelial nitric oxide synthase (eNOS) gene expression in EPCs was similar to that in mature endothelial cells. Transplantation of EPCs derived from AT after the acute phase was applied in rats with traumatic brain injury (TBI). Transplanted EPCs participated in the neovascularization of injured brain. Improving functional recovery, reduction of deficiency volume of brain, host astrogliosis and inflammation were found. These results suggest that adult AT derived stem cells can be induced to functional EPCs and have beneficial effect on cell therapy.

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Endothelial progenitor cells (EPCs) have recently been demonstrated to be responsible for postnatal vasculogenesis in physiological and pathological neovascularization [1]. Although proliferation of the cerebral endothelial cells (ECs) ceases in the adult brain, both angiogenesis and vasculogenesis appear in the formation of new blood vessels following a stroke [20]. Circulating EPCs can be recruited and contributed to neovascularization after stroke, furthermore transplanted bone marrow (BM)-derived EPCs can also participate in cerebral neovascularization following focal cerebral ischemia [3,10]. EPCs are the main cells contributing to neovascularization and promoting angiogenesis by the secretion of growth factors. However, since the number of self-mobilized EPCs is small, ex vivo expansion of autologous EPCs in the treatment of adult injuries may prove to be advantageous.

Adult EPCs have often been isolated from adult peripheral blood [6] and BM [20]. However, a recent study demonstrated that peripheral blood mononuclear cells (MNCs) are not an ideal source of adult EPCs for cell therapy, because EPCs from peripheral MNCs retain its typical monocytic functions and show little proliferative capacity [19]. Adult BM is difficult to obtain clinically, moreover, the quantity of it is limited. In this study, we induced EPCs from adult AT, which, like BM, is derived from the embryonic mesenchyme and contains an easily isolated stroma, with an aim of identifying a better source of adult autologous cells for cell therapy.

All animal procedures were performed under protocols conforming to National Institutes of Health Guidelines. MSCs from AT were isolated from 2-month-old adult male Wistar rats weighing 220–280 g. Bilateral lumbar subcutaneous AT (white AT) was carefully dissected and minced using sterile eye scissors, then enzymatically dissociated with 0.15% (w/v) collagenase type I (Invitrogen). Cells were harvested from femurs and tibias of the same rats, then enzymatically dissociated with 0.15% (w/v) collagenase type I (Invitrogen) at 37 °C for 60 min. The suspension was neutralized with DMEM/F12 (Hyclone, Thermofisher Scientific, USA) containing 10% FBS (Hyclone, US) and then centrifuged at 1500 rpm for 10 min to separate the floating adipocytes from the stromal vascular fraction. The pellet of stromal cells was resuspended in DMEM/F12 containing 10% FBS. Cultures were kept in a 37 °C incubator with 5% CO\textsubscript{2}. When the cells became 80% confluent, they were passaged with 0.25% trypsin/EDTA (Invitrogen).

BM cells were harvested from femurs and tibias of the same animals used for isolation of MSCs from AT. Nucleated BM cells were isolated by Ficoll-Paque gradient. Cells were incubated at 37 °C in
5% CO2 with minimal disturbance. After 24 h, cells were washed with PBS to remove unattached cells.

EPCs from AT and BM were induced according to modifications of previously reported protocols [3]. Briefly, cells were cultured in a differentiation medium (DMEM low-glucose supplemented with 10^{-8} mol/l dexamethasone, 5% FBS, 20 ng/ml vascular endothelial growth factor (VEGF), 5 ng/ml basic fibroblast growth factor, 5 ng/ml insulin-like growth factor), at 37 °C in 5% CO2. We changed the medium to fresh differentiation medium every 3 days and passaged every 5–7 days. Seven days after initial differentiation, the cells developed a cobble-like morphology (Fig. 1A and B), called EPCs by many researchers [3,10]. No obvious differences were observed in the morphologies between EPCs derived from two different tissues.

To detect the proliferative abilities of EPCs, the two types of EPCs were plated at 1000 cells/cm² and harvested at 3, 7, 10, and 14 days respectively. The cells at each time point were counted using a hemocytometer. Cumulative population doublings were calculated using the formula:

\[ x = \frac{\log_{10}(N_H) - \log_{10}(N_1)}{\log_{10}(2)} \]

where \( N_1 \) is the inoculum cell number and \( N_H \) is the harvest cell number [7]. EPCs retained the relatively strong proliferative capacity demonstrated by the MSCs before induction. However, the proliferative capacity decreased as cultivation progressed; from the same initial cell densities, BM-EPCs and AT-EPCs at passage one increased almost fivefold when cultured for 3 days, while only

Fig. 1. The morphologies of endothelial progenitor cells derived from adipose tissue (A) and bone marrow (B) (magnifications ×200). Mean values of the cumulative population doubling times are also shown (C).
a twofold increase was observed between days 10 and 14. This could be related to the age of the EPCs. But also could be related to the phenomenon of contact inhibition when they reached 90% confluence; we noted that these cells always grew in monolayers and never overlapped, and we found out that the proliferation rate of EPCs passed every 5–7 days seemed more large than that of EPCs cultured continually. No significant difference was found between the proliferative capacities of the EPCs from the two different sources (Fig. 1C).

The development of surface markers on the cultured MSCs was evaluated by flow cytometry analysis at passage three, and EPCs from the two sources were cultured for 14 days. Briefly, the cells were detached as described above and washed with PBS. They were then incubated with primary monoclonal antibodies to CD133, vWF, Flk-1, CD34 (Santa Cruz, USA), CD45, CD90 (BioLegend, San Diego, CA), and CD44 (Chemicon, USA). Fluorescent-conjugated secondary antibodies (Invitrogen, Eugene) were used for quantitative fluorescence analysis. Although there is no consensus...
regarding a definitive antigenic phenotype for MSCs in culture, we found that MSCs from BM and AT were negative for CD34 and CD45, indicating a lack of cells of hematopoietic origin. Both types of MSCs were positive for the typical MSC marker proteins CD44 and CD90 (Fig. 2A). Our results were in agreement with the previous studies of MSC surface phenotypes [7,8,16,21]. The similarities in phenotypes between MSCs derived from AT and BM support the hypothesis that the former could differentiate into EPCs under appropriate conditions like the latter.

EPCs were further characterized by determination of surface protein expression from three donors at day 14. Each sample was analyzed at passage three. EPCs derived from the two sources displayed no expression of hematopoietic marker CD34. However, more than 70% of both types of EPCs expressed the stem cell marker CD133, and the EC marker VWF. About 90% of EPCs expressed the EC marker Flk-1 (Fig. 2B). The phenotypic characteristics of EPCs derived from both sources of tissues were similar. These results suggest that AT could be induced to differentiate into EPCs like MSCs from BM. The percentage of CD133+ cells increased by more than 70% after culture in differentiation medium for 7 days, suggesting that these cells could retain their immature properties at suitable condition.

Phagocytic function of EPCs was assayed as previous protocol [19]. Briefly, cells were cultured in medium supplemented with 10 μg/ml of Dil labeled acetylated LDL (Dil-Ac-LDL) for 4 h. The medium was washed off with PBS and replaced by medium with Indian ink (10 μl/ml), and then incubated for another 4 h. Hoechst 33342 counterstain of nuclear was used to quantify the cell number. The cells were observed under a phase-control fluorescence microscope. More than 95% of EPCs took up Dil-Ac-LDL, but no cells took up Indian ink (Fig. 3A and B), showing that these cells had the characteristics of EPCs, but not of monocyt/macrophages.

Growth factor reduced basement membrane matrix (BD MatrigelTM, MA, USA) was used to examine the ability of the cells to undergo angiogenesis in vitro. A mixture of 800 μl of DMEM/F12 medium and 200 μl of Matrigel solution was prepared and kept on ice to avoid solidification. Fifty microliters of the mixture was transferred into one well of a pre-cooled 96-well plate. The plate was pre-incubated at 37 °C for at least 1 h to allow the matrix solution to solidify. A total of 1 × 104 cells in 150 μl of differentiation medium were added to each well. The morphological characteristics of cells cultured for 48–72 h were assayed using a phase-control microscope. In vitro angiogenesis assays indicated that both BM-EPCs and AT-EPCs had the ability to undergo angiogenesis for relatively long periods (Fig. 3C and D).

The expression of eNOS in BM and AT derived EPCs cultured for 4, 7, 10, and 14 days was determined by quantitative real-time polymerase chain reaction (qRT-PCR), as previously reported [7,8]. Mature ECs derived from rat cardiac microvasculature were used as a positive control. The cells were rinsed twice in 1× PBS and total RNA was extracted using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. The CDNA was generated from 1 μg of total RNA and PCR was performed with 1 μl of CDNA using Taq DNA polymerase (Promega, Wisconsin, USA). PCR of 185 rRNA was used as an internal control for the amount of input RNA. Initial cDNA denaturation was performed at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 15 s and measuring the fluorescent dye Sybr green I, labeled of double-stranded DNA, at 72 °C of each cycle. Use of rat eNOS primers, 5′-ATC AGG AAC GCC ACC AGG AG-3′ upstream and 5′- AGA TGG TCA GGA ACC AGG TG-3′ downstream, generated a 167-bp product. QRT-PCR was carried out using the LightCycler System (Roche, Mannheim, Germany). Relative gene expression was analyzed using the 2−ΔΔC(T) method, as described by Schmittgen [13].

According to the 2−ΔΔC(T) method, relative gene expression of mature ECs is 20 = 1. At 4, 7, and 10 days, the relative eNOS gene expressions of BM-EPCs and AT-EPCs were similar to the levels of mature ECs, and there were no significant differences between the two types of cells. At 14 days, the eNOS gene expression level in EPCs from both sources increased to more than three times that of the eNOS gene expression level of mature ECs (Fig. 3F).

A controlled cortical impact model in rat was used to determine whether culture-expanded EPCs were contributed to neovascularization during traumatic injury healing [5]. Thirty adult female Wistar rats weighing 250–300 g, purchased from Shanghai Institutes for Biological Sciences were used. Six millimeter diameter craniotomies were performed over the left hemisphere, the center of the bone-hole was positioned 2 mm anterior and 2.5 mm lateral to the bregma. Injury was induced by impacting the left cortex with a footplate of a 4.5 mm diameter tip at force of 20 × 30 cm (force is expressed as weight × distance dropped) and 2 mm of compression. At 7 day post-injury, randomly selected animals received transplantation. Grafts were injected into the cavity caused by injury using a 10 μl Hamilton syringe. Twenty experimental animals (ten for each group, AT-EPCs transplantation group and BM-EPCs transplantation group) received a 10 μl 0.5% peptide hydrogel solution (BD Biosciences, Cambridge, MA) suspending with EPCs derived from AT or BM (1 × 106 cells/ml, labeled with Dil). The other 10 rats received the same volume of saline as the control. In all animals, limb use asymmetry test [2] was performed before TBI and at the 4 weeks after transplantation. And at the end of 4 weeks after transplantation, the animals were killed and perfused by transcandial injection of normal saline and subsequently with 4% paraformaldehyde/PBS. Rat brains were isolated and were embedded in paraffin. A series of 5-μm-thick sections with 100-μm interval were cut from a 5-mm thick block containing the injury site in the coronal plane and stained with hematoxylin and eosin (H&E). Volume of cerebral tissue deficiency was measured. Survival and distribution of transplanted EPCs were examined under fluorescence microscopy.

Examination of the behavior revealed significantly lower neurological severity scores in AT-EPCs and BM-EPCs transplanted groups 4 weeks after transplantation, in comparison with that in the saline group, and there was no significantly difference between AT-EPCs and BM-EPCs transplanted groups (Fig. 4I). Calculation of the total deficiency volume showed that the EPCs transplanted groups had a significantly smaller volume than the control group, and still no significantly difference was noted between AT-EPCs and BM-EPCs transplanted groups (Fig. 4I). The transplanted cells as dye labeled cells accumulated mainly in the injury site; however, some EPCs migrated into the boundary zone (Fig. 4B). Many of these migrated Dil-labeled EPCs were found to have been incorporated into varying calibers of capillaries (Fig. 4C and D). Analysis of the immunofluorescence demonstrated that astroglisis and inflammation reactions were reduced by EPCs transplantation, AT-EPCs seemed to inhibit astroglisis more effectively than BM-EPCs, but no significant difference between two groups was shown (Fig. 4K and L). In another test, EPCs were not observed to colocalize with GFAP, Neun, CD68, and NF200 (Fig. 4E–H).

The results of our study suggested that inducing EPCs from MSCs could have the advantages of a high yield rate, low cost, and simplicity of operation compared with the method of selection of cells using magnetic microbeads or flow cytometry. However, VEGF-2, CD34 or CD133 are usually used to isolate EPCs [6], but all these antigens are shared by hematopoietic stem cells. Moreover, CD34 expression is not required for normal vascular development, since CD34-deficient mice demonstrate no vascular abnormalities [4,15]. CD133 is regarded as an important and more specific marker for identifying immature cells, but several observations have demonstrated that CD133 expression is very low in MSCs derived from peripheral blood, BM, AT and cord blood [7,8,11,16]. Moreover, EPCs...
with no expression of CD34 and CD133 have been isolated from cord blood [9].

EPCs derived from BM expressing eNOS were demonstrated by several researches [9,17]. Moreover, Zhang et al. observed that the EPCs from human peripheral blood expressed a lower level of eNOS than mature ECs [19]. In our study, we found that within 10 days of culture, the relative level of eNOS gene expression in EPCs was similar to that in mature ECs, suggesting that expression of eNOS may be a functional characteristic of EPCs. However, interestingly, the eNOS gene expression level in the EPCs had increased more than three times as compared with that of the mature EC at 14 days of culture. We speculate that this sharp increase in eNOS expression may be due to a response to the long-term VEGF stimulation. However, it also might be the age (time in vitro) of the EPCs influence the eNOS expression. Further studies are needed to clarify this. Based on the results of the current study and several previous studies [12], the following three functional characteristics appear to be unique to EPCs in vitro: Uptake of Ac-LDL but not of Indian ink; forming tube-like structures in vitro; and eNOS expression.

In the present study, traumatic injury local administration of adult EPCs derived from AT and BM revealed that these cells participated in adult brain neovascularization, and promoted tissue reconstruction. Transplantation of EPCs resulted in improvement in behavioral performance and reduction of volume of injury cavity. These results are consistent with the previous studies showing beneficial effects of BM derived EPCs on cerebral ischemia [3]. The major mechanism of these benefits may be that these cells can participate in and promote neovascularization, providing a fitful environment for neurogenesis and reducing the apoptosis, just as the case that can enhance the neuroplastic effect and functional angiogenesis in the ischemic brain [14].

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