Differential characteristics and *in vitro* angiogenesis of bone marrow- and peripheral blood-derived endothelial progenitor cells: evidence from avian species

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Abstract

Objectives: This study was conducted to compare phenotypes and *in vitro* angiogenic capacity of putative endothelial progenitor cells (EPCs) derived from bone marrow (BM) and peripheral blood (PB), from an avian species.

Materials and methods: Mononuclear cells were isolated from chicken BM and PB (BMMNCs and PBMNCs) and cultured in EGM-2 medium. Cells at days 7–14 were used for the experiments. Expression of progenitor and endothelial markers, number of Dil-ac-LDL/lectin dual-positive cells and adipogenic and osteogenic differentiation were determined. Migration and *in vitro* angiogenic ability between BMMNC- and PBMNC-derived cells were compared.

Results: PBMNCs developed typical EPC appearance, with initial spindle shape followed by a cobblestone form, whereas BMMNC-derived cells retained their constitutive spindle-like morphology throughout the study. Cells derived from both sources expressed CD133, CD31 and VEGFR-2, although PBMNCs-derived cells had lower CD133 expression. Nevertheless, number of Dil-ac-LDL/lectin dual-positive cells did not differ between groups. Adipogenic and osteogenic lineages were verified in BMMNC- but not in PBMNC-derived cells. PBMNC-derived cells formed tubular networks on Matrigel. However, BMMNC-derived cells formed few tube-like structures, which were

not morphologically comparable to those developed by their counterparts.

Conclusion: Our results suggest that so called EPCs derived from BMMNCs are not ‘true’ EPCs, supporting previous findings on mammals that BM may not serve as an optimal isolation source of EPCs.

Introduction

Endothelial progenitor cells (EPCs) compose a cell population in the peripheral circulation capable of differentiation into endothelial cells. Accumulating evidence suggests that EPCs play a critical role in neoangiogenesis and in endothelial maintenance and repair (1–4), and EPC-based cell therapy is emerging as a promising novel therapeutic approach for various cardiovascular disorders (5).

It is most commonly accepted that EPCs in the circulation originate from the bone marrow (BM), and that vascular injury triggers mobilization of EPCs from it to the peripheral blood (PB) (6,7). Accordingly, both BM and PB are widely used for isolation and culture of EPCs. Although both BM- and PB-derived EPCs (BM-EPCs and PB-EPCs, respectively) have been shown to incorporate into the vasculature of experimentally induced wounds or ischaemic areas (8,9), opinion on therapeutic potential of BM-EPCs remains conflicting (7).

Angiogenic potential of putative EPCs isolated from BM and PB has been extensively investigated in various animal models. Although both BM- and PB-EPCs isolated from rats (10) or pigs (11,12) have shown ability to form capillary-like tubular structures, more recent investigation by Amini *et al.* (13) demonstrated that BM-EPCs from rabbit failed to display vasculogenic activity, whereas PB-EPCs exhibited high angiogenic potential. In addition, it was found that BM-EPCs from humans had poor angiogenic potential *in vitro* and did not form blood vessels *in vivo* (14). Thus, whether BM represents an opti-

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mal source for EPC isolation with respect to cell-based therapy and tissue engineering is debatable (13). There is need and scope for use of as many different species, including non-mammals, to better understand ideal sources for EPC isolation, as possible.

The present study was conducted to compare phenotypes and *in vitro* angiogenic capacity of EPCs derived from BM and PB from an avian species, fast-growing meat providing chickens. With regard to *in vivo* significance, we chose the chicken model for our study, as it is used to investigate cardiovascular pathologies such as atherosclerosis (15,16) and pulmonary arterial hypertension (17,18).

Materials and methods

Cell culture

Protocols described here were approved by the Zhejiang University Animal Care and Use Committee. EPCs were obtained from BM and PB as previously described (13). Briefly, BM cells were harvested by flushing femurs and tibias of 1-week-old chickens and PB was collected from jugular veins of 4- to 6-week-old ones. All animals used were totally free of any significant disease. Mononuclear cells (MNCs) from BM and PB (BMMNCs and PBMNCs, respectively) were obtained by gradient centrifugation using Ficoll medium, density of 1.078 g/ml (Tianjin Haoyang Biological Manufacture Co., Ltd, Tianjin, China), and washed twice in 0.1 M phosphate-buffered saline (PBS). Cells were counted and their viability was checked by trypan blue exclusion.

The MNC fraction was then cultured in endothelial cell growth medium (EGM)-2 (Lonza, Walkersvil, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and fibroblast growth factor-B (FGF-B), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), ascorbic acid and heparin, according to instruction provided by the medium manufacturers. Cells were then seeded on rat tail type 1 collagen-coated 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA) at 1.6×10^6 per well. Plates were incubated at chicken internal body temperature of 39 °C, in a humidified environment with 5% CO₂. Non-adherent cells were removed after 48 h culture. Medium was then refreshed every 2–3 days.

Cell proliferation assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, BMMNCs (5×10^5 /well) and

PBMNCs (5×10^5 /well) were seeded in 24-well plates and unattached cells were removed after 48 h plating. At the appropriate time point, 40 µl MTT (5 mg/ml) was added to each well and cells were incubated for a further 4 h. Supernatant was removed and 200 µl dimethyl sulphoxide (DMSO) was added into each well (to dissolve the blue formazan) for 15 min in the dark, followed by measurement of optical density (OD) at 570 nm. Cell densities achieved on indicated days were plotted as mean MTT OD value of triplicates.

Reverse transcription-polymerase chain reaction (RT)-PCR

Total RNA was prepared from cultured cells on days 7 and 14 by acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated by reverse transcription using cDNA synthesis kit (Fementas, Shenzhen, China), which was used as template in subsequent PCR analysis with the following primers: CD133: 5'-AGG AGC AAA GAA ACT GGT CAA C-3' (forward) and 5'-CCA AGC AAA GGT CCA ACA TTA T-3' (reverse); CD34: 5'-ACA TCT CAA CTG CTG TGT CCT TGG -3' (forward) and 5'-TGG CTG GTC TGA ATG CTG GTC T -3' (reverse); CD31: 5'-AAG GGG ATG ATA TTC TGC AAT TT-3' (forward) and 5'-TTG TCT GAT CTC GTA CAC TGC TC-3' (reverse); and VEGFR-2: 5'-GGT CGC ATG AAC ATG AAG AA-3' (forward) and 5'-TTG GTA GGG TTT GTA AGG AC-3' (reverse). Expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-TAA AGG CGA GAT GGT GAA AG-3'; reverse: 5'-ACG CTC CTG GAA GAT ATG GAT-3') was used as internal control for RNA loading.

PCR was performed in 20 µl consisting of 0.4 µl dNTP Mix (20 mM), 1.2 µl MgCl₂, 0.4 µl each primer, 1.0 µl first-strand template cDNA, 0.3 µl Ex-Taq (TaKaRa Biotechnology Co., Ltd, Dalian, China), 2 µl 10× PCR buffer and 14.3 µl ddH₂O. Cycling conditions were 35 cycles denaturation at 94 °C for 30 s, annealing temperature was adjusted according to the manufacturer's instructions and PCR product was extended at 72 °C for 2 min. PCR products were detected by 1.3% agarose gel electrophoresis and visualized using Gold-view staining.

Dil-ac-LDL and lectin staining

On day 7 after plating, cells were incubated in Dil-ac-LDL (20 µg/ml; Invitrogen) at 39 °C for 4 h, washed twice in PBS (pH 7.4) and fixed in 4% paraformaldehyde for 15 min. Then they were incubated in

FITC-UEA-1 lectin (10 µg/ml) for 1 h. Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) and images were acquired with fluorescence microscopy (Nikon Ti-FL; Nikon Cooperation, Tokyo, Japan). Cells stained positively by both DiI-ac-LDL and lectin were considered to be EPCs and were counted using Photoshop software (19). Resulting EPC count was expressed as percentage of total DiI-ac-LDL/lectin double-positive cells to DAPI-positive cells.

Adipogenic and osteogenic differentiation

Oil red O staining was performed to identify adipocytes, as previously described (20). Briefly, cells were fixed *in situ* in 10% paraformaldehyde for 45 min, washed in 60% isopropanol and stained in oil red O solution for 5 min, at room temperature. They were then rinsed in water until it became clear. Finally, pure water (500 µl) was added to each well. Osteogenic differentiation was determined by alizarin red S staining as previously described (14). Briefly, cells were fixed in 70% ice-cold ethanol for 1 h and stained with alizarin red S, 10 mg/ml in dark for 45 min. The stain was subsequently removed and 0.5 ml PBS was added to each well for 30 min. Cells were then washed in distilled water and allowed to dry.

Cell migration assay

In vitro migration was measured using the Transwell system (Corning Costar, Acton, MA, USA) with 6.5-mm diameter polycarbonate filters (8-µm pore size). Briefly, cell suspension (3×10^4 cells/well) in serum-free medium was placed in the upper chamber, and the lower chamber was filled with medium containing 5% FBS. The chamber was incubated for 24 h at 39 °C. Non-migrating cells were completely removed from the top surface of the membrane by washing. Cells that had migrated, adhering to the undersurface of filters were fixed in 4% paraformaldehyde and stained in Giemsa reagent before the filters were gently removed and mounted on glass slides. Migratory activity was evaluated by counting cells that had passed through, in 4 random low-power (original magnification $\times 100$) microscope fields (2.54 mm²/field). Mean count per field was used to calculate total number of migrated cells on the whole filter. All groups were studied in triplicate.

In vitro tube formation assay

BD Matrigel™ Basement Membrane Matrix (BD Biosciences) was thawed and placed in 96-well plates at room temperature for 30 min to solidify. Cells (1×10^4 /well) were seeded to tops of solidified gels

and incubated at 39 °C for 48 h. Tube formation was observed using a phase-contrast microscope. Tube formation was defined as composed of structures at least 6 cells in length.

Statistical analysis

Mean data are presented with standard deviation (SD); statistical comparisons between groups were performed using Student's *t*-test SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Values of *P* < 0.05 were considered significant.

Results

Differing morphology of cells derived from BMMNCs and PBMNCs

Under conditions favouring endothelial cell expansion, cells derived from BMMNCs proliferated more rapidly than their counterparts from PBMNCs. After 2–3 days culture, BMMNCs gave rise to colonies composed of round cells with radiating elongated spindle-like cells at peripheries. These reached confluence by 4–5 days culture, when they were further passaged. Cells continued to form colonies consisting of spindle-shaped cells after 3 weeks culture. PBMNCs began to modify their spherical to spindle shape 3–4 days post plating, then proliferated to form colonies and gradually displayed characteristic homogeneity and cobblestone-like morphology by around day 14 of culture (Fig. 1).

Proliferation of BMMNC- and PBMNC-derived cells

Next, we performed MTT assay to monitor proliferation capacity of each source of cells over 14 days culture. As shown in Fig. 1, numbers of PBMNCs progressively reduced before day 6 and in the order of 85% cells were lost at this time point relative to day 2. Remaining cells showed no signs of growth up to day 12 after plating. Similarly, BMMNCs reduced by nearly 30% between days 2 and 6. However, they displayed high proliferative ability in the next 2 days, followed by a slow expansion before day 14 (Fig. 2). It was interesting to note the significantly higher number of attached cells of BMMNC origin than those of PBMNC culture on day 2 (*P* < 0.01); these subsequently differentiated into more cells than their counterparts.

Both BMMNC- and PBMNC-derived cells expressed endothelial lineage cell markers

Gene expression profiles revealed that both BMMNC- and PBMNC-derived cells expressed CD133, a typical

Figure 1. Morphology of cells derived from (a) chicken bone marrow (BM) and (b) peripheral blood (PB). Mononuclear cells (MNCs) from BM and PB (BMMNCs and PBMNCs, respectively) were plated on 6-well tissue plates and cultured in EGM-2 medium. Unattached cells were removed after 48 h plating. Note that cells derived from PBMNCs were homogeneous with cobblestone-like morphology by around day 14, but BMMNC-derived cells retained a spindle-like shape even after 3 weeks culture.

Figure 2. Cell proliferation determined by MTT assay read at 570 nm. Note attached cells in 2-day old cultures subjected to a MTT assay. Results are given as mean $\pm$ SD of triplicates. BMMNCs, bone marrow mononuclear cells; PBMNCs, peripheral blood mononuclear cells. ** P < 0.01 and *** P < 0.001 versus PBMNCs.

marker of progenitor cells, and CD31 and VEGFR-2, each of which is a marker of endothelial lineage cells. However, mRNA level of CD133 in cells derived from PBMNCs was lower than that from BMMNC-derived cells (Fig. 3). CD34, another haematopoietic progenitor marker, was undetectable in cells raised from either source (images not shown).

Number of Dil-ac-LDL/lectin dual staining cells did not differ between BMMNC- and PBMNC- derived cells

As shown in Fig. 4, both populations derived from BMMNCs and PBMNCs displayed the capacity to take

Figure 3. Reverse transcriptase (RT)-PCR products of surface markers CD133, CD31 and VEGFR-2 of cells derived from (a) mononuclear cells (MNCs) of bone marrow (BMMNCs) and (b) peripheral blood (PBMNCs) on days 7 and 14 after plating. Fragments were fractionated on a 1.3% agarose gel and stained with Gold-view reagent. Images are representative of three different experiments.

up Dil-ac-LDL and bind lectin. Percentage of Dil-Ac-LDL/lectin dual-positive cells was not significantly different between cells raised from BMMNCs and PBMNCs (P > 0.05).

Differentiation of BMMNCs into adipocytes and osteoblasts

As shown in Fig. 5, BMMNCs cultured in endothelial cell-favouring medium differentiated into adipocytes, as shown by oil red O staining of lipid droplets contained. BMMNCs were positive for alizarin red S staining as well, indicating presence of a process of osteogenic differentiation. However, PBMNCs did not display adipocyte or osteoblast differentiation.

Figure 4. Uptake of DiI-ac-LDL (red fluorescence) and binding of lectin (green fluorescence) by cells derived from (a) the mononuclear cells (MNCs) of bone marrow (BMMNCs) and (b) peripheral blood (PBMNCs). Merged images in right panel. Nuclei visualized by DAPI staining. Images are representative of three different experiments. Cell counting was performed by dividing microscope fields in 108 subfields (c), and percentage of DiI-ac-LDL/lectin double-positive cells was calculated (d). Results are expressed as mean $\pm$ SD and are representative of five replicates. NS, no significant difference ($P > 0.05$).

BMMNC- and PBMNC-derived cells displayed similar capacity of migration

On days 7 and 14 of culture, cells derived from BMMNCs and PBMNCs were subjected to transwell cell migration assay. As shown in Fig. 6, both BMMNC- and PBMNC-derived cells had higher capacity for migration on day 14 than on day 7 ($P < 0.001$). Number of BMMNC-derived cells traversing the porous membrane did not differ from that of PBMNC-derived cells either on day 7 or day 14.

BMMNC-derived cells poor ability to form tube-like structure in vitro

On day 14, BMMNC- and PBMNC-derived cells were seeded on to Matrigel matrix and examined microscopically for tube formation. Within 24 h of incubation, PBMNC-derived cells gave rise to tightly adherent cords and capillary-like structures, assembled in branching

reticular networks (Fig. 7a). By contrast, cells raised from BMMNCs formed clusters or individual spindle-shaped and elongated cells after 24 h of plating. Very few cords and only incomplete and sparse tube-like structures were observed after 4 days culture; these were morphologically different from those formed by PBMNC-derived cells (Fig. 7b), indicating poor angiogenic potency.

Discussion

Separation and induction of EPCs can be achieved by culturing adherent MNCs (21). Here, MNCs freshly isolated from chicken BM and PB were seeded on collagen-coated plates and cultured under conditions favouring endothelial-specific differentiation. During culture, the PBMNCs differentiated into spindle-shaped cells succeeded by cobblestone-like cells, consistent with morphological signs of human PBMNC-derived early

Figure 5. Microscopic view of oil red O and alizarin red S stains. (a) Adipogenic cells identified by oil red O staining (lipid appeared red); (b) Cells negative for oil red O staining; (c) Osteogenic cells confirmed by alizarin red S staining (calcium deposits appear orange); (d) Cells are negative for alizarin red S staining. BMMNCs, bone marrow mononuclear cells; PBMNCs, peripheral blood mononuclear cells.

Figure 6. Transwell migration assay of cells derived from chicken bone marrow mononuclear cells (BMMNCs) and peripheral blood mononuclear cells (PBMNCs). Numbers of migrated cells counted from four, 100× microscope fields per filter and total number of migrated cells was calculated. Results shown as average number of migrated cells per filter $\pm$ SD from triplicate experiments. *** $P < 0.001$; NS, no significant difference ($P > 0.05$).

and late EPCs, respectively (22). Supporting previous findings (22), our results showed that early EPC-predominant cultures did not proliferate, whereas late EPC-enriched cultures did. Spindle-shaped and elongated cells were also obtained by culturing our chicken BMMNCs, which had similar appearance to EPCs isolated from rat and rabbit (23). However, in contrast to PBMNCs, BMMNCs did not differentiate into cobblestone-like cells during this experiment. Nevertheless, spindle-shaped cells raised from this source displayed

high proliferative capacity. In this context, our results suggest distinct differentiation and proliferative types of behaviour between PBMNC- and BMMNC-derived cells.

In mammals, BM- and PB-derived EPCs share some markers such as CD133, CD34, VEGFR-2 and CD31, while EPCs isolated from PB have reduced expression of CD133 (24). Due to lack of availability of commercial chicken-specific antibodies against these markers during this study, we did not immunocytochemically determine numbers of cells positive for any EPC markers. However, our PCR results showed that both BMMNC- and PBMNC-derived cells expressed CD133, VEGFR-2 and CD31 regardless of culture stage, and confirmed reduction in CD133 mRNA level in PBMNC-derived cells compared to those derived from BMMNCs. Interestingly, in the present study, *CD34* gene could not be detected in either population even with the sensitive RT-PCR technique, suggesting deficiency of 'classic' 133+/CD34+ EPCs in the cultures. Nevertheless, results here do not allow us to argue that cells we obtained were incapable of differentiating into 133+/CD34+ EPCs, as 133+/CD34- subpopulations in MNCs have been found to be possible precursors of 133+/CD34+ EPCs (25).

Due to a lack of definitive surface markers for EPCs, numerous studies have defined them as DiI-ac-LDL/lectin double-positive cells (26–28). By judging EPCs in this way, we found that numbers of EPCs in BMMNC-derived cells did not differ significantly from those in PBMNC-derived cells. It should be noted though, that

Figure 7. Tube formation of the cells derived from (a) peripheral blood mononuclear cells (PBMNCs) and (b) bone marrow mononuclear cells (BMMNCs) on a Matrigel matrix surface. Images representative of three different experiments.

the feature of DiI-Ac-LDL and lectin uptake is not necessarily unique for EPCs (29). Not in accordance with the data of EPC count, we found BMMNC-derived cells had limited *in vitro* angiogenic potential compared to those raised from PBMNCs. Supporting our findings, it has been reported that BM-EPCs isolated from rabbit (13), goat (30) and human (14) also have failed to display vasculogenic activity.

More recent evidence has shown that culture of BMMNCs in EGM-2 medium gave rise to bone and adipose tissues rather than EPCs (14). In the present study, cells derived from chicken BMMNCs also readily differentiated into adipogenic and osteogenic lineages. These results, along with our findings that BMMNC-derived cells had limited potential of tube-formation and that their tube-like structures were not morphologically similar to those formed by PB-EPCs, suggest that cells derived from BMMNCs were not 'true' EPCs. In addition, lack of typical endothelial appearance might serve as alternative evidence that the BMMNC-derived cells were not 'true' EPCs. It is known that both EPCs and mesenchymal stem cells have high capacity for migration (31), which might explain why there was no significant difference in migratory activity between cells derived from BMMNCs and PBMNCs. Taken together, our results support previous findings that BM may not serve as an optimal isolation source of EPCs (13).

Mechanisms underlying distinct differentiation potential of BMMNCs and PBMNCs into mesenchymal cells under endothelial growth-promoting conditions remain unknown, but which might be associated with different components of the MNC fraction between BM and PB. It is known that MNCs in BM might have a large number of cells readily available to differentiate into mesenchymal stem cells (32,33), whereas those in PB have limited differentiation potential to mesenchymal stem cells (34). In particular, the CD133⁺ fraction of MNCs from BM and mobilized PB (not normal circulation) can readily differentiate into various mesenchymal cells such as adipocytes, osteocytes, chondrocytes and neuronal/glial cells, under appropriate conditions (35,36). Although induction media used for isolation of

EPCs and mesenchymal stem cells are basically different, they share some cytokines such as bFGF and IGF-1. In this context, it is not surprising to find that BMMNCs in EGM-2 medium can differentiate into mesenchymal cells, whereas PBMNCs cannot.

Nevertheless, our results do not support the claim of one previous study that EPCs do not arise from BM (14). In contrast to findings that cells derived from human BMMNCs in EGM-2 medium were devoid of endothelial markers (14), we observed expression of CD31 as well as VEGFR-2 in our chicken BMMNC-derived cells. In many studies, however, cells derived from BMMNCs in EGM-2 demonstrated not only endothelial phenotypes but also highly angiogenic potential (37,38). Taken together, discrepancy between different groups probably reflects an uncertainty, but not improbability, EPC differentiation of BMMNCs in EGM-2. Effects of various growth factors in EGM-2 medium might partly contribute to this uncertainty. It is also notable that PB contamination often occurs in BM aspirates, which might, to some extent, account for presence of EPCs in BMMNC cultures.

The main limitation of the piece of research described above is lack of investigation related to *in vivo* angiogenic activity of PBMNC- and BMMNC-derived cells. Although formation of a tubular network on Matrigel currently represents a defining feature of endothelial lineage, caution is needed to directly correlate such a morphological assay with endothelial functionality *in vivo*. Indeed, BM-derived mesenchymal stem cells have been found to differentiate into endothelial cells that directly contribute to vascular repair (39), in addition to stimulating *in vivo* angiogenesis by acting through paracrine mechanisms (40). Thus, the present study can not rule out *in vivo* vasculogenic capacity of cells raised from BMMNCs in EGM-2 medium.

In summary, the present study has shown that culturing PBMNCs under endothelial growth-promoting condition gave rise to an EPC population with the ability to form tube-like networks on Matrigel. Under identical culture conditions, BMMNCs-derived cells expressed endothelial markers, but failed to develop typ-

ical EPC appearance and showed only limited *in vitro* vasculogenic activity. In addition, they displayed characteristics of mesenchymal cells during differentiation in EGM-2 medium. Our results from this avian species has thus confirmed previous study on mammals that BM may not serve as an optimal source of EPC isolation (13).

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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