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Short Communication

Isolation and characterization of peripheral blood-derived endothelial progenitor cells from broiler chickens

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ABSTRACT

Peripheral blood-derived endothelial progenitor cells (EPCs) have been extensively studied in mammals but the isolation and characterization of EPCs in avian species have not been reported. In this study, chicken peripheral blood mononuclear cells (PBMNCs) were cultured under conditions favoring endothelial-specific differentiation for 2 weeks. One heterogeneous cell population dominated by spindle-shaped cells (early EPCs) and one homogeneous cell population exhibiting cobblestone-like morphology (endothelial outgrowth cells, EOCs) appeared sequentially.

Quantitative polymerase chain reaction (PCR) showed the expression of several progenitor and endothelial cell markers such as CD133, VEGFR-2 and CD31 in both cell populations. However, CD34, another progenitor marker, was undetectable in either freshly isolated PBMNCs or cultured cells. The endothelial phenotype of the EOCs was further identified by acetylated low-density lipoprotein/lectin double staining, and in vitro tube formation. Collectively, these data demonstrate that chicken EPCs can be isolated and cultured from PBMNCs and suggest that EPCs obtained from peripheral blood may originate mainly from the CD34– subpopulation.

Endothelial progenitor cells (EPCs) are bone marrow-derived immature cells that are able to differentiate into endothelial cells and are involved in endothelial homeostasis as well as physiological and pathological angiogeneses (Asahara et al., 1999). So far, two morphologically distinct EPC subpopulations, namely early EPCs (eEPCs) and endothelial outgrowth cells (EOCs, also called late-outgrowth EPCs or endothelial colony-forming cells), have been identified in mammals. eEPCs are spindle-shaped cells that lack tube-forming capacity unlike EOCs that have cobblestone-like morphology, display tubulogenic potential in vitro and can directly incorporate into the resident vasculature in vivo (Medina et al., 2010).

The characteristics of EPCs in avian species remain unknown. In the present study, EPCs from broiler chickens were isolated and cultured from peripheral blood mononuclear cells (PBMNCs) and were confirmed by morphological and genotypic analyses. We also assessed their potential for angiogenesis in vitro.

PBMNCs from clinically healthy 4- to 6-week-old Arbor Acres broilers were prepared by density-gradient centrifuging as previously described (Mukai et al., 2008) and resuspended in EBM-2 medium supplemented with growth factors as provided in the EGM-2 BulletKit (Lonza), 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded onto 6-well tissue plates coated with rat tail collagen (5 μg/cm^2, Sigma-Aldrich) at 1×10^7 cells/well, and incubated at 39 °C in 5% CO_2. Non-adherent cells were removed after 48 h of plating and the medium was refreshed every 2 days thereafter. Cell morphology was monitored daily using a phase contrast microscope.

Total RNA was prepared from PBMNCs on day 0 (before plating) and days 2 (immediately after the non-adherent cells were removed), 7 and 14 of plating using TRIzol reagent (Invitrogen Life Technologies). The cDNA was generated with 2 μg RNA (Fermentas). Conventional polymerase chain reaction (PCR) was performed using TaKaRa PCR Amplification Kit (TaKaRa) in a 20 μL volume containing 1 μL of cDNA and 0.5 μmol/L of each primer designed for the particular genes of interest (Table 1). PCR was performed for 40 cycles

Table 1
Sequences of the primers used in the conventional PCR and SYBR-green-based quantitative PCR (qPCR).

Tested genes	Primer sequence (5′→3′)
CD133	CTGCCAACCAACACTTAACTAGCCA (sense)
	TTCTCTGATTGCTCCTGCCATTGTC (antisense)
CD34	AGCCACTCGCATCCAGAGAACA (sense)
	TCCGCTGTTGCTCACGTCTTTG (antisense)
VEGFR-2	AAGTTGAGCTGGCGGTAGGAGAA (sense)
	TCATGTTCATGCGACCACTGGATG (antisense)
CD31	GAAGAAAGAGATGAGCAGGCAGGAC (sense)
	TGTAGCAAGGCAGATCGAGACA (antisense)
GADPH	TAAAGGCGAGATGGTGAAAG (sense)
	ACGCTCCTGGAAGATATGGAT (antisense)

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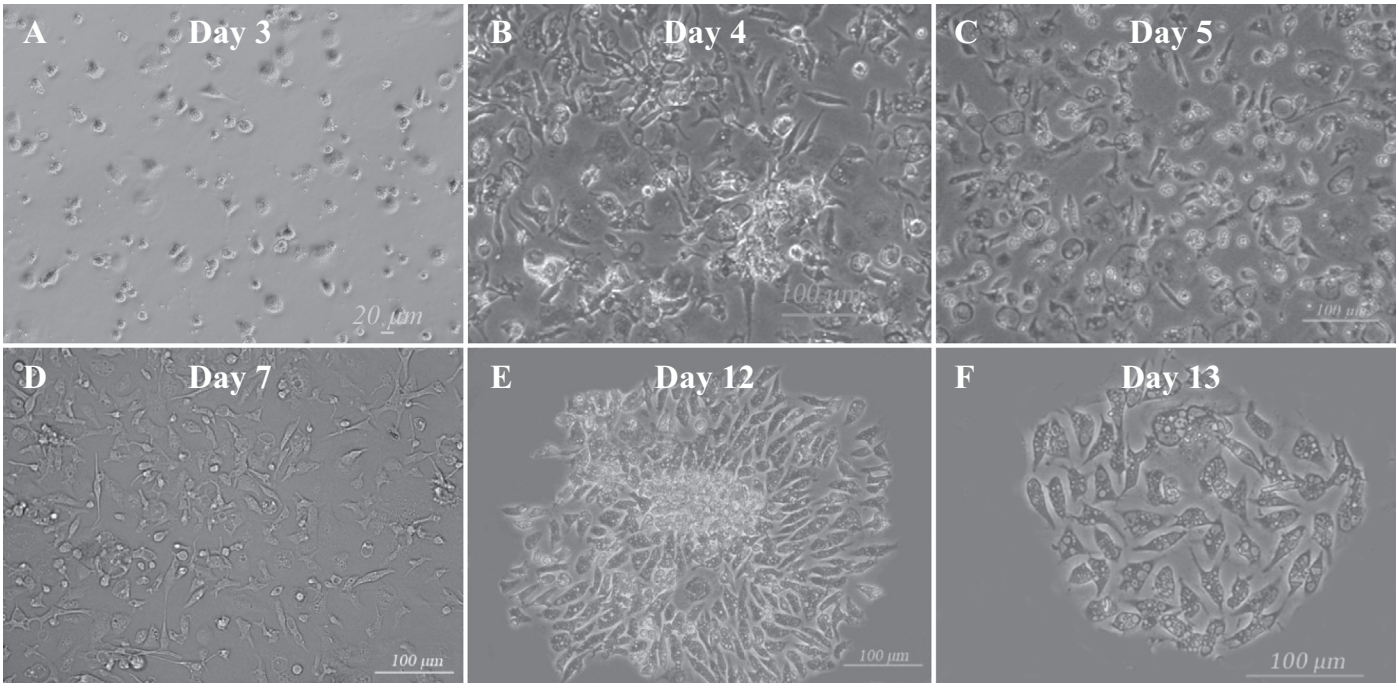

Fig. 1. Endothelial progenitor cells (EPCs) derived from chicken peripheral blood mononuclear cells (PBMNCs). PBMNCs were plated on collagen-coated 6-well plates and cultured in EGM-2 medium. Unattached cells were removed after 48 h of plating. (A) On day 3 of plating, attached cells displayed a small, rounded morphology; (B) small clusters with a core of rounded cells and radiating flat cells appeared on day 4 of plating; (C and D) cells differentiated into spindle-like morphology during days 5–7 of culture; (E) cell colonies consisting of a central cluster of rounded cells surrounded by multiple spindle cells appeared on day 12 of plating; (F) cells differentiated into characteristic homogeneity and cobblestone-like morphology on day 13.

(30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C). SYBR-green based quantitative PCR (qPCR) reactions were performed in a 20 μL volume with 2 μL of 1:20 diluted cDNA template, 0.4 μmol/L each primer, and 10 μL of SYBR Green Master Mix (Roche Diagnosis). The real-time qPCR reaction was run on the ABI PRISM 7300 System (Applied Biosystems) using standard conditions (2 min at 95 °C, 40 cycles of 15 s at 95 °C and 45 s at 60 °C). Relative quantification of the mRNA levels of the target genes was determined using the comparative CT (threshold cycle values) method ($2^{-\triangle CT}$ method). The results are presented as fold change to GAPDH gene expression.

As an alternative approach, cell phenotype was characterized by 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-ac-LDL, Kalen) uptake and fluorescein isothiocyanate-conjugated (FITC) *Ulex europaeus* lectin (FITC-UEA-l, Sigma-Aldrich) binding (Rehman et al., 2003). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology). Cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. On day 14 of plating, cells were submitted to an in vitro angiogenesis assay on BD Matrigel Basement Membrane Matrix (BD Biosciences) (Li et al., 2008).

Statistical analysis was performed using SPSS 16.0 for Windows. Data were presented as means ± standard deviation (SD) and were analyzed using independent-sample *t* test procedure. $P < 0.05$ was considered statistically significant.

Consistent with the time-dependent appearance of two morphologically distinct EPC subtypes derived from human PBMNCs (Hur et al., 2004), culture of chicken PBMNCS gave rise sequentially to spindle-shaped eEPCs and cobblestone-shaped EOCs by around days 6 and 10 of plating, respectively (Fig. 1). In addition to their

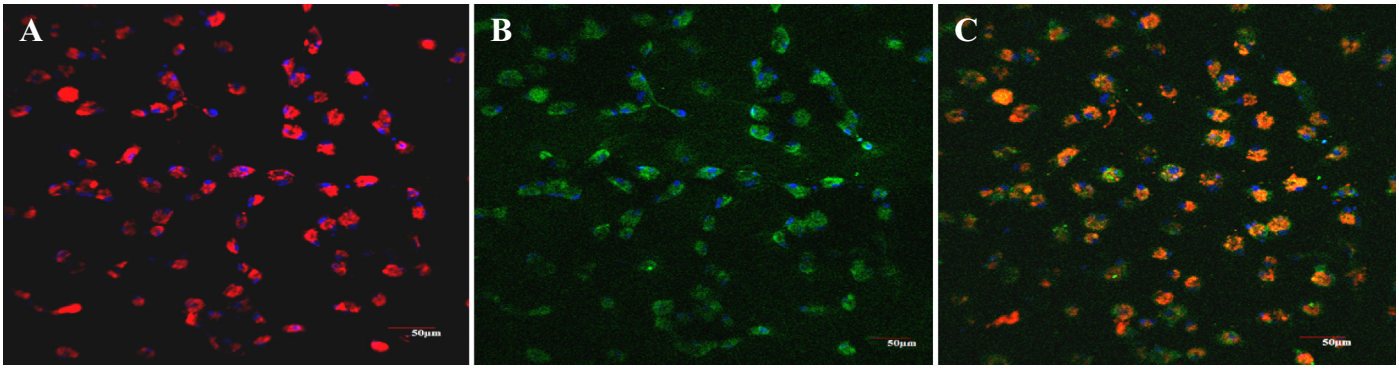

Fig. 2. Dil-Ac-LDL uptake and FITC-UEA-1 lectin binding. (A) Uptake of Dil-Ac-LDL (red fluorescence); (B) binding of FITC-UEA-l lectin (green fluorescence); (C) double-positive cells for Dil-ac-LDL incorporation and FITC-UEA-1 lectin binding. Blue labeling represents nuclear staining by DAPI. Photographs were taken under a confocal microscopy (Leica).