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Co-culture with endothelial progenitor cells promotes survival, migration, and differentiation of osteoclast precursors

Hao Pang [1], Xue-Hui Wu [1], Sheng-Long Fu, Fei Luo, Ze-Hua Zhang, Tian-Yong Hou, Zhi-Qiang Li, Zheng-Qi Chang, Bo Yu, Jian-Zhong Xu *

Department of Orthopaedics, Southwest Hospital, The Third Military Medical University, Chongqing, China

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ABSTRACT

In this study, we report the effect of endothelial progenitor cells (EPCs) on the biological behavior of osteoclast precursors in vitro by establishing an indirect co-culture system of mice EPCs and RAW 264.7 monocyte cells. Results show that the survival, migration, and differentiation of osteoclast precursors were greatly enhanced when co-cultured with EPCs. These phenotypic changes coincide with the upregulation of multiple genes affected cell behavior, including phospho-VEGFR-2, CXCR4, phospho-Smad2/3, phospho-Akt, phospho-ERK1, and phospho-p38 MAPK. The results collectively suggest that EPCs could modulate the survival, migration, and differentiation potential of osteoclast precursors, thus providing new insights in understanding of correlation between angiogenesis and bone homeostasis.

1. Introduction

Bone homeostasis is maintained by a dynamic balance between bone-resorbing osteoclasts and bone-forming osteoblasts [1,2]. The osteoclast is a bone-degrading macrophage polykaryon generated from the cell–cell fusion of monocyte/macrophage precursors at or near the bone surface [3]. Old or necrotic bones must be removed by osteoclasts to provide space for osteogenic precursors before new bone formation. Osteoclast-based bone resorption precedes and leads to growth of the neovasculature [4]. Therefore, osteoclast activity is critical in regulating bone homeostasis.

Endothelial progenitor cells (EPCs) are a specialized source of pluripotent progenitors that are capable of inducing vasculogenesis and angiogenesis [5]. Vasculogenesis or angiogenesis is an important fundamental process during skeletal development, repair, and regeneration because the bone is a highly vascularized tissue [6]. Recent studies have shown that EPCs crosstalk with osteoblast precursors and tightly couple with osteoblastic bone formation [7,8]. Whether or not EPCs can also affect the function and activity of osteoclast precursors has yet to be examined so this topic is the focus of our present study.

2. Materials and methods

2.1. Isolation and culture of EPCs

All animal procedures were approved by the Institutional Animal Care and Use Committee of The Third Military Medical University. Murine bone marrow cells harvested from the tibias and femurs of C57BL/6 mice (8 wk to 12 wk old) were subjected to density gradient centrifugation in Percoll (density = 1.077 g/mL; Sigma, USA) at 1500 rpm for 20 min at room temperature. The mononuclear cells were isolated from the buffy coat between the Percoll solution and the blood plasma and cultured in endothelial cell growth medium-2 (EGM-2, Cambrex, USA) with Single Quots growth supplements (Cambrex, USA) on fibronectin-coated dishes. Cells were maintained in a humidified atmosphere at 37 °C, incubated with 5% CO_2, and digested with 0.25% trypsin plus 0.01% ethylenediaminetetraacetic acid for subculture until they reached about 80% to 90% confluence.

EPCs were characterized by immunohistochemical staining for vascular endothelial growth factor receptor 2 (VEGFR-2) (Abcam, CA, UK) and von Willebrand Factor (vWF) (Santa Cruz, USA). The cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton X-100 for 5 min. After blocking with 5% goat

Abbreviations: VEGFR-2, vascular endothelial growth factor receptor-2; CXCR4, C-X-C chemokine receptor type 4; TGF-β1, transforming growth factor beta 1; Smad2/3, mothers against decapentaplegic homolog 2/3; p38MAPK, p38 mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinases1/2.

* Corresponding author. Fax: +86 023 65340297.
E-mail address: xujianzhong1962@163.com (J.-Z. Xu).

[1] These authors contributed equally to this work.

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serum at room temperature for 30 min, cells were stained with specific primary antibodies at 4 °C overnight. After rinsing with PBS three times and incubating with secondary antibodies for 2 h at 37 °C, EPCs were mounted with Vectashield mounting medium (Vector Laboratories) before observation.

Bone marrow-derived EPCs were also identified by the uptake of DiI-labeled acetylated low-density lipoprotein (DiI-ac-LDL; Molecular Probes, USA) and fluorescein isothiocyanate (FITC)-labeled Ulex Europaeus Agglutinin 1 (FITC-UEA-1; Vector, USA). Bone marrow-derived EPCs were treated with 10 mg/L Dil-ac-LDL for 4 h at 37 °C according to the manufacturer's instructions. After subsequent washing with PBS, the cells were fixed with 2% paraformaldehyde for 10 min at 4 °C. Following 1 h incubation with 10 mg/L FITC-UEA-1 at room temperature and subsequent PBS washing, cells were observed under a fluorescence microscope (Leica, Germany).

The angiogenic capacity of the early EPCs was determined by Matrigel tube-like formation assay. Matrigel (Sigma) was diluted 1:1 in 500 μL EGM-2 media in 96-well plates and polymerized by incubating at 37 °C for 1 h. EPCs (2×10^4 cells/well) were seeded onto the Matrigel and incubated in a humidified atmosphere at 37 °C with 5% CO_2. After 24 h, 3 d, and 7 d representative digital micrographs were taken.

2.2. RAW 264.7 cell culture

RAW 264.7 monocyte/macrophage cells (American Type Culture Collection, USA) were used as a cell model for osteoclast precursors and cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The RAW 264.7 cells were kept at 37 °C in a 5% CO_2 incubator, and the medium was changed every 2 d.

2.3. EPC and RAW 264.7 cell co-culture

The co-culture system was established by inserting a transwell chamber (Millipore, Bedford, MA, USA) with 3 μm pores into 12-well plates. RAW 264.7 cells were seeded on the plates (Costar, NY, USA) at a density of 1×10^3 cells/well. The same amount of EPCs was seeded on the transwell chamber. The chamber was inserted into the wells of the plate to allow crosstalk between the two cell types. DMEM (1 mL) was added to cover both upper-layer EPCs and lower-layer RAW 264.7 cells. Half of the culture medium was changed every 2 d.

2.4. RAW 264.7 viability assay

The survival of RAW 264.7 cells was analyzed using a cell counting kit-8 (CCK-8, Beyotime, China) on days 1 through 7 and measured by microplate reader scanning at 450 nm. The attached RAW 264.7 cells co-cultured with EPCs were washed twice with PBS, carefully removed from the 12-well plates using 0.25% trypsin, and then collected by centrifugation at 1000 rpm for 5 min. For the cell survival assays, RAW 264.7 cells were cultured in 24-well plates at 2×10^3 cells per well for 24 h, treated with 10 μL of CCK-8 in every well, and incubated for 1 h at 37 °C. Measurement of the cell absorbance was performed at 450 nm.

2.5. Transwell migration assay

The migration of RAW 264.7 cells was measured using transwell inserts with a pore size of 8 μm (Costar, Corning, NY). RAW 264.7 cells were placed in the upper chamber and EPCs were seeded on the lower chamber. The cells were incubated at 37 °C and 5% CO_2 for 5 d, rinsed with PBS, and then fixed with 10% formalin for 10 min. Migrated cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000) for 15 min before fluorescent images of the lower surface of the transwell membrane were captured using a microscope (Leica, Germany) at 200 × magnification. The number of cells in three 2.4 mm^2 images per membrane was respectively quantitated by counting cells with blue-stained nuclei to calculate the number of cells per mm^2, as previously described by Gordillo et al.[9].

2.6. Osteoclastic differentiation

To confirm the effect of EPCs on the osteoclastogenesis of osteoclast precursors, the number of multinucleated osteoclast-like cells in the co-culture group was compared with those in a negative control group that had RAW 264.7 cells cultured in DMEM medium with 10% fetal bovine serum (FBS) and those in a positive control group that had RAW 264.7 cells cultured with 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) and 25 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, NJ, USA). The multinucleated osteoclast-like cells were detected by the enzyme tartrate-resistant acid phosphatase (TRAP) and identified as TRAP-positive multinucleated ($\geqslant$3 nuclei) cells. The number of multinucleated osteoclast-like cells formed in each well was quantified after a 7 d culture and presented as the mean of six wells to evaluate the osteoclast-formation activity.

2.7. Western blot

For analysis of the changes in protein abundance, protein was extracted from RAW 264.7 cells with or without EPC co-culture for Western blot. Primary antibodies for Western blot included CXCR4 (ABcam, Cambridge, MA, USA), phospho-VEGFR-2, phospho-Akt, phospho-ERK1/2, phospho-Smad2/3, phospho-p38MARK, and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz, CA, USA). Western blot was performed according to the manufacturer's instructions.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The release of vascular endothelial growth factor-A (VEGF-A), stromal cell-derived factor-1 (SDF-1), and transforming growth factor beta 1 (TGF-β1) was measured in 10 × concentrated culture supernatants by ELISA (R&D Systems, USA), as previously described by Urbich et al.[10]. The medium of RAW 264.7 cells, EPCs, and co-culture system was respectively concentrated 10 × by centrifugation at 10,000 rpm for 20 min at 4 °C after EPCs were cultured with equal amounts of medium without supplements and FBS for 24 h.

2.9. Statistical analysis

All data are expressed as mean ± SEM. Student's *t* test was used to assess statistical significance for paired observations. Data of multiple comparisons were analyzed using ANOVA and Dunnett's post hoc test. All tests were performed by SPSS and the level of significance was defined at $p < 0.05$.

3. Results

3.1. Identification and characterization of EPCs

After 1 wk in vitro culture, bone marrow-derived EPCs exhibited a spindle-shaped morphology and tended to form cluster-like colonies (Fig. 1A). Immunohistochemistry demonstrated that EPCs were positive for VEGFR-2 (Fig. 1B) and vWF (Fig. 1C) after culturing for 7 d. EPCs were also defined by the binding of FITC-UEA-1