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Endothelial progenitors enhanced the osteogenic capacities of mesenchymal stem cells *in vitro* and in a rat alveolar bone defect model

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ABSTRACT

Objective: Transplantation of mesenchymal stem cells (MSCs) may promote bone healing. Endothelial progenitor cells (EPCs) may enhance the osteogenic properties of MSCs by improving their microenvironment. We aimed to investigate whether EPCs can enhance the osteogenic properties of MSCs *in vitro*, and whether transplantation of EPC-MSC cell sheets could promote bone regeneration in a rat model of alveolar bone defect.
Design: MSCs and EPCs were obtained from 2-week-old Sprague-Dawley rats. Cell sheets were prepared using MSCs and MSCs co-cultured with EPCs. Morphological characteristics of cell sheets were observed by H&E staining. Osteogenic differentiation capacities of the cell sheets were assessed by alkaline phosphatase (ALP) staining, Alizarin Red S staining and qRT-PCR. Cell sheets were transplanted into alveolar bone defects in 8-week-old rats. Six weeks later, bone formation was assessed by micro-CT.
Results: EPC-MSC sheets exhibited faster osteogenesis than MSC sheets. Six weeks after implantation, alveolar bone defects transplanted with EPC-MSC sheets exhibited a better bone reconstruction. MSC sheets generated new bone that partially covered the defect areas, while EPC-MSC sheets exhibited more robust osteogenic activity, with continuous new bone that almost covered the entire defect area.
Conclusions: Transplantation of cell sheets containing EPCs and MSCs promoted bone regeneration.

1. Introduction

The repair of alveolar bone defects is a major concern for clinicians (Zhang et al., 2012). Mesenchymal stem cells (MSCs) are a promising candidate for bone tissue engineering. Indeed, MSCs are easy to harvest, readily expand *in vitro*, and can be induced to differentiate into different mesenchymal lineages (Derubeis & Cancedda, 2004). Studies showed that an effective method of new bone formation could be achieved by combining MSCs with porous osteoconductive scaffolds (Ciapetti, Granchi, & Baldini, 2012). However, some issues remain such as inadequate neovascularization of implanted grafts and limited application of the scaffolds (Anderson, Rodriguez, & Chang, 2008; Badylak & Gilbert, 2008). These issues need to be solved before the possibility of using this approach in the clinical setting.

The use of endothelial progenitor cells (EPCs) might be part of the strategy for enhancing vascularization for bone engineering (Rouwkema, Rivron, & van Blitterswijk, 2008). EPCs were first described by Asahara et al. in 1997 (Asahara et al., 1997) and contribute to the formation of new blood vessels (Rafii & Lyden, 2003). More specifically, EPCs have the capacity to differentiate into cells that line the lumen of blood vessels (Luttun, Carmeliet, & Carmeliet, 2002). Therefore, EPCs are able to promote neovascularization and, indirectly, new bone formation during bone healing (Atesok, Li, Stewart, & Schemitsch, 2010). Beside direct neovascularization, transplanted EPCs also release chemotactic factors to recruit host EPCs, further enhancing neovascularization (Keramaris et al., 2012; Seebach, Henrich, Wilhelm, Barker, & Marzi, 2012).

Scaffolds can be used as vectors to support tissue generation, but they can induce inflammatory responses that interfere with tissue generation and repair (Elloumi-Hannachi, Yamato, & Okano, 2010; Yang et al., 2005). In addition, the use of enzymatic digestion to detach cells from the culture plate disrupts the valuable extracellular matrix (ECM) (Kang, Kim, Fahrenholtz,

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Khademhosseini, & Yang, 2013). Therefore, an approach circumventing the problems of scaffolds and digestion would be the transplantation of cell sheets. Indeed, the layered and condensed cell sheets would preserve cell–cell interactions and enrich the ECM. Since the ECM provides signals that determine tissue morphology, structure, and function (Nelson & Bissell, 2006), an enhanced tissue regeneration should be achieved using a tissue engineered graft capable of preserving and generating ECM (Wei et al., 2012). Previous studies have already shown that cell sheets can be used to repair myocardium, blood vessels, and peridontal ligaments (Matsuura et al., 2012; Pirraco et al., 2011; Rayatpisheh et al., 2014; Zhou, Li, Mao, & Peng, 2012). In addition, several methods for preparing cell sheets have been described (Harimoto et al., 2002; Kim, Lim, Donahue, & Lowe, 2005). Induction using vitamin C could be used to achieve intact cell sheets with standard culture dishes and cell scrapers only (Nakamura et al., 2010; Sakai et al., 2013).

In the present study, we aimed to establish a co-cultured EPC-MSC composite cell sheet to improve the vascularization of the graft in order to improve bone healing. These sheets were assessed *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals

Sprague-Dawley (SD) rats were from the laboratory animal research center of the Fourth Military Medical University (Xi'an, China). Two-week-old rats were used as donors for cell preparation, and 8-week-old rats (250–300 g body weight) were used as recipients. All animals were housed in a temperature-controlled environment with a 12-h light-dark cycle, and provided with food and water ad libitum.

All experiments were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of the Fourth Military Medical University and approved by the Animal Ethical Committee of the Fourth Military Medical University.

2.2. Isolation, culture, and identification of MSCs

MSCs were prepared and cultured as previously described (Zhang, Wei, Zhu, & Han, 2008). Briefly, 2-week-old SD rats were used to obtain bone marrow-derived mononuclear cells (BMNCs). Femurs and tibias were aseptically removed after sacrifice and bone marrow was collected by flushing the diaphysis with Minimum Essential Medium alpha Medium (α-MEM) (Gibco, Rockville, MD, USA). Histopaque-1077 (1.077 g/mL, St. Louis, MO, Sigma) density gradient centrifugation at 2000 rpm for 20 min was used to separate BMNCs from the bone marrow. BMNCs were cultured in α-MEM (Gibco) supplemented with 10% fetal bovine saline (FBS; Gibco) and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) at 37 °C in a humidified 5% CO_2 incubator. The culture medium was first changed on day 3 to remove non-adherent cells, and then every three days thereafter. Cells were passaged when they reached 80–90% confluence using 0.25% trypsin in 1 mM EDTA (Sigma). Cells were used for subsequent experiments at the third passage.

MSC: characteristics were assessed using anti-rat CD29-FITC, CD45-PE, CD90-PE-Cy5 (all from eBioscience, San Diego, CA, USA) and CD34-PE (Abcam, Cambridge, MA,USA) antibodies followed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). In addition, differentiation into adipogenic and osteoblastic lineages was used to further characterize the MSCs by culturing the cells in adipogenesis induction medium (α-MEM, supplemented with 10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 0.5 mM IBMX, and 10 μM insulin, Sigma), or osteogenesis induction medium (α-MEM supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid, Sigma). Alizarin Red S was used to confirm osteogenic differentiation, while Oil Red O was used to confirm adipogenic differentiation (Gala, Burdzinska, Idziak, Makula, & Paczek, 2011).

2.3. Isolation, culture, and identification of EPCs

BMNCs were washed twice with PBS, and then suspended in Endothelial Cell Basal Medium-2 supplemented with EGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza Walkersville, MD, USA). After 48 h at 37 °C and in 5% CO_2, non-adherent cells were collected and re-suspended in EGM-2 medium (Lonza). The medium was changed every 3 days.

Flow cytometry was performed to characterize the phenotype of EPCs using anti-rat CD34-PE (Abcam), VEGFR2-PE, and CD133-FITC antibodies (both from Bioss, Beijing, China). EPCs were characterized according to their ability to take up DiI-labeled acetylated low-density lipoprotein (DiI-ac-LDL) and to bind fluorescein isothiocyanate-labelled *Ulex europaeus* agglutinin-1 (FITC-UEA-1). For this, EPCs were incubated with 2.5 mg/L DiI-ac-LDL (Molecular Probes, Grand Island, NY, USA) for 2 h at 37 °C, then incubated with 10 mg/L of FITC-UEA-1 (Vector Laboratories, Burlingame, CA, USA) for 1 h at 37 °C and analyzed using a fluorescent microscope (FV1000, Olympus, Japan). EPCs were stained positively for both markers.

2.4. Cell sheet formation

To determine the optimal cell proliferation conditions, preliminary experiments were performed to confirm the ratio and numbers of EPCs and MSCs for the co-culture system. EPCs and MSC were co-cultured at a 1:2 ratio (4×10^3 EPCs and 8×10^3 MSCs); at 1:1 (6×10^3 EPCs and 6×10^3 MSCs); and at 2:1 (8×10^3 EPCs and 4×10^3 MSCs) in 96-well plates. The MTT assay was used to assess cell proliferation. The MTT assay showed that the proliferative activity was achieved when EPCs and MSCs were cultured at a 1:1 ratio (optical density = 0.63 ± 0.01 compared with 0.59 ± 0.02 for the 1:2 ratio and 0.46 ± 0.04 for the 2:1 ratio; $P < 0.05$). Therefore, the 1:1 ratio was selected for the subsequent experiments.

Cell sheets were created by culturing MSCs alone (3×10^5 cells/well) or with an equal number of EPCs (1.5×10^5 cells/well of each cell type) in α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin at a density of 3×10^5 cells/well in 6-well plates until reaching 80–90% confluence. Cells were induced to form cell sheets by supplementing the medium with 50 μg/mL of vitamin C.

2.5. Morphological observations of cell sheets

The cell sheets were detached after 10 days using a scraper. For histological observation, the sheets were fixed with 4% paraformaldehyde, dehydrated through a series of ethanol solutions, embedded in paraffin, and sectioned for H&E staining.

2.6. Osteogenic differentiation of the cell sheets

Cell sheets were incubated with osteogenic induction medium to induce osteogenic differentiation. After 7 days, alkaline phosphatase (ALP) staining was measured with the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Haimen, China), according to the manufacturer's instructions. After 21 days, the cell sheets were washed with PBS and fixed in 10% formalin solution for 30 min at room temperature, washed