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Influence of co-culture on osteogenesis and angiogenesis of bone marrow mesenchymal stem cells and aortic endothelial cells

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ABSTRACT

Co-culture of bone forming cells and endothelial cells to induce pre-vascularization is one of the strategies used to solve the insufficient vascularization problem in bone tissue engineering attempts. In the study, primary cells isolated from 2 different tissues of the same animal, rat bone marrow stem cells (RBMSCs) and rat aortic endothelial cells (RAECs) were co-cultured to study the effects of co-culturing on both osteogenesis and angiogenesis. The formation of tube like structure in 2D culture was observed for the first time in the literature by the co-culture of primary cells from the same animal and also osteogenesis and angiogenesis were investigated at the same time by using this co-culture system. Co-cultured cells mineralized and formed microvasculature beginning from 14 days of incubation. After 28 days of incubation in the osteogenic medium, expression of osteogenic genes in co-cultures was significantly upregulated compared to RBMSCs cultured alone. These results suggest that the co-culture of endothelial cells with mesenchymal stem cells induces both osteogenesis and angiogenesis.

1. Introduction

Although bone heals itself, bone defects which are caused by infection, trauma, cancer, or diseases such as osteoporosis usually result in fracture non-unions and require tissue grafts. On the other hand, allo or xenografts have a high complication rate including the risks of infection and immune rejection (Salgado et al., 2004). Bone tissue engineering strategies address the problem of healing critical size bone defects by combining cells to regenerate the tissue, growth factors to guide cell behavior and scaffolds to provide a support for the cells to form the ultimate 3D shape of the targeted tissue (Yarlagadda et al., 2005; Chan and Leong, 2008; Lee and Atala, 2014). The main problem in these traditional bone tissue engineering attempts is the insufficient vascularization of newly formed bone tissue (Hofmann et al., 2008; Santos et al., 2009; Amaral et al., 2009; Ghanaati et al., 2011; Liu et al., 2013). Without proper vascularization and blood supply, cells in the tissue engineered construct suffer from hypoxia, depletion of nutrients and accumulation of waste products. In addition, biochemical signaling is disrupted, affecting tissue homeostasis and eventually making tissue regeneration difficult (Ghanaati et al., 2011; Aguirre et al., 2010).

In bone, a highly vascularized tissue, blood vessels play especially important roles in fracture healing by satisfying the oxygen and nutrient requirements, delivering hormones and directing inflammatory signals and cells to the wound site (Bai et al., 2013).

In most of the tissues, cells typically can survive up to a distance of 200 μm from the nearest capillary network and those further away from the capillaries suffer because they depend on simple diffusion for the transport of the nutrients and oxygen, and for waste removal (Lovett et al., 2009). Diffusion, however, is not adequate for thick and dense tissues like bone without proper vessel network in regeneration process and tissue engineering constructs can only rely on the ingrowth of host vessels which might not be early enough (Rouwkema et al., 2006). On the other hand, the contribution of infiltration of the local blood vessels is quite limited since it is very slow and only possible up to a depth of several hundred micrometers from the implant surface (Jabbarzadeh et al., 2008; Wang et al., 2010) and it is even harder in mineralized tissues such as bone. Thus, for the successful integration of tissue engineered constructs formation of a stable and functional vascular network is essential (McFadden et al., 2013). In order to obtain a fully functional, vascularized bone construct, bone tissue engineering researchers began including pre-vascularization of the construct as a

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step before implantation. One of the strategies to achieve pre-vascularization involves seeding the scaffolds with endothelial cells (ECs) that can spontaneously form vessel-like networks before seeding the bone forming cells (Levengood et al., 2011).

Another strategy is to use a co-culture of endothelial cells with bone forming cells to achieve vascularization simultaneously with osteogenesis. Since bone is a tissue formed by several cell types, co-cultures of heterogeneous cells can better mimic the *in vivo* microenvironment than monotypic cell cultures (Goubko and Cao, 2009). Besides, co-culture of ECs with bone forming cells allow researchers to study how the cellular crosstalk between these cell types affect functionality of the others (Kirkpatrick et al., 2011).

Interactions between heterogeneous cells provide the cues essential for differentiation, organization, and homeostasis as soluble signals and *via* cell-to-cell interactions. In bone, ECs in addition to being a component of angiogenesis, secrete regulatory molecules such as bone morphogenic proteins (BMPs), endothelins and prostaglandins that control the differentiation and activity of osteoblasts (Bai et al., 2013). Osteoblasts, in turn, influence EC activity by secreting angiogenic factors such as VEGF and bFGF (Santos et al., 2009). In direct co-cultures, cell-to-cell interactions between two cell types have also been shown to constitute the niche that influences cell fate including osteogenic differentiation (Villars et al., 2002; Saleh et al., 2011) and angiogenesis (Loibl et al., 2014).

In this study, mesenchymal stem cells isolated from rat bone marrow (BMSC) were directly co-cultured with endothelial cells isolated from rat aorta to study the effect of this co-habitation on angiogenesis and bone formation. Cell proliferation was studied to assess the compatibility of these cells. Differentiation to osteogenic lineages was assessed by ALP activity as an early osteoblast marker, von Kossa staining to observe mineralization and quantitative PCR to detect the expression of osteogenic genes by the BMSC. In addition, angiogenic tube formation was studied by confocal microscopy. This study involves co-culturing of two primary cell types of the same animal and the effects of this co-culture strategy on both osteogenesis and angiogenesis were investigated since there is a need for the literature to look the events not only from osteogenesis or angiogenesis point of view but also from both sides. It was also aimed to obtain an appropriate protocol for the successful primary endothelial and mesenchymal stem cells co-culture through seeding techniques and media compositions for a better osteogenesis and vascular structure formation.

2. Materials and methods

2.1. Isolation and growth of cells

This study was conducted after approval by Yeditepe University Animal Research Local Ethics Committee (YÜDHEK). Rat aortic endothelial cells (RAECs) were isolated from inside lumen of abdominal aorta of rats according to Kobayashi et al. (2005). Briefly, blood perfusion was applied to anesthetized 4-week-old Sprague Dawley male rats. When the perfusion was ended, aorta was exposed and dissected from the aortic arch to abdominal aorta. Connective and adipose tissue around the aorta was removed with a fine forceps and scissors under the stereomicroscope. Aorta was incubated in a collagenase Type II (Gibco, Invitrogen, USA) (2 mg/mL) solution at 37 °C for 45 min. RAECs were removed from aorta by flushing the lumen of the aorta with DMEM (Gibco, Invitrogen, USA) containing 20% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and plated in collagen Type I (Roche, Germany) coated tissue culture flasks. In order to prevent any cross contamination by smooth muscle cells or fibroblasts, medium was removed after 1.5 h incubation at 37 °C. Attached cells were washed with warm PBS (Gibco, Invitrogen, USA) and rat aortic endothelial cell growth medium was added (Cell Applications, USA). Medium was changed twice a week.

RBMSCs were isolated from the same rats by flushing the bone marrow of the femur and the tibia. RBMSCs were cultured in DMEM supplemented with 10% FBS (Gibco, Invitrogen, USA) and 100 units/mL penicillin-streptomycin-fungicide mixture (Pan Biotech, Germany). Medium was changed twice a week.

Cell passages of 3–5 were used for all of the experiments performed.

2.2. Characterization of cells

Specific cell surface antigens of passage 3 RBMSCs were analyzed by flow cytometry (FACSCalibur – BD Pharmingen, USA). Cell surface markers analyzed for RBMSCs were: a) rat specific hematopoietic lineage markers, CD 45 (BD Pharmingen, USA) and CD 11a (BD Pharmingen, USA), and b) rat specific MSC markers, CD 90 (BD Pharmingen, USA) and CD 29 (BD Pharmingen, USA). Briefly, 5×10^5 RBMSCs were incubated for 1 h at 4 °C with conjugated antibodies and then washed with PBS (Gibco, Invitrogen, USA) twice to remove excess antibodies. Cells were then resuspended in 400 μL PBS, assayed using FACSCalibur (Becton Dickinson, USA) and the data was analyzed using Cell Quest software (Becton Dickinson, USA).

RAECs were incubated with CD31 (PECAM-1) primary antibody (LSBio, USA) for 1 h at 4 °C followed by incubation with FITC conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 h at 4 °C and observed by fluorescence microscope (Nikon, Eclipse TE200, USA). To further characterize RAECs, Angiogenesis Assay (Cell Biolabs, USA) was performed according to the company's instructions in order to test the ability of the isolated endothelial cells to form angiogenic tubes on extracellular matrix (ECM) gel. Formed tubes were observed by both bright field and fluorescence microscopes after being stained with Calcein AM in the Angiogenesis Assay kit (Cell Biolabs, USA).

2.3. Co-culture and preparation of co-culture media

RBMSCs and RAECs were mixed in 5:1 ratio prior to seeding. Cell suspensions containing 5×10^4 RBMSCs and 1×10^4 RAECs were transferred to 6-well plates and then medium was added into each well. Only RBMSC and only RAEC containing wells were included as controls. Since the media for the co-culture groups had to support both cell types, growth media of RBMSCs and RAECs were mixed in 1:1 ratio. In the differentiation studies, supplements required for the differentiation of RBMSCs, such as ascorbic acid (Sigma-Aldrich, USA) (50 μM) dexamethasone (Sigma-Aldrich, USA) (100 nM) and β-glycerophosphate (Sigma-Aldrich, USA) (10 mM), were added to each respective medium. Media and supplements added are listed in Table 1.

2.4. Assessment of cell proliferation

MTS test (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA) was used to determine cell proliferation (Kose et al., 2003). Briefly, MTS reagent (200 μL) was added to each well of the 6-well plate and incubated for 2 h at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader (BIO-TEK, ELx800, USA).

2.5. Determination of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured spectroscopically using ALP kit (RANDOX Laboratories, Ireland). Cells were first lysed with Tris (Sigma-Aldrich, USA) buffer (0.1 M, pH 9.0) containing 0.01% Triton® X-100 (Sigma-Aldrich, USA). Cell lysates were subjected to 3 successive freeze-thaw cycles by freezing at −20 °C for 10 min and thawing at 37 °C for 10 min. Then, samples were sonicated for 10 min on ice with 30 s breaks every minute. Each sample (100 μL) was mixed with 20 μL of *p*-nitrophenyl phosphate solution supplied by ALP kit (RANDOX Laboratories, Ireland). Absorbance was measured every minute at 405 nm for 10 min using Elisa Plate Reader (BIO-TEK, ELx800, USA). ALP activity was calculated using a calibration curve