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Cyclic tensile strain promotes the osteogenic differentiation of a bone marrow stromal cell and vascular endothelial cell co-culture system

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ABSTRACT

Mechanical stimuli and neovascularization are closely coupled to osteogenic differentiation and new bone formation. The purpose of present study was to detect the effect of cyclic mechanical strain on a co-culture system of bone marrow stromal cells (BMSCs) and vascular endothelial cells (VECs) and to clarify the related mechanisms. Primary BMSCs and VECs were isolated from Sprague-Dawley rats and co-cultured at various ratios (1:0, 1:2, 1:4, 4:1, 2:1, 1:1, and 0:1). To determine optimized loading conditions, the cells were then subjected to various cyclic tensile strains (0%, 3%, 6% and 9%) using a Flexcell 5000 mechanical loading system. A protocol of 6% strain on the co-cultured cells at a 1:1 ratio was selected as the optimized culture conditions based on the best osteogenic effects, which included increased ALP activity, matrix mineralization and the expressions of VEGF, Runx-2 and Col-1. The VEGF-R inhibitor tivozanib was used to analyze the paracrine role of VEGF, and the osteogenesis-promoting effects of 6% tensile strain were abrogated in the co-cultured cells treated with tivozanib. These results demonstrate that cyclic tensile strain promotes osteogenic differentiation in BMSC/VEC co-culture systems, possibly via a VEC-mediated paracrine effect of VEGF on BMSCs.

1. Introduction

It is well-established that neovascularization plays a crucial role in the bone formation process during development and fracture healing [1,2]. Newly formed vasculature provides bone tissue with nutrients, oxygen, and growth factors and removes wastes. Substantial evidence has indicated that vascular endothelial cells (VECs) promote the osteogenic differentiation of bone marrow stromal cells (BMSCs) via direct cell-to-cell contact or diffusible molecules [3,4]. Thus, the co-culture of VECs and BMSCs has become a common approach to achieve pre-vascularization for tissue-engineered constructs [2]. In addition to vascularization, mechanical loading is also closely coupled to the rate and quality of bone formation [5–7]. Bone mass and architecture are dynamically adapted to external loading. Previous studies have analyzed the responses of osteoprogenitors, including BMSCs, under cyclic mechanical strain and have demonstrated that such tensile loading enhances BMSC osteogenesis [8–10].

Because both VECs and mechanical stimuli are involved in bone formation and remodeling, the combined treatment of both factors may have a more significant impact on bone regeneration compared to a single factor alone. Several previous studies have focused on this combined effect and suggested that the presence of VECs with mechanical stimuli could further enhance BMSC osteogenesis [7,11]. However, optimized culture conditions and the intrinsic mechanism of this combined effect remain to be further elucidated.

Current concepts suggest that paracrine and autocrine mechanisms involving various diffusible molecules are essential in regulating the metabolic activities of co-cultured cells [4]. Among these diffusible molecules, vascular endothelial growth factor (VEGF) is a potent angiogenic and osteogenic promotor that activates its receptor 2 (VEGFR2) both in vitro and in vivo [12]. Moreover, several studies have noted VEGF level elevations induced by various mechanical stimuli in mono-cultured BMSCs, osteoblasts or other osteoprogenitors [10,13,14].

Based on the aforementioned, we hypothesize that VEGF is a key regulator in the cross-talk between BMSCs and VECs under cyclic mechanical strain. The goal of this study was to investigate the combined effect of VECs and cyclic mechanical strain on BMSC osteogenesis in vitro while elucidating the regulatory mechanism

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of this co-culture system. To accomplish this, a 2-dimensional (2D) direct contact co-culture system of rat BMSCs and VECs was established and then subjected to equibiaxial cyclic tensile strain. The optimal co-culture cell ratio and mechanical loading magnitude were determined based on the best osteogenic effect. The specificity of BMSC differentiation was assessed by examining markers of osteogenic differentiation. Furthermore, the role of VEGF as a candidate regulator was evaluated via specific receptor inhibitors. The results confirmed that cyclic tensile strain further enhanced BMSC osteogenesis in the BMSC/VEC co-culture system and indicated that VEGF plays an essential role in the cross-talk between VECs and BMSCs under dynamic loading conditions via paracrine regulation.

2. Material and methods

2.1. Cell isolation and culture

2.1.1. Culture of BMSCs

Rat BMSCs were isolated and expanded according to previously reported methods [15]. Bone marrow was obtained from the femora of 4-week-old male Sprague-Dawley (SD) rats. The animals were sacrificed by cervical dislocation, and the bones were excised aseptically. The ends of the femora were removed, and the bone marrow was flushed out using 10 ml of Dulbecco's modified Eagle medium (DMEM, HyClone, US) supplemented with 10% fetal bovine serum (FBS, Gibco, US) and 1% penicillin/streptomycin (HyClone, US). The collected cells were plated on a 100-mm culture dish with culture media changed twice a week. After 1 week in primary culture, the BMSCs were regularly trypsinized and subcultured. Cells between the second and the fourth passages were used for further experiments.

2.1.2. Culture of VECs

Rat VECs were isolated according to a previously established method [16]. In brief, primary VECs were isolated from the aortas of 4-week-old male SD rats using an explant culture model. The descending aortas were dissected aseptically and sliced into 1–2 mm pieces. The explant tissues were placed endothelial surface down in a 60-mm culture dish with 5 ml endothelial cell growth medium-2 (EGM-2, Lonza, US). After a 9-day incubation in a humidified atmosphere (37 °C, 5% CO_2), the explant tissues were removed, and the monolayer cells were maintained in culture for another 14 days. Then, the cells were regularly trypsinized and expanded. Cells between the second and the fourth passages were used for cell type identification and for further experiments.

2.1.3. Co-culture of BMSCs and VECs

BMSCs and VECs were co-cultured in direct contact at various ratios (BMSCs: VECs, 1:0, 1:2, 1:4, 4:1, 2:1, 1:1, 0:1). Cell suspensions in DMEM were prepared and seeded on culture plates at the density of 5×10^4 cells/ml. The optimal co-culture ratio was determined based on best osteogenic effect.

2.2. Alkaline phosphatase (ALP) staining and ALP activity assay

ALP staining was performed using a BCIP/NBT Alkaline Phosphatase Color Development kit (Beyotime, China). In brief, the culture medium was discarded, and the cells were fixed in 4% PFA for 30 min. Then, the cells were washed twice with ice-cold PBS and stained with 1.5 ml of BCIP/NBT solution for 30 min prior to light microscopic observation.

For the assessment of ALP activity, cells were washed twice with PBS and lysed in 450 μl ALP buffer containing 0.2% Triton-100. The total protein was then quantified using a BCA Kit (Thermo, US). Subsequently, 100 μl of cell lysate was assayed by adding 100 μl of p-nitrophenylphosphate (dNPP, Sigma, US) as a substrate for 30 min at 37 °C. The absorbance was read spectrophotometrically at 405 nm, and the enzyme activity was expressed as OD value/mg protein.

2.3. Alizarin red staining (ARS) and semi-quantitative assessment

Alizarin red staining was performed using previously established methods [17]. The culture medium was discarded, and the cells were fixed in 4% PFA for 30 min. Then, the cells were washed twice with ice-cold PBS and stained for 5 min with Alizarin red (Cyagen, US) prior to light microscopic observation. Then, the cells were incubated in 10% cetylpyridinium chloride (CPC, Sinoreagent, China) at room temperature for 1 h. The absorbance of supernatants was examined at 590 nm for the semi-quantitative assessment.

2.4. Application of cyclic tensile strain

According to the osteogenic activity of the co-cultured cells, a 1:1 ratio of BMSCs: VECs was selected for further experiments. The cells were seeded on 6-well Bioflex culture plates coated with Collagen I (Flexcell, US) at the BMSCs: VECs ratios of 1:0, 1:1 and 0:1. Subsequently, the plates were cultured for 3 days until cell confluence and then subjected to equibiaxial cyclic tensile strain (0.5 Hz, semi-sinusoidal wave form) using a Flexcell Tension System (Flexcell 5000, US). To optimize the cyclic tensile strain, the cells were subjected to various mechanical strains (0%, 3%, 6% and 9%) for 48 h. The optimal loading magnitude was determined based on the best ALP activity and selected for further experiments.

2.5. ELISA

The supernatants collected from the loading experiments and static controls were subjected to ELISA assays. The VEGF production in each group was quantified using a rat VEGF ELISA Kit (Biotechwell, China) according to the manufacturer's instructions.

2.6. qRT-PCR

The total RNA was extracted using RNAiso Plus reagent (Takara, Japan), and the RNA concentration was measured according to the absorbance at 260 nm (NanoDrop1000, Thermo, US). A total of 2 μg RNA was used as a template for single-strand cDNA synthesis performed using a PrimeScript RT reagent Kit (Takara, Japan). After reverse transcription, real-time PCR was performed using a Bio-Rad IQ5 Real-Time PCR System (Bio-Rad, US) with an SYBR PrimeScript RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. The qRT-PCR results were analyzed and expressed as the relative RNA levels of the cycle threshold (CT) value, which was then used to calculate the fold-change according to the value of each control sample ($2^{-\Delta\Delta CT}$ method). β-actin was used as an internal normalized reference. The following primers were used:

VEGF-F, 5′-GGCTCTGAAACCATGAACTTTCT-3′;
VEGF-R, 5′-GCAATAGCTGCGCTGGTAGAC-3′;
Runx2-F, 5′-ATCCAGCCACCTTCACTTACACC-3′;
Runx2-R, 5′-GGGACCATTGGGAACTGATAGG-3′;
Col1-F, 5′-CAGGCTGGTGTGATGGGATT-3′;
Col1-R, 5′-CAAGGTCTCCAGGAACACC-3′;
β-actin-F, 5′-GTAAAGACCTCTATGCCAACA-3′;
β-actin-R, 5′-GGACTCATCGTACTCCTGCT-3′.