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Osteogenic differentiation and angiogenesis with cocultured adipose-derived stromal cells and bone marrow stromal cells

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ABSTRACT

The purpose of this study was to determine the influence of cocultured adipose-derived stromal cells (ASCs) in enhancing the osteogenic differentiation and angiogenesis of bone marrow stromal cells (BMSCs) as well as the underlying mechanism and the optimal ratio. Two *in vitro* coculture models, segregated cocultures using transwell and mixed cocultures, were employed to assess the indirect and direct effects of coculture respectively. Coculture was carried out for 14 days using 1×10^5 BMSCs and ASCs of variable number. BMSCs, ASCs, or both were seeded in PLGA scaffold and implanted in the subcutaneous tissue of 25 nude mice for *in vivo* analysis of angiogenesis. To evaluate the orthotopic bone formation, critical size calvarial defects were created on 20 mice, and implanted with hydroxyapatite/β-tricalcium phosphate granules plus BMSCs, ASCs, or both. From both transwell and mixed coculture model, 1×10^5 BMSCs cocultured with 0.5×10^5 ASCs showed significantly greater osteogenic differentiation and mineralization than BMSCs alone. The mixed ASC/BMSC coculture at or above a ratio of 0.5/1 showed increased secretion of vascular endothelial growth factor (VEGF), and induced effective tube formation from human umbilical vein endothelial cells, which were comparable to ASCs. Cytokine profiling assay and gene expression study showed elevated levels of angiogenic factors VEGF and CXCL1, osteogenic factor Wnt5a as well as transforming growth factor (TGF)-βR1 and SMAD3 from BMSCs when cocultured with ASCs. After 5 weeks of implantation, polylactic-co-glycolic acid (PLGA)-ASCs-BMSCs had a number of vascular structures comparable to PLGA-ASCs and significantly greater than PLGA-BMSCs. Calvarial defects treated with ceramic/BMSCs/ASCs had greater area of repair and better reconstitution of osseous structure than the defects treated with ceramic/ASCs or ceramic/BMSCs after 10 weeks. In conclusion, ASCs added to BMSCs promoted osteogenesis and angiogenesis at the optimal ASC/BMSC ratio of 0.5/1.

1. Introduction

Bone has a capacity for self-repair without scar formation, a property uncommon in adult tissues. Most fractures heal spontaneously or with the help of surgical procedures [1]. However, despite the inherent ability of the bone to regenerate itself, there are a number of clinical situations in which complete bone healing fails to occur [2]. Typical examples are extreme conditions in trauma or malignant tumor resection, which result in critical-size bone defects (spanning>2 cm) [3]. Besides, osteonecrosis of the femoral head (ONFH), a destructive disease of the hip joint caused by inadequate blood supply, affects young adults. While total hip arthroplasty (THA) is usually required for these patients [4], the longevity of artificial joint is limited in young patients who underwent THA, posing them at higher risk for failure during their life span. Therefore, therapeutic measures that enable regeneration of the affected bone rather than replacing it are necessary for ONFH patients.

Adult mesenchymal stem cells can be isolated from bone marrow [5], skeletal muscles [6], adipose tissues [7], synovial membranes [8], or trabecular bone [9]. Stromal vascular fraction (SVF) within adipose tissue contains multi-potent mesenchymal progenitor cells called adipose-derived stromal cells (ASCs). While bone marrow stromal cells (BMSCs) have been extensively investigated as the most suitable cell source for bone tissue engineering due to their excellent osteogenic potential, ASCs have also been given attention due to their easy accessibility and abundance [10]. ASCs are easily acquired through minimally invasive techniques

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from abdominal fat or from other sites such as infra-patellar fat [11]. ASCs can differentiate into not only mesenchymal lineage cells but also vascular lineage cells such as endothelial, smooth muscle and circulating cells [12,13].

Autologous subcutaneous adipose tissue is known to be associated with therapeutic angiogenesis, a strategy to treat tissue ischemia by promoting the proliferation of collateral vessels [14,15]. ASCs secrete multiple angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) at bioactive levels [16–18]. ASCs injected locally or systemically were shown to stimulate angiogenesis and recovery of muscle tissues after an ischemic insult [13]. While BMSCs also promote angiogenesis, their pathway to promote angiogenesis is known to be different from ASCs [19].

Clinical situations that require cell transplantation for bone regeneration are usually associated with poor vascular supply. On the other hand, high concentration of cells is usually necessary to achieve rapid new bone formation, which paradoxically demands a good vascular supply. Consequently, rapid revascularization is very important for the survival of implanted cells and bone formation. Addition of ASCs to BMSCs may enhance the revascularization of implanted BMSCs, and thus the success of cell transplantation. However, considering the lower osteogenic potential of ASCs as compared with BMSCs [10], co-treatment of ASCs to BMSCs may reduce the efficiency of desired new bone formation at the implanted site. Thus, to apply the concept of co-treatment of ASC and BMSC to clinical situations, it is important to determine whether ASCs and BMSCs, when treated together, will have beneficial or detrimental effects in terms of both osteogenic differentiation and angiogenesis as well as the optimal ratio of ASCs to BMSCs. The purpose of this study was to investigate the influence of cocultured adipose-derived stromal cells (ASCs) in enhancing the osteogenic differentiation and angiogenesis of bone marrow stromal cells (BMSCs) as well as the underlying mechanism and the optimal ratio to promote these desired functions. To prove the concept, we used transwell and mixed cocultures for the *in vitro* model, and the subcutaneous implantation and critical size calvarial defect model in nude mice for the *in vivo* demonstration of enhanced angiogenesis and osteogenesis respectively.

2. Materials and methods

2.1. Cell isolation and cultivation

The bone marrow samples used to isolate BMSCs were obtained from 3 patients (mean age: 62 years, range: 50–82 years) undergoing THA due to osteoarthritis. Informed consent was obtained from all donors. The ASCs were isolated from the lipoaspirates generated during elective liposuction of 3 patients (mean age, 32 years; range, 31–33 years). BMSCs and ASCs were cultured according to the previously established method (See Supplementary Text) [11]. Both types of cells had been also characterized in our previous study [11]. However, flow cytometry for CD31 was performed to exclude the presence of endothelial cells. Only 3.1% of ASCs and 0.3% of BMSCs were positive for CD31.

2.2. Colony-forming unit-fibroblasts (CFU-F) assay

For assay of CFU-F, 150 cells were plated into 10 cm petri-dish in 10 ml minimum essential medium-α supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and 20% lot-selected FBS. On day 14, cultures were fixed and stained with 0.5% crystal violet. The colonies containing 50 or more cells were counted. The colony-forming efficiency was determined by number of colonies per 150 cells plated. BMSCs had 26.8 ± 5.5 colonies and ASCs had 22.8 ± 1.9 colonies ($N = 5$).

2.3. In vitro coculture of ASCs and BMSCs

For osteogenic induction, the cells were plated and cultured for 14 days in osteogenic media (OM; α-MEM media containing 1% antibiotics/antimycotics, 10% FBS, 100 nM dexamethasone, 50 μM L-ascorbate 2-phosphate, 10 mM β-glycerophosphate). *In vitro* segregated cocultures using transwell were carried out in OM using BMSCs and ASCs in passage 3 in the following 4 ways: #1, 1×10^5 BMSCs and 0.25×10^5 ASCs; #2, 1×10^5 BMSCs and 0.5×10^5 ASCs; #3, 1×10^5 BMSCs and 0.75×10^5 ASCs; #4, 1×10^5 BMSCs and 1×10^5 ASCs (Fig. 1A). These settings had BMSCs cultured on the floor of the culture plate (lower well) and ASCs on the transwell insert (upper well). *In vitro* mixed cocultures were also performed in the same proportion (#5, 1×10^5 BMSCs plus 0.25×10^5 ASCs; #6, 1×10^5 BMSCs plus 0.5×10^5 ASCs; #7, 1×10^5 BMSCs plus 0.75×10^5 ASCs; #8, 1×10^5 BMSCs plus 1×10^5 ASCs) in ordinary 6-well culture plates (Fig. 1B). As controls, 1×10^5 BMSCs and 1×10^5 ASCs were also cultured in monolayer. The media were changed every third day. Alkaline phosphatase (ALP) assay was performed to detect the early osteogenesis from BMSCs, and Von Kossa staining and calcium quantification to detect mineralized nodule formation after 14 days of osteogenic induction. The cells were also recovered and used for other analyses to detect the expression of osteogenic genes and proteins after 14 days. Serum-free OM was used for sets of mixed coculture to measure VEGF. Culture media removed on day 3 were sent for enzyme-linked immunosorbent assay (ELISA) to quantify VEGF.

2.4. ALP Staining and activity

For ALP staining, the induced cells were fixed in 10% formalin for 10 min. The cells were permeabilized for 30 min with 0.1% Triton X-100 in PBS, and treated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 10–30 min. After rinsing, the sections were counterstained with nuclear fast red for 5 min. In addition, to quantify ALP, Ostase® BAP Immunoenzymetric Assay (Immunodiagnostic Systems Ltd, Fountain Hills, AZ, USA) was used according to the manufacturer's instructions. All values are expressed as fold changes over the control: 1×10^5 BMSCs cultured in monolayer.

2.5. Von Kossa staining and calcium quantification

To observe calcium deposition in the extracellular matrix, induced cells were washed twice with PBS and fixed in 70% ethanol for 1 h. After washing thrice with PBS, they were incubated with 1% silver nitrate solution under ultraviolet light for 20 min. They were then washed three times with distilled water, and un-reacted silver was removed with 5% sodium thiosulfate for 5 min. Following this, they were rinsed in distilled water and counterstained with methyl green for 5 min. Images were captured with a light microscope. To quantify the degree of mineralization, Calcium Colorimetric Assay Kit (BioVision, Inc, San Francisco, CA, USA) was used according to the manufacturer's instructions. All values are expressed as fold changes over the control: 1×10^5 BMSCs cultured in monolayer.

2.6. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using the standard guanidine isothiocyanate Tri-Reagent (Sigma) protocol and quantified using the Quant-iT RNA assay kit and the Qubit Fluorometer system (Invitrogen, Grand Island, NY, USA). Using the ReverAid (Fermentas Inc., Hanover, MD, USA) H Minus first strand synthesis for RT–qPCR, 1 μg of total RNA was reverse-transcribed with 0.5 μg of oligo (dT) primer. All PCR reactions were performed in standard 10 μl reactions: 4.5 μl (10 ng) cDNA, 0.5 μl 100 μM sense and 0.5 μl 100 μM antisense primers, and 4.5 μl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics, Penzberg, Germany). *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control for PCR amplification and the relative normalization ratio of PCR products derived from each target gene was calculated using the software of the LightCycler System (Roche, Indianapolis, Indiana, USA). All experiments were performed in triplicate for mRNA expression of *ALP, type I collagen* (*COL1A1*), *osteocalcin* (*OCN*), and *bone sialoprotein* (*BSP*), *VEGF, chemokine ligand 1* (*CXCL1*), *Wnt5a, matrix metalloproteinase (MMP)-1, transforming growth factor* (*TGF*)*-β1, transforming growth factor β receptor 1-β1* (*TGFβR1*), *SMAD3*. The primer pairs used are listed in Table 1.

2.7. ELISA

Wells of microtiter plates were coated (overnight, 4°°C) with 5 μg of total soluble protein in 100 μl of PBS, washed three times with PBS Tween-20 (PBST) and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After blocking, the samples were incubated with specific primary antibodies for 2 h at room temperature, washed, and incubated with secondary antibodies for 1 h at room temperature, then washed again. The reaction was visualized by addition of 50 μl of chromogenic substrate (3,3′,5,5′-tetramethylbenzidine) for 20 min. The reaction was stopped with 100 μl of 1 M H_2SO_4 and its absorbance at 450 nm was measured with reduction at 630 nm using an ELISA plate reader. The primary antibodies used were COL1A1 polyclonal antibody, OCN polyclonal antibody and BSP polyclonal antibody (Abcam, Cambridge, UK) diluted at 1:10,000 in blocking solution. The secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (Abcam) and goat anti-mouse IgG (Abcam) diluted at 1:2000 in blocking solution. As a reference for quantification, standard curves were established by serial dilutions of recombinant COL1A, BSP and OCN proteins. All values are expressed as fold changes over the control, which were BMSCs treated with the standard OM.

2.8. VEGF quantification

From the mixed coculture settings, the medium was taken from the culture plate after 3 days of *in vitro* culture and assayed to measure the level of VEGF. A human