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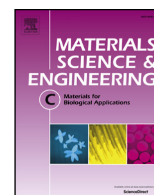

Vascular extracellular matrix and fibroblasts-coculture directed differentiation of human mesenchymal stem cells toward smooth muscle-like cells for vascular tissue engineering

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ABSTRACT

Construction of an artificial vascular graft is widely considered a promising strategy in vascular tissue engineering. However, limited sources of functional vascular smooth muscle cells (VSMCs) remain a major obstacle in vascular tissue engineering. In this study, we innovatively developed an approach to obtain functional VSMCs by onsite differentiating human bone marrow-derived mesenchymal stem cells (MSCs) directed by decellularized extracellular matrix (ECM) and fibroblasts. The resulting cells and ECM-cells constructs were characterized by real time RT-PCR, immunofluorescence staining, cell contractile functions, and migration capacity. Our results showed both ECM and fibroblasts induced MSCs differentiation toward VSMC-like cells with increased transcription of marker genes, upregulated expression of contractile apparatus proteins, and enhanced functional activity of VSMC phenotype. Interestingly, our findings revealed that native ECM and fibroblasts-coculture had a higher potential to promote MSCs differentiation into VSMCs than growth factors cocktail (GFC) supplemented culture, thereby providing a potential source of VSMCs for blood vessel constitution.

1. Introduction

Successful constitution of tissue-engineered blood vessels possessing natural structure and functions of native arteries remains a daunting challenge in vascular tissue engineering, in spite of many efforts have been made in this field [1,2]. Vascular cells and biological scaffolds are two essential elements that constitute robust bioartificial vessels [3]. Although various novel biomimetic materials were developed over the past decades [4], limited availability of endothelial cells and smooth muscle cells for vessel constitution persists as a bottleneck problem in vascular tissue engineering [5]. Many efforts have been made on the procurement of endothelial cells [6–8], but the necessity of obtaining functional vascular smooth muscle cells (VSMCs) is largely neglected [3]. Present in the media layer, VSMCs are critical to maintaining the structural and functional integrity of blood vessels by providing physical support and regulating the blood flow and pressure by contracting and relaxing in response to exogenous stimulus [9]. However, the access to autologous VSMCs from biopsies is limited due to their difficulty in acquirement of pure populations, restricted proliferation potentials and rapidly declining cell functions during *in vitro* expansion [3,10]. Therefore, obtaining sufficient functional VSMCs is a prerequisite for developing vessel substitutes in vascular tissue engineering.

To address the challenge in VSMCs source restrictions, smooth muscle-like cells differentiated from embryonic stem cells (ESCs) [11,12], mesenchymal stem cells (MSCs) [13–17], and induced pluripotent stem cells (IPS) [1,18] have gained great attention and exhibited highly promising results. Maturation of VSMCs is a complicated process driven by multiple chemical, physical, and biological cues. Currently, the differentiation of stem cells toward VSMCs is often guided by growth factor stimulation, such as transforming growth factor-beta 1 (TGFβ1), platelet-derived growth factor BB (PDGF-BB) and bone morphogenetic protein-4 (BMP4) on 2D substrates [15,16] and/or 3D synthetic biomaterials [2,10,19,20]. Nevertheless, chemical induction initiated by growth factors might cause unspecialized differentiation [10] or undesirable cell apoptosis [3]. Natural decellularized extracellular matrix (ECM) exhibited excellent biological

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properties and biocompatibility [21,22], and has been applied in the reconstruction of different tissues, such as ligaments, tendons, and abdominal wall with desired shape and mechanical properties of the tissues from which they were derived [23,24]. More interestingly, cell-matrix and cell-cell interactions have been recognized to play crucial roles in various biological events including cell adhesion, proliferation, migration, and differentiation [25,26]. The microenvironment, both ECM and a number of different types of stromal cells such as fibroblasts surrounding VSMCs, provide many benefits for facilitating the differentiation of stem cells toward VSMCs and their maturation during vessel formation [27].

In this study, we tested our hypothesis that microenvironmental signals (ECM or fibroblasts-coculture) may have the capability to direct stem cell differentiation toward a matured VSMC phenotype similar to the ability of soluble growth factors. We tested the effect of natural decellularized blood vessel ECM scaffold, indirectly cocultured fibroblasts, and growth factor cocktail (GFC) composed of TGFβ1, PDGF-BB and BMP4 on human bone marrow-derived MSCs differentiation toward VSMCs. Human VSMCs were used as the control VSMCs lineage. In addition, a 3D coculture model was developed to evaluate the combined effect of the three major factors mentioned above on differentiation of MSCs into VSMCs.

2. Materials and methods

2.1. Preparation of decellularized ECM scaffolds

Fresh porcine carotid arteries (Fig. S1a) were kindly provided as gift by Alumend (Sioux Falls, SD). After trimming excess connective tissues and fat, the carotid arteries were washed completely with phosphate buffered saline (PBS), and then decellularized according to the protocol described previously with minor modifications [28]. Briefly, carotid arteries were incubated in 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer (8 mM CHAPS, 1 M NaCl, and 25 mM EDTA) for 22 h, followed by brief washes with PBS three times. Next, carotid vessels were treated for 24 h with sodium dodexyl sulfate (SDS) buffer composed of 1.8 mM SDS, 1 M NaCl, and 25 mM EDTA, followed by complete washes with PBS ten times. After that, the carotid vessels were incubated with 0.2 mg/ml DNase and 1 mg/ml RNase in PBS for 16 h at 37 °C, and then washed thoroughly with PBS ten times. Lastly, the decellularized ECM (Fig. S1b) were cut into small discs with 5 mm diameter (Fig. S1c) with tissue punch and stored at − 80 °C. All decellularization steps were performed with agitation and under sterile conditions. All reagents were purchased from Sigma Aldrich (Sigma, St. Louis, MO).

2.2. Cell maintenance and differentiation

Human bone marrow-derived MSCs (P_1–P_5, ATCC, Manassas, VA) were maintained in MSC culture medium (ATCC) supplemented with MSC growth kit (ATCC). Human VSMCs (P_1–P_3, ATCC) were grown in VSMC culture medium (ATCC) supplemented with VSMC growth kit (ATCC). Human fibroblasts (P_6–P_{10}, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, ATLANTA Biologicals, Lawrenceville, GA). All cells were subcultured at about 90% confluency and the culture media was refreshed every 2 days.

To evaluate the effects of decellularized vessel ECM and fibroblasts on the differentiation of MSCs into VSMCs, MSCs were cultured in different conditions as follows: (1) MSCs maintained in complete MSC culture medium were defined as MSC group; (2) MSCs were seeded in the upper side of 12-well Transwell (Cat. No. 3460, Corning) with a final concentration of 3×10^4 cells/well, and 1×10^5 fibroblasts were seeded in the lower side of Transwell. The indirect cocultures of MSCs and fibroblasts were conducted with complete MSC culture medium in the apical compartment and fibroblast culture medium in the basolateral compartment; (3) Decellularized vessel ECM scaffolds were immersed in 70% ethanol for 60 min and followed by washes with PBS 3 times, and then equilibrated at 37 °C for another 48 h in complete MSC medium. The residual medium were removed with sterile gauze before MSCs were seeded into adventitial surface of the scaffolds (6×10^4 cells/scaffold) and grew in complete MSC medium; (4) MSCs cultured in complete MSC medium supplemented with growth factor cocktail (GFC) composed of 10 ng/ml TGFβ1, 25 ng/ml PDGF-BB, and 2.5 ng/ml BMP4 (all from Invitrogen) were defined as GFC group; (5) MSCs cultured in the 3D system (Fig. S1d), which combined all three factors that examined in this work, *i.e.*, ECM, coculture with fibroblast, and GFC, were defined as combined 3D coculture model group. Briefly, SMCs seeded in ECM scaffolds were placed in the upper chamber of Transwell and cocultured with fibroblasts that were seeded in the lower chamber. The cocultures were carried out with complete MSC medium containing GFC in the apical compartment and fibroblasts medium in the basolateral compartment; (6) VSMCs maintained in VSMC culture medium were set as positive control. All the cells were maintained at 37 °C in a 5% CO_2 humidified incubator, and changed media every other day.

2.3. Histological analysis and scanning electron microscopy (SEM) examination

Fresh carotid arteries, decellularized vessel ECM scaffolds, and the ECM-MSCs reconstituted tissue prepared by seeding MSCs in the ECM scaffold and differentiated in the combined 3D coculture models were fixed with 4% paraformaldehyde (Affymetrix, CA) at 4 °C overnight, followed by washes with PBS three times. The paraffin-embedded sections (5 μm thickness) were prepared by Sanford Research (Sioux Falls, SD) and stained with hematoxylin and eosin dyes (Sigma) or Masson's trichrome dyes (Sigma). Images were acquired using an inverted phase contrast microscopy (IX83, Olympus microscope).

The fresh carotid arteries, decellularized ECM scaffold, and the ECM-MSCs reconstituted tissue in combined 3D coculture models were fixed with 2.5% glutaraldehyde (Sigma) overnight at 4 °C, and dehydrated in a series of ethanol with increasing concentration (25%, 50%, 75%, 95% and 100%) before lyophilization. The dried samples were then mounted onto stubs, sputtered with gold, and visualized with SEM (FEI Quanta 450).

2.4. Cell viability evaluation and F-actin staining

To assess the effect of ECM, fibroblasts, and GFC on MSCs viability, the ECM-MSCs reconstituted tissue in combined 3D coculture model on day 1, 7, and 14 were stained with calcein-AM/EthD-1 solution (Invitrogen) as described in previous publication [29]. Live cells were stained green, whereas dead cells were stained red. The samples were observed under confocal microscopy (IX83 FV1200, Olympus).

To evaluate the effect of ECM, fibroblasts, GFC, and the combined effect of all the three factors on F-actin expression in MSCs, the cells harvested from each experimental condition were visualized by phalloidin-FITC staining. Briefly, the samples were fixed with 4% paraformaldehyde at 4 °C overnight, followed by staining with phalloidin-FITC and Hoechst 33342 (Abcam, Cambridge, MA) for F-actin and nucleus, respectively. The fluorescence images were acquired using confocal microscopy.

2.5. Real-time RT-PCR analysis

To compare the transcript level of VSMC characteristic genes between different experimental groups, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction, and quantified by spectrophotometric analysis at 260 nm wavelength. Thereafter, a portion of 200 ng RNA underwent reverse transcription in a 20 μL reaction mixture using High Capacity cDNA Reverse Transcript

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