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Isolation and trans-differentiation of mesenchymal stromal cells into smooth muscle cells: Utility and applicability for cell-sheet engineering

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Abstract

Background. Bone marrow (BM)-derived mesenchymal stromal cells (MSCs) have shown potential to differentiate into various cell types, including smooth muscle cells (SMCs). The extracellular matrix (ECM) represents an appealing and readily available source of SMCs for use in tissue engineering. In this study, we hypothesized that the ECM could be used to induce MSC differentiation to SMCs for engineered cell-sheet construction. *Methods.* Primary MSCs were isolated from the BM of Wistar rats, transferred and cultured on dishes coated with 3 different types of ECM: collagen type IV (Col IV), fibronectin (FN), and laminin (LM). Primary MSCs were also included as a control. The proportions of SMC (a smooth muscle actin [aSMA] and SM22a) and MSC markers were examined with flow cytometry and Western blotting, and cell proliferation rates were also quantified. *Results.* Both FN and LM groups were able to induce differentiation of MSCs toward smooth muscle–like cell types, as evidenced by an increase in the proportion of SMC markers (aSMA; Col IV 42.3 ± 6.9%, FN 65.1 ± 6.5%, LM 59.3 ± 7.0%, Control 39.9 ± 3.1%; $P = 0.02$, SM22; Col IV 56.0 ± 7.7%, FN 74.2 ± 6.7%, LM 60.4 ± 8.7%, Control 44.9 ± 3.6%) and a decrease in that of MSC markers (CD105: Col IV 64.0 ± 5.2%, FN 57.6 ± 4.0%, LM 60.3 ± 7.0%, Control 85.3 ± 4.2%; $P = 0.03$). The LM group showed a decrease in overall cell proliferation, whereas FN and Col IV groups remained similar to control MSCs (Col IV, 9.0 ± 2.3%; FN, 9.8 ± 2.5%; LM, 4.3 ± 1.3%; Control, 9.8 ± 2.8%). *Conclusions.* Our findings indicate that ECM selection can guide differentiation of MSCs into the SMC lineage. Fibronectin preserved cellular proliferative capacity while yielding the highest proportion of differentiated SMCs, suggesting that FN-coated materials may be facilitate smooth muscle tissue engineering.

Key Words: *cell culture, co-culture, flow cytometry, scanning electron microscopy*

Introduction

Heart failure remains a frequent and life-threatening disorder despite recent medical and surgical advances. Interest in myocardial regenerative therapy as a means to improve left ventricular (LV) function in patients with end-stage heart disease is growing. We previously reported that a cell-sheet engineering approach using co-cultured smooth muscle cells (SMCs) and endothelial progenitor cells (EPCs) induced functional recovery of distressed myocardium in a small animal heart failure model. Recapitulating the natural interaction between EPCs and SMCs created structurally mature, functional microvasculature. This method, however, required that SMCs be obtained from the thoracic aorta, which is not possible in the clinical arena [1].

To resolve this problem, bone marrow (BM)-derived mesenchymal stem cells (MSC) have shown potential to differentiate into various cell types, including SMCs [2]. Additionally, it is known that the extracellular matrix (ECM) represents a powerful regulator of SMC phenotypic modulation for tissue engineering [3,4].

Tissue engineering has become an essential field of research for effective regenerative therapy [5]. In the past decade, tissue engineering therapeutic products created from cells and scaffolds have been

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widely investigated, and several products have become commercially available. Researchers have explored scaffold-based tissue engineering, such as the biodegradable scaffold [6,7], decellularized tissues [8], hydrogel and cell mixtures [9], bioprinting technology [10] and fiber-based tissue engineering [11]. Conversely, our group has used scaffold-free cell-sheet technology for clinical translation [12]. The cell-sheet is created on, and removed from specialized dishes that are covalently grafted with a temperature-responsive polymer, poly (N-isopropylacrylamide; PIPAAm), which changes from hydrophobic to hydrophilic by simply lowering the temperature without any enzymes [13]. The cell-sheet technology fabricates three-dimensional (3-D) tissues from densely adherent cells without an artificial scaffold and enzymatic digestion. The cell-sheet is easily manipulated and has a robust ability to integrate with native tissues by retaining cell-cell junctions, as well as the ECM on the basal surface of the cell-sheet.

In this study, we hypothesized that the ECM could stimulate transdifferentiation of MSCs into SMCs to facilitate construction of an SMC supported, angiogenic bi-level cell-sheet.

Materials and methods

Isolation of MSCs and EPCs

The carotid artery of male Wistar rats (8 weeks old, 250–300 g; Charles River) was dissected and transected under isoflurane anesthesia. BM mononuclear cells were isolated from the long bones (8 weeks old, 250–300 g; Charles River), filtered through a 40 um cell strainer (Falcon), and centrifuged at 300*g* for 7 min. Red blood cells (RBCs) were excluded using 1x RBC lysis buffer (eBioscience, #00-4337-57) for 10 min at 4°C. Remaining cells were cultured in a medium with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, #11995-040) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and gentamicin on non-coated culture dishes for 24 h at 37°C. Following incubation, the adherent cells were washed and then cultured in a medium with DMEM containing 10% FBS and gentamicin. A purified population of MSCs was obtained 10 to 14 days after the initiation of culture. MSC was determined in accordance with the criteria of the International Society for Cellular Therapy [2].

EPCs were isolated and cultured, as described previously [1]. Briefly, BM mononuclear cells were isolated from the long bones of Wistar rats by density gradient centrifugation with Histopaque 1083 (Sigma-Aldrich) and cultured in endothelial basal medium-2 supplemented with EGM-2 SingleQuot (Lonza) containing human epidermal growth factor, 5% FBS, vascular endothelial growth factor (VEGF), basic human fibroblast growth factor, recombinant human long R3 insulin-like growth factor-1, ascorbic acid and gentamicin on vitronectin (Sigma-Aldrich, V0132-50VG)–coated dishes. The combination of endothelium-specific media and the removal of non-adherent BM mononuclear cells were intended to select for the EPC phenotype.

ECM-driven trans-differentiation of MSCs into SMCs

The primary rodent MSCs were transferred and cultured in a medium with DMEM and 10% FBS on 60-mm culture dishes coated with 1 of 3 different types of ECM: fibronectin (FN group, BD Biosciences), Collagen IV (Col IV group, BD Biosciences) and laminin (LM group, BD Biosciences) at 37°C in a humidified atmosphere of 5% CO_2 in air. Primary MSCs were also included in this study (Control group). MSCs were plated at a density of $4–6 \times 10^3$ cells/cm^2. MSC growth medium was used as the nutrient medium and all media were exchanged every 48–72 h.

Phenotypes of trans-differentiated SMCs and cultured MSCs assessed with flow cytometry

To elucidate the phenotypes of cultured MSCs and trans-differentiated SMCs, flow cytometry was employed using markers specific for MSCs, EPCs, and SMCs. Cell assessment was performed after 10 to 14 days' culture on each plate. Single-cell suspensions of 10^6/mL were fixed with Fixation/Permeabilization Diluent (eBioscience, 00-5223-56) for 30 min on ice. Following washing with 10% FBS in phosphate-buffered saline (PBS), cells were incubated with an optimal concentration of rabbit polyclonal anti-alpha smooth muscle actin antibody (Abcam, ab5694, 1:100), rabbit polyclonal anti-SM22 alpha antibody (Abcam, ab14106, 1:100), rabbit monoclonal anti-Caldesmon antibody (Abcam, ab32330, 1:100), mouse monoclonal anti-CD105 antibody (Abcam, ab156756, 1:100), rabbit polyclonal anti-CD73 antibody (Abcam, ab175396, 1:100) or rabbit polyclonal anti-CD45 antibody (Abcam, ab10558, 1:100) diluted in 10% FBS in PBS for 2 h on ice. After washing 2 times with 10% FBS in PBS, cells were incubated with donkey anti-rabbit immunoglobulin (Ig)G heavy and light chains (H&L) (Alexa Fluor 488) preadsorbed (Abcam, ab150065), and donkey anti-mouse IgG H&L (Alexa Fluor 488; Abcam, ab150105) for 2 h on ice. The percentage of cells expressing each cell surface antigen was analyzed with a Becton Dickinson FACSCalibur flow cytometer. Data analysis was performed using FlowJo vX (Tree Star Inc) [1]. Control samples consisted of cells with fluorescein isothiocyanate (FITC)-conjugated rat IgG_{2b} κ isotype control (BD Pharmingen, #556923, 1:100) or Alexa Fluor 647 rat IgG IgG_{2b} κ isotype control (BD Pharmingen, #557691, 1:100) diluted in 10% FBS in PBS.