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Differentiation of Adult Stem Cells into Smooth Muscle for Vascular Tissue Engineering

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Background. **Herein we evaluate the potential of adipose-derived stem cells (ASC) to differentiate into smooth muscle cells (SMC) and their potential for use in a tissue-engineered vascular graft.**

Materials and Methods. **We isolated ASC (CD13 + 29 + 90 +) from the peri-umbilical adipose tissue of patients undergoing vascular surgery, and cultured them in media containing angiotensin II (AngII), sphingosylphosphorylcholine (SPC), or transforming growth factor-beta 1 (TGFβ1) for up to 3 weeks. SMC differentiation was assessed by (1) expression of early (calponin, caldesmon) and late (myosin heavy chain, MHC) SMC markers by RT-PCR, qPCR and Western blot, and (2) contraction upon plating on collagen gel. Differentiated ASCs were seeded onto a vascular graft (decellularized saphenous vein) within a bioreactor, and cell attachment was determined using confocal microscopy.**

Results. **Prior to differentiation, ASC expressed low levels of all three molecular markers. After culture in each differentiating medium, the extent of up-regulation of calponin, caldesmon, and MHC was variable across all cell lines. After seeding onto collagen gel, ASCs differentiated in SPC and TGFβ1 exhibit contractile properties, similar to smooth muscle cell controls. Differentiated stem cells adhered and proliferated on the vascular graft.**

Conclusion. **These data suggest that human adipose-derived stem cells (1) exhibit variable expression of SMC molecular markers after differentiation, (2) exhibit a contractile phenotype after differentiation with SPC and TGFβ1, and (3) proliferate on a vascular graft scaffold. Thus, ASCs are potentially useful in the construction of autologous arteries.**

Key Words: **adult stem cells; adipose tissue; smooth muscle cells; vascular tissue engineering.**

INTRODUCTION

Autologous vascular tissue remains the gold standard conduit for small-diameter (<6 mm) vascular bypass. Many patients afflicted with peripheral arterial disease (PAD) lack suitable autologous tissue needed for bypass due to systemic disease or prior vein harvest [1]. The current alternative for these patients is bypass using a prosthetic graft or cryopreserved allograft, both of which produce inferior results [2, 3]. As a result of this problem, tissue engineering strategies have emerged in an attempt to create a more suitable alternative conduit. The ultimate goal is to create a graft composed of endothelial cells (EC) and smooth muscle cells (SMC) with structure and function similar to native vessels.

Many tissue engineering strategies for creating a bypass graft have used adult stem cells harvested from bone marrow or blood [4–8]. While both of these stem cell populations have shown potential to differentiate into mature cell lineages, cell harvest can be difficult, and availability of these cells is limited by advanced patient age and the presence of comorbidities [5–10]. Adipose tissue has been shown to be an abundant source of stem cells that are easily obtainable [9, 11, 12]. Prior work in our laboratory has shown that adipose-derived stem cells (ASC) are able to differentiate into an endothelial cell lineage that can be seeded onto a bypass graft scaffold [9, 13]. One next step toward the production of a tissue engineered bypass conduit is to focus on ASC differentiation toward a SMC lineage.

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Smooth muscle cells are defined by both molecular markers and function. There are five molecular markers commonly used in the literature to assess for SMCs. These markers are α-smooth muscle actin (ASMA), SM22, calponin, caldesmon, and myosin heavy chain (MHC). α-Smooth muscle actin and SM22 are early markers of developing smooth muscle. They are not specific to a smooth muscle cell lineage and have been shown to be transcribed in pluripotent stem cells. Calponin, caldesmon, and MHC are intermediate to late markers of SMC differentiation and are more specific to a SMC lineage. Smooth muscle cell function includes contraction and production of extracellular matrix.

SMC differentiation has been described in the literature using many different adult stem cell sources, including bone marrow, adipose, and neural crest [14–18]. The majority of these studies have used soluble growth factors to induce differentiation. Factors, such as transforming growth factor β-1 (TGFβ1) [14–16, 18], transforming growth factor β-3 (TGFβ3) [17], angiotensin II (ANG) [18], sphingosylphosphorylcholine (SPC) [17], ascorbic acid [16], and platelet-derived growth factor BB [15, 16], have been used to induce varying degrees of SMC differentiation. Culture conditions differ across the studies, including the concentration of the growth factor and the type of media used. Different stock media are used with varying concentrations of glucose and fetal bovine serum (FBS). Occasionally, the media is enhanced with other supplements in addition to the differentiating agent being evaluated. Additionally, the various studies use different molecular markers to assess differentiation. This lack of consistency across studies makes it difficult to compare and interpret results.

The purpose of this study is to investigate the influence of certain soluble factors on SMC differentiation of ASC. By using a single stem cell type and consistent, standardized culture conditions, our goal is to compare the potential of the soluble factors ANG, SPC, and TGFβ1 to drive ASC toward a SMC phenotype. The SMC phenotype will be assessed on a molecular level by the expression of calponin, caldesmon, and MHC, and functionally based on contractility. We will also explore the potential for use of these differentiated ASC in the creation of a tissue-engineered vascular bypass graft.

MATERIALS AND METHODS

The Thomas Jefferson University Institutional Review Board (IRB) approved of all human studies. All patients gave informed consent prior to tissue donation.

ASC Isolation

Human adipose tissue was collected from the peri-umbilical region of patients undergoing elective vascular surgical procedures *via* liposuction aspiration (Table 1). The adipose tissue was digested in Collagenase I (4 mg/gm tissue; Worthington, Biochemical Corp., Lakewood, NJ) for 1 h at 37°C. Next, it was centrifuged at $1500 \times g$ to separate the cellular component. After discarding the supernatant, the resultant stromal-vascular pellet was suspended in non-differentiating media (Media-199; Mediatech, Herndon, VA) supplemented with 13% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), antibiotic-antimycotic solution (12 mL/L; Mediatech) and heparin (7.5 U/mL). Finally the stromal vascular fraction, containing our ASC population (CD13 + 29 + 90 +), was plated on gelatin-coated culture flasks (1×10^6 cells per flask) at 37°C in 5% CO_2. Previous analysis has shown that greater than 98% of the cell population is CD13 + 29 + 90 + , and that ASC cultures were morphologically homogeneous [13].

Differentiation of ASC

ASC were differentiated toward a smooth muscle cell lineage *via* exposure to soluble factors. Differentiating media consisted of Media-199 (Mediatech), 13% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), antibiotics (12 mL/L; Mediatech), heparin (7.5 U/mL), and one of the following supplements: (1) 1 μM angiotensin II (Sigma-Aldrich, St. Louis, MO), (2) 2 μM sphingosylphosphorylcholine (Matreya, LLC; Pleasant Gap, PA),or (3) 2 ng/mL TGF-β1 (R and D Systems, Inc., Minneapolis, MN) [17, 18]. ASC were cultured in differentiating media for up to 3 wk. Media was changed twice weekly throughout the culture period.

Cell Proliferation Assay and Doubling Time

Cell counts were performed every 24 h for 7 d using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) for ASC ($n = 3$) grown in each of our four culture medias: non-differentiating media (no supplements added), ANG, SPC, and TGFβ1. Starting cell counts were 2×10^4 for each cell population. Doubling time (T_d) was calculated using the following formula: $T_d = (t_1 - t_0)\log 2/\log(N_1/N_0)$ where t_1 is the time of the second cell count, t_0 is the time of the first cell count, N_1 is the number of cells in the second cell count, and N_0 is the number of cells in the first cell count.

Evaluation of Differentiation

Differentiation of ASC was evaluated by (1) expression of SMC markers, and (2) assessment of contractile function. Human arterial smooth muscle cells (American Type Culture Collection, Manassas, VA) were used as positive control and undifferentiated ASC were used as negative control.

Expression of SMC Specific Markers: RT-PCR

Expression of smooth muscle cell markers were identified by isolating total RNA from differentiated cells after 1 and 2 wk in culture. Total RNA was extracted through RNeasy mini columns (Qiagen, Valencia, CA). RNA concentration was obtained *via* spectrophotometer analysis. Reverse transcription was then performed by using the Promega reverse transcription system (Promega, Madison, WI). One μg of total RNA was used in each reaction. The following primer pairs (Operon, Huntsville, AL) were used: Calponin (forward 5′ATGTCCTCTGCTCA CTTCA3′; reverse 5′TTTCCGCTCCTGCTTCTCT3′), Caldesmon (forward 5′AGATTGAAAGGCGAAGAGCA3′; reverse 5′TTCAAGCCAGC AGTTTCCTT3′), MHC (forward 5′GGACGACCTGGTTGTTGATT3′; reverse 5′GTAGCTGCTTGATGGCTTCC3′). Gel electrophoresis was then performed on a 2% agarose gel treated with ethidium bromide and visualized using a UV light box.

Expression of SMC Specific Markers: Quantitative PCR

Total RNA was isolated from differentiated cells after 1 and 2 wk in culture using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. TaqMan gene expression assays targeting the smooth muscle cell markers calponin, caldesmon and MHC were obtained