Fibroblast growth factor and vascular endothelial growth factor play a critical role in endotheliogenesis from human adipose-derived stem cells

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Objective: **Adipose-derived stem cells (ASCs) are a potential adult mesenchymal stem cell source for restoring endothelial function in patients with critical limb ischemia. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) play a major role in angiogenesis and wound healing. This study evaluated the effects of FGF and VEGF on the proliferation, migration, and potential endothelial differentiation of human ASCs with regards to their use as endothelial cell substitutes.**

Methods: **ASCs were isolated from clinical lipoaspirates and cultured in M199 medium with fetal bovine serum (10%), FGF2 (10 ng/mL), VEGF (50 ng/mL), or combinations of FGF2 and VEGF. Cell proliferation rates, viability, and migration were measured by growth curves, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and scratch assays. For cell attachment determinations, ASCs were seeded onto a scaffold of small intestinal submucosa for 5 days. Endothelial differentiation capabilities of ASCs were confirmed by expression of endothelial cell-specific markers using quantitative polymerase chain reaction, immunofluorescence staining, and cord formation on Matrigel (BD Biosciences, San Jose, Calif). PD173074, a selective inhibitor of FGF receptor, was used to confirm the importance of FGF signaling.**

Results: **ASCs treated with FGF or combinations of FGF and VEGF showed increased proliferation rates and consistent differentiation toward an endothelial cell lineage increase in platelet endothelial cell adhesion molecule (CD31), von Willebrand factor, endothelial nitric oxide synthase, and vascular endothelial cadherin message, and in protein and cord formation on Matrigel. FGF and VEGF stimulated ASC migration and increased the attachment and retention after seeding onto a matrix graft of small intestinal submucosa. Blockade of FGF signaling with PD173074 abrogated ASC endothelial cell differentiation potential.**

Conclusions: **These results indicate that FGF and VEGF are ASC promoters for proliferation, migration, attachment, and endothelial differentiation. FGF and VEGF have a costimulatory effect on ASC endotheliogenesis. These results further suggest that ASCs with enhanced FGF signaling may potentially be used for tissue engineering and cell-based therapies in patients with critical limb ischemia. (J Vasc Surg 2016;■:1-10.)**

Clinical Relevance: **Endothelial dysfunction is a primary mediator of vascular disease and critical limb ischemia. Treatment of this disease state is limited by available conduit and the inherent thrombogenicity of bypass grafts. Development of phenotypically and functionally similar endothelial cells from adipose-derived stem cells may allow for the creation of vascular conduits that are readily available and provide increased patency rates. Our study differentiated a cell line from human adipose that could be manipulated into endothelial cells that exhibit characteristics of endothelial cells, providing further information to already existing literature.**

Critical limb ischemia is a clinical condition driven by endothelial dysfunction and injury. Traditional therapy for these patients has been aimed at vascular bypass grafts and endovascular stenting. Conduits for vascular bypasses include autologous vein graft or prosthetic conduit. However, autologous vein is limited, and synthetic conduits are less durable.[1] More recently, cell therapy has been developed as a potential angiogenic therapy.[2] With the advent of cell-based regenerative medicine, a new approach to the treatment of these patients may be at the forefront of future therapeutic options.

Adipose-derived stem cells (ASCs) are able to differentiate into multiple cell lineages, including endothelial, adipogenic, osteogenic, chondrogenic, and myogenic cell lines.[2-13] This has previously been reported to be the result of a cellular milieu of various soluble factors produced by the ASCs themselves.[14] This secretory profile of ASCs is regulated by exposure to different agents.[15,16]

With the prominent role the cellular environment of ASCs plays, in vitro studies have focused on manipulating

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Author conflict of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214

http://dx.doi.org/10.1016/j.jvs.2016.04.034

the culture medium of the ASCs in an effort to direct differentiation patterns in a lineage-specific pattern.[17,18] Studies have shown that by treating ASCs with endothelial growth medium (EGM2), ASCs could be differentiated into endothelial-like cells. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) have both been implicated in this differentiation pathway.[18-20]

FGF is a growth factor involved in angiogenesis, wound healing, embryonic development, and various endocrine-signaling pathways. ASCs cultured in medium supplemented with FGF express morphologic changes of mature endothelial cells and also express endothelial markers.[4] FGF has proliferative effects in multipotent human stem cells,[21-24] and more importantly, FGF controls differentiation of ASCs and is involved with endothelial cell migration.[25]

VEGF is a growth factor that promotes endothelial survival, proliferation, and migration.[26-28] Research has shown that adult stem cells in culture medium supplemented with VEGF express endothelial-specific markers and that inhibition of the VEGF pathway leads to decreased messenger (m)RNA levels of these markers.[28]

In the field of vascular surgery, this multipotency of ASCs that have been treated with growth factors may allow for their application in cell-based regenerative therapies.[2,29,30] To date, some groups have attempted seeding prosthetic graft material but have reported minimal success secondary to the thrombogenic nature of these grafts. This has been partly attributed to the inability of these stem cell-seeded grafts to produce endothelial nitric oxide synthase (eNOS), a critical protein produced by endogenous endothelial cells.[31]

Given the previous findings of the role of growth factors on vasculogenesis and endotheliogenesis, this study was conducted to determine the role of FGF and VEGF in the endothelial behavior of ASCs and contribution of these growth factors to the ability of ASCs to differentiate to endothelial-like cells. This study hypothesized that treatment of cells with FGF or VEGF would stimulate the ASCs to differentiate to endothelial-like cells that are phenotypically and functionally similar to true endothelial cells. Furthermore, the combination of FGF and VEGF (FGF/VEGF) would have a synergistic increase in the activation of stem cells.

METHODS

Isolation and culture of human ASCs. In accordance with an Investigational Review Board-approved protocol, informed consent was obtained from all patients before collection of adipose samples. Adipose tissue was obtained from the abdomen and breast of patients undergoing cosmetic liposuction and breast reduction. Liposuctioned adipose tissue was washed and incubated in collagenase I solution with bovine serum albumin (1 mg/mL + 4 mg/mL; Worthington, Lakewood, NJ) for 1 hour at 37°C. Cells were centrifuged at 1500*g* for 10 minutes and washed with phosphate-buffered solution (PBS). The stromal vascular cells were cultured at 37°C in 5% CO_2 in medium M199 (Mediatech Inc, Manassas, Va) and supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution (Gemini Bio-Products, West Sacramento, Calif). Nonadherent cells were removed after 24 hours, and the culture medium was replaced twice weekly. Cells were split 3:1 when ~80% confluence was reached.

Cell proliferation and doubling time. Proliferation was assessed by constructing growth curves over an 8-day period. ASCs (n = 6) were plated into 24-well plates at 5×10^3 cells/cm^2 with M199 medium and treated with 10 ng/mL FGF, 50 ng/mL VEGF (Gemini Bio-Products), or FGF/VEGF for 8 days with replenishment of medium every 3 to 4 days. At various times, cells were trypsin-released and counted using a Coulter counter (Beckman Coulter Inc, Fullerton, Calif). Doubling time was calculated for the log phase of growth (between days 0 and 8) using the formula doubling time = $(t - t_0)\log 2/(\log N - \log N_0)$, where t and t_0 are the times at which the cells were counted, and N and N_0 are the cell numbers at times t and t_0, respectively. Cells cultured in M199 medium were used as the control. All assays were done in triplicate.

MTT assay. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assay was also used to evaluate the effect of FGF and VEGF on ASC proliferation. ASCs (n = 4) were plated at a density of 1×10^4 cells/well in a volume of 1 mL in 24-well plates. At day 2 and day 6, the culture medium was replaced with a medium containing MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, Mo) and incubated at 37°C in 5% CO_2 for 4 hours. The wells were decanted, and the purple formazan crystals that formed were dissolved in 200 mL dimethyl sulfoxide (Sigma-Aldrich). The absorbance of the plate was read on a microplate reader at 570 nm. Fresh cells were used as controls. All assays were done in triplicate.

Endothelial differentiation and gene expression. ASCs (n = 4) were cultured in M199 medium supplemented with 10% FBS and FGF (10 ng/mL), VEGF (50 g/mL), or FGF/VEGF for up to 10 days. ASCs cultured in M199 medium and EGM2 medium (Lonza, Basel, Switzerland) were used as controls. The medium was exchanged with fresh medium every 3 days. Total RNA was extracted using TRIzol reagent. (Life Technologies, Grand Island, NY). For each sample, 1 μg of RNA was converted to complementary DNA using reverse transcription via the SuperScript II First-Strand Synthesis System (Life Technologies) according to the manufacturer's protocol.

To detect the specific genes expressed, reverse transcription polymerase chain reaction (PCR) was performed using the following primers:

eNOS: 5-primers (5′-TCCCCCAGAACTCTTCCT T-3′) and 3-primers (5′-CTCATTCTCCAGGTGC TTCA-3′);