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The effect of urine-derived stem cells expressing VEGF loaded in collagen hydrogels on myogenesis and innervation following after subcutaneous implantation in nude mice

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ABSTRACT

Impairment of sphincter muscles or their neural and vascular support leads to stress urinary incontinence. The aim of this study was to determine the role of urine-derived stem cells (USCs) over-expressing vascular endothelial growth factor (VEGF) in collagen-I gel on angiogenesis, cell survival, cell growth, myogenic phenotype differentiation of the implanted cells and innervations following implantation in vivo. USCs were infected with adenovirus containing the human $VEGF_{165}$ and green fluorescent protein genes. A total of 5×10^6 cells, USCs alone, or plus endothelial cells or human skeletal myoblasts (as control) suspended in collagen-I gel were subcutaneously implanted into nude mice. Extensive vascularization and more implanted cells was noted in VEGF-expressing USCs groups compared to the non-VEGF groups in vivo. Numbers of the cells displaying endothelial markers (CD 31 and von Willebrand's factor) and myogenic markers (myf-5, MyoD and desmin), and regenerated nerve fibers displaying neural markers (S-100, GFAP and neurofilament) significantly increased in the grafts of VEGF-expressing USCs. Improved angiogenesis by VEGF-expressing USCs enhanced grafted cell survival, recruited the resident cells and promoted myogenic phenotype differentiation of USCs and innervation. This approach has important clinical implications for the development of cell therapies for the correction of stress urinary incontinence.

1. Introduction

Autologous cells or mesenchymal stem cell injection therapy for stress urinary incontinence (SUI) has recently offered a promising alternative for repair of urethral sphincter function. Major advantages of autologous cells are avoiding adverse events related to the rejection of implanted allogenous tissues, and bypassing ethical concerns about use of embryonic stem cells [1]. Ear chondrocytes [2], muscle-derived progenitor cells [3], bone marrow-derived mesenchymal stromal cells [4], and adipose-derived stem cells [5] are regarded as candidates for this therapy. However, harvesting stem cells from these tissues causes potential complications such as donor site morbidity. Moreover, local anesthesia or general anesthesia is needed. To eliminate these complications and to generate a large amount of cells with low medical costs, an autologous stem cell source obtained with non-invasive procedures would be desirable.

Recently, we have successfully established a primary culture system to isolate and repopulate stem cells from urine, called urine-derived stem cells (USCs) [6–12]. These cells can grow up from a single cell to a large amount of cells (1×10^{14} cells) at passage 14 after 8 weeks [10]. After optimizing our methods, up to 140 USC clones/24 hour's urine can be consistently obtained from each individual [6]. In a previous report, 5×10^7 skeletal muscle-derived stem cells in 4 ml of solution were required to treat SUI with an endoscopic injection [13]. Therefore, 3.7×10^8 cells generated from one urine sample (containing 10–15 USC clones/200 ml urine) at

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low passage (<$p4$) within 3 weeks [10] is enough for use in cell injection for SUI [12]. Additionally, these cells exhibit pericyte/mesenchymal stem cell markers and detectable levels of telomerase. USCs also can give rise to mesoderm cell lineages, like osteocytes, chondrocytes, adipocytes, myocytes, and endothelial cells [14]. These cells can be obtained non-invasively at a low cost, using a simple technology to harvest good-quality cells that can be expanded to the required quantity [7]. To retain high rates of donor cell viability and function in vivo, angiogenic gene manipulation is frequently used in cell-based therapy. Several studies have reported that both mesenchymal stem cells and differentiated cells that express VEGF can improve cell survival, angiogenesis, and myogenesis [15]. As mesenchymal stem cells possess angiogenic and anti-fibrotic effects, they are a good cell source for treatment of SUI.

The injectable hydrogels applied for tissue repair are classified into synthetic and natural biomaterials, including (1) synthetic materials (i.e. Poly(organophosphazene) hydrogels); (2) the body's own polymers (i.e. collagen gel, hyaluronic acid); (3) polymers in nature (i.e. hydrated chitosan gels); (4) self-assembled peptides (i.e. peptide amphiphile system); and (5) new innovations and combinations (i.e. coating a poly lactic acid microcarrier with collagen) [16,17]. Collagen as a natural extracellular matrix proteins is widely distributed in the human body [18]. As a hydrogel with high water content, collagen type I (collagen-I) gel is most commonly used as a cell delivery vehicle in cell injection therapy because of its advantages in being biodegradable, non-cytotoxic, easily injectable and highly versatile in tissue repair [19].

Improving local blood circulation, promoting muscle tissue engineering, and recovering peripheral nerve function are three critical criteria for correction of urethra sphincter muscle defects, particularly with sever scarred tissues or lack of blood supply in the neighboring tissue of urethral sphincter. Our hypothesis for this study is that VEGF-expressing USCs plus endothelial cells in collagen-I gel can contribute to a better outcome in all three elements. The objective of this study was to systematically evaluate angiogenesis in VEGF-expressing USCs in collagen-I hydrogel and its effect on the myogenic differentiation and neo-innervation in quantification fashion and to determine whether improved revascularization could improve injection therapy for potential in the correction of SUI.

2. Materials and methods

2.1. Ethical approval

Protocols for human urine collection, umbilical cord, and human skeletal muscle samples used in this study were approved by the Wake Forest University Health Sciences Institutional Review Board. Written informed consents have been obtained and were approved by Wake Forest University institutional review board. Experiments using nude mice were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

2.2. USCs isolation and expansion

Twenty-two voided urine samples (100–400 ml/sample) were collected from five healthy individuals (male, 25–40 years old). Each fresh urine specimen was immediately transferred to the laboratory for isolation and culture as reported previously [7]. Briefly, urine samples were centrifuged at 500 × g for 5 min and the supernatant was removed. The cell pellet was gently resuspended in mixed media composed of embryo fibroblast medium (EFM) and keratinocyte serum-free medium (KSFM) (1:1 ratio) [7] and plated in 24-well plates (p0). Individual single USCs appeared 3–5 days after initially plated and reached a confluence of 70–80%, 3–4 days late. The cells were trypsinized, and transferred into 6-well dishes (p1). Finally, cell cultures were transferred to a 100 mm culture dish (p2) for expansion. For most experiments, USCs at p3–4 were used.

2.3. Human umbilical cord endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated by brief perfusion of enzyme solution into umbilical cord veins [20]. HUVECs were then cultured on fibronectin-coated plates (Millipore, Billerica, MA) using Endothelial Growth Medium-2 (EGM2) (Lonza Biologics, Portsmouth, NH) containing 2% fetal bovine serum (FBS) and supplements, including VEGF (2 ng/ml), epidermal growth factor, hydrocortisone, GA-1000 (gentamicin, amphotericin-b), fetal bovine serum, basic fibroblast growth factor, insulin-like growth factor, ascorbic acid, and heparin at manufacturer concentrations at 37 °C in a 5% CO_2 cell incubator.

2.4. Human skeletal myoblast culture

Human myoblasts were isolated from chopped skeletal muscle tissue (1 mm × 1 mm) by incubation in 10 ml of Collagenase-II (0.1% w/v)-Dispase (4 mg/ml) solution prepared in DMEM for 1 h at 37°C with constant shaking (60 rpm). The liberated cells were collected (400 × g) and washed with DMEM medium containing 10% horse serum and plated into a 6-well tissue culture dish. Two hours after, the supernatant from the dish was transferred to another well and the process repeated. Five days after culture, the media was changed to SkGM2 (Lonza Biologics, Portsmouth, NH) containing 10% FBS at 37 °C in a 5% CO_2 cell incubator.

2.5. Infection of cells with adenoviral vectors

The recombinant E1-deleted adenoviral vector encoding green fluorescent protein (GFP) and human $VEGF_{165}$ under the cytomegalovirus promoter (Ad-GFP/VEGF) was obtained from the Harvard Human Gene Therapy Initiative (Boston, MA). Multiplicity of infection was optimized by using the virus at different dilutions as we have described previously [12]. USCs were seeded in a 6-well plate at 2000/cm^2, and infected with the adenoviral vector expressing VEGF (Ad-GFP/VEGF) at multiplicities of infection (MOI) from 50 to 1000. The MOI that generated maximum transfected efficiency with minimum cellular toxicity was chosen for further experiments.

2.6. Angiogenic growth factors secreted by USCs

To measure angiogenic trophic factors secreted by USCs before VEGF gene transfection, totally 5 × 10^5 USCs at passage 2 were seeded at 6-well plates and were incubated with serum-free DMEM under normal condition (5% CO2, 37°C) for 24 h. The conditioned medium was analyzed by human angiogenesis array kit (R&D Systems) according to the manufacturer's instructions. Briefly, the membrane containing 55 angiogenesis-related antibodies was blocked with bovine serum albumin for 1 h on a rocking platform at room temperature. Membrane was then incubated with culture supernatants from USCs, along with detection antibody cocktail overnight on a rocking platform at 4 °C. The membrane was incubated with streptavidin-horseradish peroxidase conjugate antibody and developed on X-ray film following exposure to chemiluminescent reagents. Pixel density was produced by Quantity One software® and was normalized to the mean pixel density from reference spots of its own membrane.

2.7. Enzyme-linked immunosorbent assay

To evaluate VEGF secreted by gene-modified USCs, culture media of VEGF-expressing USCs were replaced every 2 days and aspirated at Days 1, 5, 10, 15, 20, 25 and 30 for VEGF assays by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, VEGF standards were also used simultaneously for generating a standard curve. Media samples were diluted (100–1000 folds) before use to remain within the range of the standard. Finally, after color development, the microplate was read at 450 nm (Molecular Devices, Spectramax-M5) to calculate VEGF concentrations.

2.8. In vivo implantation

For preparation of cell injection, USCs (p3) from 5 donors were trypsinized and 4 × 10^6 cells were seeded in a 15 cm^2 dish, respectively. To infect cells with Ad-GFP/VEGF, adenovirus was added into 5 ml of DMEM high glucose (serum free) culture media and incubated for 2 h at 37 °C in a 5% CO_2 incubator. After the virus-containing medium was removed, the USCs were incubated in normal culture medium for another 14–18 h before collecting for injection.

Table 1
Research design.

Groups(G)	Injections of cell − collagen-I gel	Number of graft/number of animal
G1	USCs-GFP/VEGF-ECs	24/12
G2	USCs-GFP/VEGF	24/12
G3	USCs-GFP	22/11
G4	Human myoblasts	10/5
G5	Cell-free	10/5