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Skeletal myogenic differentiation of urine-derived stem cells and angiogenesis using microbeads loaded with growth factors

Guihua Liu [a,1], Rajesh A. Pareta [a,1], Rongpei Wu [a,b], Yingai Shi [a], Xiaobo Zhou [c], Hong Liu [d], Chunhua Deng [b], Xiangzhou Sun [b], Anthony Atala [a], Emmanuel C. Opara [a], Yuanyuan Zhang [a,*]

[a] Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA
[b] Department of Urology, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, GuangDong 510080, China
[c] Radiology/Translational Biology Department, The Methodist Hospital Research Institute, Houston, TX 77030, USA
[d] Center for Bioengineering and School of Electrical and Computer Engineering, University of Oklahoma, Norman, OK 73019, USA

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ABSTRACT

To provide site-specific delivery and targeted release of growth factors to implanted urine-derived stem cells (USCs), we prepared microbeads of alginate containing growth factors. The growth factors included VEGF, IGF-1, FGF-1, PDGF, HGF and NGF. Radiolabeled growth factors were loaded separately and used to access the *in vitro* release from the microbeads with a gamma counter over 4 weeks. *In vitro* endothelial differentiation of USCs by the released VEGF from the microbeads in a separate experiment confirmed that the released growth factors from the microbeads were bioactive. USCs and microbeads were mixed with the collagen gel type 1 (2 mg/ml) and used for *in vivo* studies through subcutaneous injection into nude mice. Four weeks after subcutaneous injection, we found that grafted cell survival was improved and more cells expressed myogenic and endothelial cell transcripts and markers compared to controls. More vessel formation and innervations were observed in USCs combined with six growth factors cocktail incorporated in microbeads compared to controls. In conclusion, a combination of growth factors released locally from the alginate microbeads induced USCs to differentiate into a myogenic lineage, enhanced revascularization and innervation, and stimulated resident cell growth *in vivo*. This approach could potentially be used for cell therapy in the treatment of stress urinary incontinence.

1. Introduction

Stress urinary incontinence (SUI) is most common in people older than 50 years of age; these are primarily women, but there are an increasing number of male patients as well [1,2]. Urinary incontinence affects up to 13 million people in the United States and 200 million worldwide. The cost of treating urinary incontinence in United States alone is $16.3 billion annually [3]. SUI is associated with the loss of various amounts of urine when intra-abdominal pressure increases due to dysfunction of the urethral sphincter or the pelvic floor muscles. Besides pharmacotherapy [4], several invasive surgical therapies, including sling surgical procedures [5] and injection of bulking agents [6], have been commonly used to treat SUI. Sub-urethral slings, such as the transvaginal or transobturator tape procedures, have about 71–72.9% success rates [5]. Although the sling procedure can enforce the weakness of pelvic floor muscles, the urethral sphincter deficiency remains [7]. Bulking procedures are particularly useful for treating SUI in patients who wish to avoid open surgical procedures [6]. A variety of biomaterials, such as bovine collagen [8], calcium hydroxyapatite, silicone [9], carbon beads [10] polydimethylsiloxane (Macroplastique), and polytetrafluoroethylene (PTFE; Teflon) [11], have been used to insert bulk around the urethra and thereby raise its outlet resistance. This provides closure of the sphincter without obstructing it, and is most effective in patients with a relatively fixed urethra. Although injection of bulking agents has provided encouraging outcomes, over time these agents are absorbed and can cause several complications, such as chronic inflammation, periurethral abscess, foreign body giant cell responses, erosion of the urinary bladder or the urethra, migration to inner organs, obstruction of the lower urinary tract with resultant urinary retention, severe voiding dysfunction, and even pulmonary embolism [6,12–14].

Cell-based therapy is an alternative to restore deficient urethral sphincter function in the treatment of SUI. Several investigations

* Corresponding author. Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA. Tel.: +1 336 713 1189; fax: +1 336 713 7290.
E-mail addresses: yzhang@wfubmc.edu, yzhang@wakehealth.edu (Y. Zhang).
[1] Both authors made equal contributions.

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have focused on autologous stem cells derived from skeletal muscle [15], bone marrow [16] or fat tissues [17], with success rates ranging from 12 to 79% [18]. To obtain these stem cells, invasive tissue biopsy procedures are usually involved, with an attendant risk of complications. We recently demonstrated that stem cells exist in human voided urine or urine drained from upper urinary tract. These cells, termed urine-derived stem cells (USCs), possess stem cell characteristics with robust proliferative potential and multi-potential differentiation [19–21]. These cells can be obtained using simple, safe, non-invasive and low-cost procedures, thus avoiding the adverse events associated with obtaining cells from other sources. Our recent studies demonstrated that adding exogenous angiogenic factors, such as transfection of the VEGF gene, significantly promoted myogenic differentiation of USCs and induced angiogenesis and innervation. However, VEGF delivered by virus caused several side effects in our animal model, including hyperemia, hemorrhage, and even animal death [22]. Thus, it is desirable to employ a safer approach in stem cell therapy to increase angiogenesis and promote muscle regeneration.

Biodegradable polymers, specifically hydrogels that deliver molecules in a controlled fashion, can be beneficial as delivery vehicles to promote regeneration and tissue healing [23]. Alginate is one of the most commonly used natural hydrogels as an aqueous drug carrier for encapsulation because of its mild gelling conditions and tunable microbead characteristics. Since alginate is a hydrophilic and negatively-charged polymer, alginate microspheres also resist protein adsorption thus making them attractive for *in vivo* studies [24]. Alginate microbeads have been shown to stably release active FGF-1 for at least 3 weeks *in vitro*, and this sustained release of FGF-1 promoted neovascularization *in vivo* without any side effects [25–27].

Our more recent data showed that USCs display myogenic and endothelial differentiation capacity when cultured in media containing the associated growth factors [28,29]. Based on these observations, we hypothesized that skeletal myogenic, anigogenic, and neurogenic growth factors released from alginate microbeads can induce USCs to give rise to a skeletal myogenic lineage, improve revascularization and innervations, and recruit resident cells to take part in tissue repair. Therefore, in the present study, we examined whether a synergistic mixture of growth factors could be released efficiently in a controlled manner from alginate microbeads, thus guiding USCs to cell differentiation and enhancing tissue regeneration for potential use in cell therapy of SUI.

2. Materials and methods

2.1. Preparation of alginate microbeads

A low-viscosity (<20 m Pas) ultrapure alginate with high guluronic acid (LVG) content (minimum 60% guluronate monomer units) was used for this study (Nova Matrix, Sandvika, Norway). LVG (1.5 wt %) was prepared in calcium free minimum essential medium (MEM) and stored at 4 °C till further use. The LVG microbeads were generated using an eight nozzle flow-focusing device at the flow rate of 1.4 ml/min and 1.5 psi air pressure. These microbeads were collected in a calcium chloride solution (1.1 wt %) and allowed to cross-link for 15 min. These microbeads were washed three times with calcium containing Hank's buffered salt solution (HBSS). The amounts of growth factors to be loaded in alginate beads were determined according to the effective dose (ED 50) provided by the manufacturer. A solution of 100 μg/ml PDGF-BB (4 μg) and 100 μg/ml HGF (10 μg) served as a skeletal myogenic promoter; 100 μg/ml VEGF (7 μg) as the angiogenesis inducer; and a combination of 1 mg/ml IGF (14 μg), 10 μg/ml NGF (0.5 μg), 300 μg/ml FGF-1 (1 μg) to promote innervation. Five units/ml heparin was added to the initial growth factor solutions. To preload the microbeads with growth factors, about 0.5 g of capsules was incubated overnight (24 h) with 0.5 ml of growth factor solutions in an Eppendorf tube on a shaker at 4 °C. The supernatant was removed and the microbeads were washed three times with HBSS (with Ca^{2+}) to remove non-incorporated growth factors. To control the release of growth factors from the microbeads we coated a semi-permeable membrane of poly-L-ornithine (PLO). Just washed growth factor loaded microbeads were incubated in 0.1 wt% PLO solution in HBSS (with Ca^{2+}) for 10 min at 4 °C followed by triple wash. Finally we incubated the microbeads in 0.2 wt% ultrapure alginate with high mannuronic acid (LVM, minimum 60% mannuronate monomer units) for 5 min at 4 °C followed by triple wash, to get alginate-PLO-alginate (APA) growth factor loaded microbeads. These will be addressed as just alginate microbeads throughout this manuscript. Each growth factor mixture was decreased to one-third of the original amount when these three parts were combined, to document synergistic effects (Table 1).

Table 1
Research design.

Groups (G)	Function groups	Injections of Cell–Microsphere beads in collagen I gel	Doses of growth factors/injection	Number of grafts/ Number of animals
G1	Control 1	Cell-free/empty beads	0	8/2
G2	Control 2	Human Myoblasts/ empty beads	0	8/2
G3	Control 3	USCs/empty beads	0	8/2
G4	Control 4	USCs-EC/empty beads	0	8/2
G5	Control 5	Cell-free + Beads with IGF/NGF/ FGF-1 + VEGF + PDGF/ HGF	IGF (43.75 ng), NGF (7.8 ng), FGF-1(15.625 ng), VEGF (21.875 ng), PDGF-BB (12.5 ng), HGF (62.5 ng)	8/2
G6	Myogenic	USCs + Beads with PDGF-BB/HGF	PDGF-BB (37.5 ng), HGF (187.5 ng)	8/2
G7	Angiogenic	USCs + Beads with VEGF	VEGF (65.6 ng)	8/2
G8	Neurogenic	USCs + Beads with IGF/NGF/FGF-1	IGF (131.25 ng), NGF (23.44 ng), FGF-1(46.875 ng)	8/2
G9	Synergetic 1	USCs + Beads with IGF/NGF/FGF-1 + VEGF + PDGF/HGF	The same dose as described in G5	16/4
G10	Synergetic 2	USCs-ECs + Beads with IGF/NGF/FGF-1 + VEGF + PDGF/HGF	The same dose as described in G5	16/4

2.2. Measurement of growth factor release in vitro

The growth factor release efficiency was evaluated *in vitro* when single, bi- or multi- combined growth factors were loaded within alginate microbeads. I-125-labeled (VEGF and IGF, Phoenix Pharmaceuticals, Inc.) and unlabeled NGF and FGF-1 (Protech) growth factors were loaded in the microbeads to investigate the *in vitro* release of growth factors. To measure the release kinetics of I-125-labeled growth factors incorporated in alginate microbeads, the microbeads were suspended in 0.5 ml of HBSS (with Ca^{2+}) and incubated at 37 °C. The supernatant was replaced fully at pre-determined time points (Fig. 1) and radioactivity between two

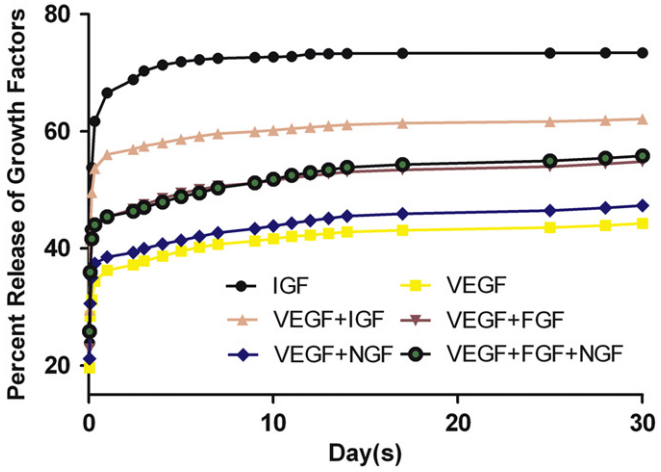

Fig. 1. Controllable release curve of alginate beads *in vitro*. The microbeads loaded growth factors, including I-125 radiolabeled VEGF, IGF and unlabeled FGF-1, NGF, were released quickly in the first few days after overnight incubation, regardless of the radiolabel. When two or more growth factors were incorporated, no significant change in the release kinetics was seen.