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Research paper

Promoted regeneration of mature blood vessels by electrospun fibers with loaded multiple pDNA-calcium phosphate nanoparticles

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ABSTRACT

Vascularization is one of the capital challenges in the establishment of tissue engineering constructs and recovery of ischemic and wounded tissues. The aim of this study was to assess electrospun fibers with loadings of multiple pDNA to allow a localized delivery for an efficient regeneration of mature blood vessels. To induce sufficient protein expression, a reverse microemulsion process was adopted to load pDNA into calcium phosphate nanoparticles (CP-pDNA), which were electrospun into fibers to achieve a sustained release for 4 weeks. Compared with pDNA-infiltrated fibers, the localized and gradual release of pDNA facilitated cell proliferation, gene transfection, and extracellular matrix secretion and enhanced the generation of blood vessels after subcutaneous implantation. Compared with commonly used pDNA polyplexes with poly(ethyleneimine), CP-pDNA nanoparticles induced significantly lower cytotoxicity and less inflammation reaction after implantation into animals. Fibers with encapsulated nanoparticles containing plasmids encoding vascular endothelial growth factor (pVEGF) and basic fibroblast growth factors (pbFGF) led to significantly higher density of mature blood vessels than those containing individual plasmid. It is suggested that the integration of CP-pDNA nanoparticles with loadings of multiple plasmids into fibrous scaffolds should provide clinical relevance for therapeutic vascularization, getting fully vascularized in engineered tissues and regeneration of blood vessel substitutes.

1. Introduction

The creation of adequate blood vessels remains an essential prerequisite for the establishment of tissue engineering constructs, which require adjacent capillary networks to provide delivery of nutrients and removal of waste products. In addition, the capability to selectively promote accumulation of precursor cells and generation of new blood vessels from existing vessels is a critical consideration in therapeutic angiogenesis for the treatment of ischemic diseases. However, such vascularization networks cannot be generated spontaneously, and vascular regeneration involves a complicated process, which includes the mobilization, adhesion, proliferation, and differentiation of progenitor cells [1]. Thus, it is essential to develop three-dimensional (3D) scaffolds by mimicking the relevant properties of extracellular matrices (ECM) and provide abundant angiogenic growth factors required to initiate and sustain neovascularization.

The soluble growth factors typically have a short half-life, leading to their rapid degradation upon direct administration, which makes it necessary to load them into tissue engineering scaffolds to achieve a localized and sustained release [2]. Anderson et al. covalently bound vascular endothelial growth factor (VEGF) to nanoparticles, leading to an increase in the endothelial cells tube branching and thickness and the total vessel network length compared to soluble growth factors [3]. Anne des Rieux et al. enhanced the efficiency of VEGF by encapsulation into nanoparticles and controlled release from 3D implants to sustain cell infiltration and organization and stimulate blood vessel formation [4]. But one major concern is the maintenance of the structural integrity and biological activity of growth factors during processing and storage of the controlled release systems, and after exposure to enzymatic and acidic microenvironment created by the acidic by-products of polymer degradation [5]. The potential activity loss usually requires high dosages loaded into the delivery system, which creates potential exposure to toxic levels of protein if unexpected dose dumping occurs. The use of plasmid DNA (pDNA) to produce growth factors in transfect cells provides a powerful alternative, is generally advantageous in long-term effects, and has low costs

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compared to purified protein delivery. Although naked DNA achieved gene expression and guided tissue regeneration in porous scaffolds [6], limitations with low gene transfer efficiency and rapid diffusion of pDNA from the scaffold motivated the use of DNA complexes instead of free pDNA. Guo et al. prepared complexes of VEGF-encoded pDNA (pVEGF) with N,N,N-trimethyl chitosan chloride, which were loaded into a bilayer porous collagen–chitosan/silicone membrane as dermal equivalents for treatment of full-thickness burn wounds. The loading of pDNA complexes resulted in a significantly high number of newly formed and mature blood vessels and fast dermal regeneration [7].

Cationic lipids or polymers have previously been introduced to condense pDNA and incubated in tissue engineering scaffolds. Liposomal systems have been frequently applied in vitro, but require strict conditions including correct cell density and components of the culture media, so it is difficult to apply this method in vivo [8]. Poly(ethyleneimine) (PEI) is a widely utilized cationic polymer for nonviral gene delivery, but suffers from noticeable cytotoxicity and immune reactions, thereby reducing the clinical relevance [9]. Calcium phosphate, the major component of hard tissues, exhibits a high binding affinity with pDNA most likely due to interactions between calcium ions and the negatively charged phosphate groups in pDNA, and thus may impart a stabilizing function to certain pDNA structures [10]. One advantage of calcium phosphate as a drug delivery vehicle is that calcium phosphate-pDNA (CP-pDNA) complexes can be carried across cell membrane via ion channel mediated endocytosis [11]. Another major advantage is that calcium phosphate is relatively insoluble at physiological pH, but has increasing solubility in the acidic environments that occur in endosome and lysosome, facilitating the intracellular delivery of pDNA [12]. Curtin et al. incorporated calcium phosphate nanoparticles containing pDNA encoding bone morphogenetic proteins into collagen-based scaffolds, and the osteogenesis was enhanced when using low levels of plasmids demonstrating their innate capacity for promoting bone formation [13].

Electrospun fibers have attracted a great deal of attention, especially in biomedical fields, as scaffolds for tissue engineering and drug delivery because of the similar skeletal structure to ECM, flexible variation in morphology, interconnected pores, and high specific surface area, and have shown to be effective for local and sustained delivery of bioactive signals [14]. To induce sufficient protein production, pDNA was incorporated into electrospun fibers through electrostatic layer-by-layer deposition of polycations and pDNA [15] or surface deposition of pDNA/chitosan nanoparticles [16]. But a significant initial release of pDNA nanoparticles led to high cytotoxicity and short-term gene expression [16]. Alternatively, emulsion electrospinning was initially applied to prepare core–sheath structured fibers with the core-loading of PEI–pDNA polyplexes [17]. The gradual release of pDNA and sufficient expression of growth factors enhanced skin wound healing in diabetic rats [18] and promoted the generation of blood vessels [19]. But significantly low cell viability, strong inflammation, and necrotic tissues were indicated for electrospun fibers with loaded PEI–pDNA polyplexes [19].

This study was aimed to assess biodegradable electrospun fibers with loadings of CP-pDNA nanoparticles to allow a localized delivery for an efficient vascularization. It was indicated that the transfection efficiency of standard preparations of CP-pDNA precipitates decreased over time as the inorganic crystals continued to grow and aggregate, becoming too large for endocytosis by cells [20]. Organic and inorganic additives and multiple-layer structures were then adopted to preserve the small size of calcium phosphate particles and to inhibit their further growth [20]. In the current study, the incorporation of calcium phosphate nanoparticles into electrospun fibers was supposed to maintain the size stability and transfection efficiency over time. Although a variety of growth factors are known to be involved, VEGF and basic fibroblast growth factor (bFGF) are the key mediators of new blood vessel growth which makes them particularly interesting for their use in blood vessel engineering and therapeutic angiogenesis [21]. Therefore, hydroxyapatite (HA) nanoparticles with encapsulated pVEGF and bFGF-encoded plasmid (pbFGF) were synthesized using reverse microemulsions. Electrospun fibers with loadings of the nanoparticles were prepared, and pDNA release was modulated to adapt to the duration for blood vessel regeneration. The cellular behaviors, including cell adhesion, proliferation, transfection efficiency, and ECM secretion, were evaluated in vitro on endothelial cells (ECs) and smooth muscle cells (SMCs). The extent of vascularization and vessel maturation was clarified after subcutaneous implantation into Sprague–Dawley rats via hematoxylin–eosin (HE) and immunohistochemical (IHC) staining.

2. Materials and methods

2.1. Materials and cells

Poly(DL-lactide)–poly(ethylene glycol) (PELA, M_w = 42.3 kDa, M_w/M_n = 1.23) was prepared by bulk ring-opening polymerization of DL-lactide using 10% (wt/wt) of dihydroxyl-poly(ethylene glycol) (PEG, M_w = 6 kDa) as macroinitiator [22]. The pVEGF, encoding fusion protein of VEGF/enhanced green fluorescent protein (eGFP), and pbFGF, encoding bFGF/eGFP fusion protein, were purchased from FulenGen Co. (Guangzhou, China). The plasmids were grown in *Escherichia coli* DH5α using LB growth media, purified using Qiagen Giga kit (Hilden, Germany), and stored in Tris–EDTA buffer (TE, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 4 °C. All the electrophoresis reagents, PEG (M_w = 2, 4, and 6 kDa), PEI (M_w = 25 kDa), bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and bisbenzimide (Hoechst 33258) were procured from Sigma (St. Louis, MO). Protein molecular weight marker and RIPA lysis buffer were from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit antihuman antibodies of collagen I, collagen IV, laminin, α-smooth muscle actin (α-SMA), and β-actin, rabbit anti-mouse antibodies of CD31, collagen IV and α-SMA, goat anti-rabbit IgG-horseradish peroxidase (HRP), IgG-fluorescein isothiocyanate (FITC), and 3,3′-diaminobenzidine (DAB) developer were purchased from Boster Bio-engineering Co., Ltd. (Wuhan, China). All other chemicals were of analytical grade and received from Changzheng Regents Company (Chengdu, China) unless otherwise indicated. Human umbilical vein ECs and human aortic SMCs were from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco BRL, Grand Island, NY).

2.2. Preparation of CP-pDNA nanoparticles

CP-pDNA nanoparticles were prepared following a published report with some modifications [23]. Briefly, two separate microemulsions (A and B) were formed with a cyclohexane/polyoxyethylene octylphenol ether (OP-10)/water system, in which cyclohexane acted as the oil phase and OP-10 was the surfactant. 0.5 mL $Ca(NO_3)_2$ aqueous solution (0.25 M) containing 250 μg pDNA was emulsified into 15 mL cyclohexane containing 10% (v/v) OP-10 by continuous stirring for 1 h to form microemulsion A. Similarly, microemulsion B was formed by emulsification of 0.5 mL $(NH_4)_2HPO_4$ aqueous solution (0.15 M) containing 250 μg pDNA into 15 mL cyclohexane containing 10% (v/v) OP-10. Microemulsion B was slowly added to microemulsion A at the rate of