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Co-delivery of FGF-2 and G-CSF from gelatin-based hydrogels as angiogenic therapy in a murine critical limb ischemic model

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Abstract

Peripheral artery disease and critical limb ischemia have become prevalent health risks in the United States due to an increasing elderly population and the prevalence of obesity and diabetes mellitus. Although highly invasive endarterectomy is the most popular method for treatment, angiogenic therapies based on growth factor administration are quickly becoming a popular alternative. Enzymatic degradation of these factors in vivo may be avoided by their incorporation in a delivery vehicle where the growth factor's release rate can be controlled by altering the vehicle's properties (i.e. cross-linking density, material selection, biodegradation, etc.). Herein, we report on the immobilization and controlled release of human recombinant basic fibroblast growth factor (FGF-2) and human recombinant granulocyte colony-stimulating factor (G-CSF) from ionic, gelatin-based hydrogel scaffolds to re-establish perfusion and induce capillary outgrowth in a murine hindlimb ischemic model. In vitro studies showed that endothelial cell proliferation was highly depended on FGF-2, whereas G-CSF stimulated migration and formation of a tubular network. When FGF-2 and G-CSF were used in combination there was an 82% increase in endothelial branch point formation compared to control groups. Leg reperfusion was assessed with laser Doppler perfusion imaging, while capillary outgrowth in the ischemic leg was evaluated using $CD31^+$ and α-SMA immunostaining. The co-delivery of G-CSF (1000 ng ml^{-1}) and FGF-2 (1000 ng ml^{-1}) from the gelatin hydrogels resulted in a 3-fold increase in the perfusion levels and a 2-fold increase in capillary density and positive α-SMA vessels compared to the empty vehicle group. In conclusion, the co-delivery of FGF-2 and G-CSF was superior to bolus administration or the delivery of either factor alone in promoting reperfusion and mature vessel formation.

Keywords: Angiogenesis; Hydrogels; Controlled delivery; Ischemia; Basic fibroblast growth factor

1. Introduction

Peripheral artery disease (PAD) afflicts more people today because of the prevalence of diabetes mellitus and the aging population [1]. Endarterectomy is currently the most available treatment option for patients; however, this method is highly invasive [2]. Therapeutic angiogenesis induced by growth factor administration is a promising non-invasive treatment option for PAD because it allows for capillary outgrowth [3], re-establishes perfusion [4] and restores tissues to normoxic conditions [5]. The delivery of exogenous growth factors, such as basic fibroblast growth factor (FGF-2) and granulocyte colony-stimulating factor (G-CSF), has shown a marked capability in restoring regional blood flow and recovering ischemic tissues [3–5]. FGF-2 is a single-chain polypeptide that is mitogenic and chemotactic for both fibroblasts and endothelial cells, which play significant roles during angiogenesis [6]. Additionally, FGF-2 stimulates wound healing and augments collateral artery development to relieve peripheral ischemia [7]. G-CSF facilitates survival, proliferation and differentiation of all cells

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within the neutrophil lineage and assists in the amelioration of infections. G-CSF is a potent mobilizer of hematopoietic stem cells from the bone marrow, has a neuroprotective effect, activates endothelial proliferation and stimulates angiogenesis via mobilizing bone marrow stem cells (BMSCs) (in particular, endothelial progenitor cells, EPCs) to the ischemic site [5,8–10]. Although administration of single growth factors may induce angiogenesis, multiple triggers may be necessary to form mature vasculature. Recent studies have suggested that by using two or more growth factors in combination may in fact promote mature vessel formation [11,12]. Growth factors administered via bolus injections can be rapidly metabolized, promote immature vasculature formation or cause systemic toxicity due to improper dosing.

An approach to circumvent problems associated with bolus delivery is the localized and controlled delivery of growth factors at the site of injury. Controlled delivery vehicles based on synthetic [13–15] and natural biomaterials [16,17] have been designed for the sustained delivery of growth factors [18–20], or plasmid DNA [21,22]. Polymer-based controlled delivery vehicles are excellent candidates for growth factor or gene delivery because they can provide spatial and temporal control of presenting the therapeutic agent at the site of interest, are non-immunogenic and can be prepared with tunable biodegradability [20,21,23,24]. In addition to polymer-based delivery vehicles, the use of plasmids or viral vectors for the delivery of angiogenic cytokine encoding genes has been employed in a number of peripheral artery disease/critical limb ischemic models with notable success [25–28]. Although viral vectors can successfully deliver angiogenic genes at the ischemic site with acceptable transfection rates, issues with patient safety, controlling the dosage of the growth factor being expressed and developing a switch-off mechanism upon repair have so far prevented the clinical use of gene therapy in therapeutic angiogenesis.

Herein, we report on the co-delivery of FGF-2 and G-CSF from ionic gelatin-based hydrogels and their synergistic effect in establishing reperfusion and mature vessel formation in a murine critical limb ischemic model. We envision that the timely release of FGF-2 and G-CSF could facilitate angiogenesis, with FGF-2 directing endothelial migration and proliferation, and G-CSF inducing endothelial progenitor cell migration to the wound site [29]. Gelatin was selected as the primary component of the delivery vehicle because of its known cytocompatibility and its ability to stabilize proteins via poly-ion complexation and assist in endothelial cell matrix remodeling [30].

2. Methods and materials

2.1. Materials

Gelatin B from bovine skin (p$I = 5.0$; ~225 bloom), poly-L-glutamic acid sodium salt (PLG; mol. wt. = 50,000–100,000 Da), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (St Louis, MO) at their highest purity. Human recombinant basic fibroblast growth factor (FGF-2; mol. wt. = 16,000 Da; p$I = 9.6$) and granulocyte colony-stimulating factor (G-CSF; mol. wt. = 18,800 Da; p$I = 6.2$) were purchased from Peprotech (Rocky Hill, NJ). Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Manassas, VA). All other chemicals were purchased from Sigma–Aldrich at their highest grade purity unless otherwise noted.

2.2. Hydrogel synthesis

Ionic, gelatin-based hydrogels were prepared from the polymerization of gelatin and poly-L-glutamic acid (PLG) via amide bond formation in the presence of the EDC/NHS activation catalysts [18]. Briefly, hydrogels were synthesized by combining 10 and 15 wt.-vol.% buffered solutions (pH7.4) of gelatin with 1 wt.-vol.% PLG. PLG was previously solubilized and activated in an aqueous buffered solution (pH 7.4) containing 1.3 mg ml^{-1} EDC and 0.44 mg ml^{-1} *N*-hydroxysuccinimide. Equal volume solutions of gelatin (150 μl) and PLG/EDC/NHS (150 μl) were merged and cross-linked for 10 min at 4 °C.

2.3. Controlled release of G-CSF and FGF-2

The release kinetics of G-CSF and FGF-2 were determined using a previously described method [31]. Briefly, G-CSF and FGF-2 were reconstituted in double-distilled water or Tris–HCl (pH 8.0), respectively, to a concentration of 0.1 mg ml^{-1}. Plastic cylinders ($h = 4.0$ mm, $D = 6.5$ mm, $V = 135.0$ mm^3) were sealed to the bottom of a 6-well culture plate using silicon glue (Dow Corning). Next, 150 μl of 10 and 15 wt.-vol.% gelatin B solution was combined with the growth factors to make a final concentration of FGF-2 or G-CSF at 1.66 μg ml^{-1}, and cross-linked with 150 μl of 2.6 mg ml^{-1} EDC, 0.88 mg ml^{-1} NHS and 2 wt.-vol.% PLG solution at 4 °C for 10 min. Release experiments were conducted on a MaxQ4000 orbital shaker (Barnstead International; Dubuque, IA) at 100 rpm and 37 °C. The hydrogels were immersed in 10 ml of PBS (pH 7.4) and samples were collected at specific time points over 14 days and stored at −20 °C prior to quantification in triplicate experiments. The amounts of G-CSF (R&D Systems; Minneapolis, MN) and FGF-2 (EMD Biosciences; San Diego, CA) released were determined using an enzyme-linked immunosorbent assay. The optical density was determined at 490 nm with a reference wavelength at 650 nm for G-CSF and at 450 nm with reference at 570 nm for FGF-2.

2.4. Activity of FGF-2 and G-CSF on endothelial cell culture

2.4.1. Proliferation

HUVECs were cultured to confluence (passage six) in T-75 flasks at 37 °C and 5% CO_2, trypsinized and transferred to 24-well plates (10,000 cells well^{-1}). Fresh endothelial cell

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