Biomaterials 35 (2014) 9635–9648

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Uncoupling angiogenesis and inflammation in peripheral artery disease with therapeutic peptide-loaded microgels

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ARTICLE INFO

Article history:
Received 7 July 2014
Accepted 5 August 2014
Available online 22 August 2014

Keywords:
Polymer
Polycaprolactone
Polyethylene glycol
Angiogenesis
Inflammation
Peptide

ABSTRACT

Peripheral artery disease (PAD) is characterized by vessel occlusion and ischemia in the limbs. Treatment for PAD with surgical interventions has been showing limited success. Moreover, recent clinical trials with treatment of angiogenic growth factors proved ineffective as increased angiogenesis triggered severe inflammation in a proportionally coupled fashion. Hence, the overarching goal of this research was to address this issue by developing a biomaterial system that enables controlled, dual delivery of pro-angiogenic C16 and anti-inflammatory Ac-SDKP peptides in a minimally-invasive way. To achieve the goal, a peptide-loaded injectable microgel system was developed and tested in a mouse model of PAD. When delivered through multiple, low volume injections, the combination of C16 and Ac-SDKP peptides promoted angiogenesis, muscle regeneration, and perfusion recovery, while minimizing detrimental inflammation. Additionally, this peptide combination regulated inflammatory TNF-α pathways independently of MMP-9 mediated pathways of angiogenesis *in vitro*, suggesting a potential mechanism by which angiogenic and inflammatory responses can be uncoupled in the context of PAD. This study demonstrates a translatable potential of the dual peptide-loaded injectable microgel system for PAD treatment.

1. Introduction

Peripheral artery disease (PAD) develops as arteries leading to the extremities become activated by inflammatory signals, followed by accumulation of plaques which limit blood flow to distal tissues. Eighty million people in the United States suffer from PAD-ranging from symptoms of intermittent pain when walking (claudication) to critical limb ischemia (CLI) [1,2]. Although surgical interventions can alleviate symptoms, these measures are not an option for over 50% of patients due to age, diabetes, or widespread blockages [2]. Several pre-clinical treatment strategies have focused on maximizing angiogenesis and arteriogenesis to restore blood flow to ischemic tissues-including gene transfer and cell delivery approaches [2]. However, these strategies overlook a causative role of inflammation in PAD progression [3–5]. Inflammatory cues activate the vascular endothelium, enabling the diapedesis of macrophages into the intima where they accumulate cholesterol to form plaques. For this reason inflammation should be minimized in PAD treatment.

Nonetheless, some level of inflammation is needed for the initiation of angiogenesis to promote collateral vessel formation and restore blood flow to ischemic tissues. On the other hand, angiogenesis can promote inflammation, meaning that therapeutics designed to promote angiogenesis can trigger an inflammatory response as well [6–11]. Therefore, independent control of inflammation and angiogenesis would be an ideal treatment for PAD. In our previous study, we demonstrated successful independent control of angiogenesis and inflammation by delivering pro-angiogenic C16 and anti-inflammatory Ac-SDKP peptides via an implantable polymer scaffold [12]. C16 is a pro-angiogenic peptide derived from laminin-1 [13] which is known to bind to the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins and increase VEGFR2 and FGFR2 production [14,15]. The tetrapeptide Ac-SDKP is derived from thymosin β-4 which can be found in platelets and wound fluid. Ac-SDKP has been identified as an anti-inflammatory and anti-fibrotic molecule which decreases macrophage infiltration and TGF-β expression [12,16].

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http://dx.doi.org/10.1016/j.biomaterials.2014.08.011
0142-9612/

Microgels were fabricated from a combination of polyethylene glycol (PEG) and poly-ε-caprolactone (PCL). PEG is a hydrophilic polymer which causes repulsion of proteins and cells [17]. PCL is a hydrophobic, slowly degrading polymer which is well-known for favorable cellular and interactions when used as tissue engineering constructs [18]. Hence, the co-polymer microgels can serve as a synthetic ECM for cell attachment and growth [19,20]. As these injectable polymers are thermo-responsive, they exist as a solution at room temperature but become a hydrogel at body temperature. This solution-to-gel (sol–gel) transition allows for easy mixing of therapeutics, including peptides with the polymer solution at room temperature before injecting into the body. Upon injection of polymers containing peptides, a stable hydrogel forms and serves as a drug depot in a site-specific manner, thereby avoiding systemic side effects [21,22]. The injectable polymer microgel system was evaluated for gelation time and biocompatibility *in vitro* before transitioning to *in vivo* experiments to evaluate the regulation of angiogenesis and inflammation in a murine model of PAD. As a comparison to these injectable microgels, solid implantable scaffolds were also loaded with peptides and used in *in vivo* experiments.

In the current work we demonstrate the ability of C16 and Ac-SDKP-loaded, injectable polymer microgel systems to successfully increase collateral vessel formation without inflammatory exacerbation in a PAD model of murine hind limb ischemia. The minimally-invasive, injectable polymer microgel approach used in this study reduces surgical injuries expected from scaffold implantation, and this is a significant advantage in the clinic as a number of PAD patients have comorbidities which prevent them from being eligible for surgical interventions.

To elucidate a mechanism of peptide-mediated decoupling of angiogenesis and inflammation, we investigated the roles of MMP-9 and TNF-α in modulating angiogenesis and inflammation. MMP-9 promotes angiogenesis by degrading collagen in the basement membrane of vasculature, enabling blood vessel remodeling and growth. Transcriptional regulation of MMP-9 is mediated by the transcription factors nuclear factor kappa beta (NF-κβ), forkhead box protein O4 (FoxO4), and activator protein (AP-1) [23–25]. Binding of these transcription factors to the promoter regions of the MMP-9 gene is stimulated by inflammatory cytokines and growth factors, such as TNF-α, IL-1α, IL-1β, PDGF and bFGF [26]. Through these inflammatory cytokine-mediated pathways of MMP regulation, angiogenesis and inflammation are highly interconnected. TNF-α is an inflammatory cytokine that is produced primarily by activated macrophages early in the acute phase of inflammatory response, and results in recruiting neutrophils to the site of inflammation [27–29]. TNF-α binds to TNF receptor 1 (TNFR1) on the cell membrane, which activates NF-κβ, MAPK pathways, or death signaling [30]. In particular, NF-κβ signaling in macrophages stimulates phagocytosis [31]. In this study we elucidated the role of TNF-α in controlling inflammation independently of MMP-9 mediated regulation of angiogenesis.

2. Materials and methods

2.1. Chemicals and reagents for injectable polymer microgel

Tin (II) ethyl hexanoate (Sn(Oct)2), ε-caprolactone, monomethoxypoly(ethylene glycol) (mPEG) (M_n = 750 Da), anhydrous tetrahydrofuran (THF), anhydrous toluene, dichloromethane, and diethyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). ε-Caprolactone was dried and distilled over CaH_2 (Alfa Aesar, Ward Hill, MA, USA) immediately before polymerization. Tin (II) ethyl hexanoate was distilled under high vacuum.

2.2. Synthesis and characterization of injectable polymer microgels

The injectable polymer is presented as 21%PEG–79%PCL (individual mole percentage) (Fig. 1A). Our previous studies have showed that this format of combinatorial polymers provides tunable degradation, mechanical, and thermal properties by changing the mole percentages [20,32]. PEG–PCL was synthesized by ring opening polymerization of ε-caprolactone according to previously published methods [20,33]. The structure and the number average molecular weight (M_n) calculated by the molar ratio of PEG and PCL was verified by NMR (Fig. S1). Injectable polymers were dissolved in H_2O to form a 13% polymer by weight solution at 25 °C and then incubated at 37 °C and observed every 10 s until a stable gel formed to determine the gelation time (Fig. 1B).

2.3. In vitro biocompatibility assay

HUVECs (ATCC) were seeded at a density of 1×10^5 cells/mL in MesoEndo media (Cell Applications) on top of pre-gelled injectable polymer microgels and cultured for 1 or 3 days at 37 °C with 5% CO_2 and stained with LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) according to the supplier's protocol ($n = 4$ per condition).

2.4. Mouse model of hind limb ischemia

All animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC) in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Wild type A/J mice were used to develop a model of PAD as described previously [34], by ligating the femoral artery and vein at one ligation below the epigastric artery and a second ligation around the artery and vein at a distal location just proximal to the deep femoral branch. The femoral artery and vein were then cut between these two sutures. A 13% by weight solution of injectable polymers in H_2O was mixed with 75 μg Ac-SDKP, C16, or the combination of Ac-SDKP and C16 at 25 °C. In order to control the hydrogel size considering the possibility that the hydrogel size may change peptide release and therefore inflammatory responses [35], a single, 10 μL bulk injection or ten, 1 μL injections of peptide-loaded polymer were made into the thigh muscle adjacent to the femoral artery ligations. The surgical incision was then closed with non-degradable sutures. As controls, femoral artery ligation surgery was performed on animals without any microgels or peptide treatment or with peptide in PBS injections into the subcutaneous tissue adjacent to femoral artery ligations. The left hind limb (unoperated) was also used as a surgical control.

2.5. In vivo peptide release from injectable microgels

A 13% by weight solution of injectable polymers in H_2O was mixed with 75 μg of FITC-labeled SDKP (GenScript) at 25 °C. A single, 10 μL bulk injection or ten, 1 μL injections of peptide-loaded polymer were made into the thigh muscle adjacent to the femoral artery ligations. After 7 days, mice were sacrificed by CO_2 inhalation and death was verified by cervical dislocation. The skin on the ischemic hind limb was removed and the adductor muscle was imaged on an IVIS 200 pre-clinical *in vivo* imaging system (Perkin Elmer, Waltham, MA) to visualize peptide retention in the tissue ($n = 4$ mice per treatment).

3. Non-invasive imaging of ischemia

3.1. LDPI

LDPI was performed on the footpad region of the hind limb of the mice using a Periscan PIM II device. This technique images surface perfusion by measuring Doppler changes in the reflectance of light due to blood flow. During imaging, ambient light and temperature were carefully controlled to avoid background variations in LDPI measurements [36]. Three scans were performed per mouse at each time point: days 0, 3, 7 and 14 after femoral artery ligation and microgel injection ($n = 6$ mice per treatment). The perfusion ratio was calculated by normalizing the average perfusion value of the ischemic footpad (right) to the average perfusion value of the control, un-operated footpad (left) using Image J (NIH).

3.2. Optical coherence tomography

Doppler OCT and speckle-variance OCT were used to non-invasively image blood vessels in the ischemic gastrocnemius muscle of mice on days 1 and 13 after femoral artery ligation, as previously described [37]. Doppler OCT detects frequency shifts in the phase-sensitive OCT signal due to flowing blood, while speckle-variance OCT tracks variation in laser speckle over time due to red blood cell movement. Doppler OCT cross-sectional scans (B-scans) were used to quantify blood flow changes over time in the hind limb, while volume intensity projections from speckle-variance OCT image volumes (C-scans) presented vessel morphology