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An injectable elastin-based gene delivery platform for dose-dependent modulation of angiogenesis and inflammation for critical limb ischemia

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ABSTRACT

Critical limb ischemia is a major clinical problem. Despite rigorous treatment regimes, there has been only modest success in reducing the rate of amputations in affected patients. Reduced level of blood flow and enhanced inflammation are the two major pathophysiological changes that occur in the ischemic tissue. The objective of this study was to develop a controlled dual gene delivery system capable of delivering therapeutic plasmid eNOS and IL-10 in a temporal manner. In order to deliver multiple therapeutic genes, an elastin-like polypeptide (ELP) based injectable system was designed. The injectable system was comprised of hollow spheres and an *in situ*-forming gel scaffold of elastin-like polypeptide capable of carrying gene complexes, with an extended manner release profile. In addition, the ELP based injectable system was used to deliver human eNOS and IL-10 therapeutic genes *in vivo*. A subcutaneous dose response study showed enhanced blood vessel density in the treatment groups of eNOS (20 μg) and IL-10 (10 μg)/eNOS (20 μg) and reduced inflammation with IL-10 (10 μg) alone. Next, we carried out a hind-limb ischemia model comparing the efficacy of the following interventions; Saline; IL-10, eNOS and IL-10/eNOS. The selected dose of eNOS, exhibited enhanced angiogenesis. IL-10 treatment groups showed reduction in the level of inflammatory cells. Furthermore, we demonstrated that eNOS up-regulated major proangiogenic growth factors such as vascular endothelial growth factors, platelet derived growth factor B, and fibroblast growth factor 1, which may explain the mechanism of this approach. These factors help in formation of a stable vascular network. Thus, ELP injectable system mediating non-viral delivery of human IL10-eNOS is a promising therapy towards treating limb ischemia.

1. Introduction

Critical limb ischemia (CLI) is a manifestation of peripheral artery disease (PAD), caused by the obstruction of blood flow to the limb [1,2]. CLI affects around 500–1000 per million of the population in Europe and North America every year. Without endovascular treatment, CLI patients are at a very high risk of amputation, leading to significant morbidity and mortality. Thus, it is considered a critical public health issue worldwide. The fundamental goal of CLI treatment is to relieve ischemic rest pain, heal ulcers, prevent limb loss and improve the quality of life, thereby extending the survival of the patient [1]. Therefore exploring new and more effective strategies for revascularization of ischemic limbs is imperative. A viable therapeutic alternative is necessary to promote angiogenesis through the delivery of proangiogenic drug (genes and growth factors) and/or cell delivery for angiogenesis [3–5].

Understanding the pathophysiology of limb ischemia is a necessity for finding an effective treatment [4]. The two major pathways contributing to pathophysiology of this disease are inflammation and angiogenesis. During an ischemic insult, most

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tissues in the body attempt to compensate for low levels of blood supply by mechanisms of angiogenesis, arteriogenesis, vascular remodeling, and hematopoiesis [3]. Among many proangiogenic growth factors, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factors (FGF) play an important role in regulating angiogenesis in an ischemic tissue [6–9]. Ischemia also induces an inflammatory response, triggered by the breakdown products of the degenerating tissue. The major proinflammatory cytokines released during this time are IL-1β, TNFα, and IL-6 [10,11]. Also, IL-10, an anti-inflammatory cytokine, has been shown to be up-regulated in the ischemic tissue [12]. Despite the endogenous up-regulation of the proangiogenic factors, this is insufficient to compensate for the progressive deterioration of the tissue, due to the lack of blood supply. Hence, delivering soluble growth factors/cytokines or therapeutic genes could help in slowing down the tissue damage and promote tissue repair [7,9,13].

Non-viral gene therapy is an evolving field [14,15]. Various cationic polymers have been developed so far to form a complexes of *p*DNA/polymer (polyplexes) to transfect the gene of interest to cells [16–18]. The polyplex helps in reduction of the size of the *p*DNA and helps it in crossing the cell membrane barrier efficiently. Also, *p*DNA micro-carriers have been developed to load and release genes of interest in a spatio-temporal manner. Recently, elastin-like polypeptide (ELP) has been used to fabricate hollow micro-spheres of a gene delivery depot [19]. ELP is used for various biomedical applications due to its biodegradable, non-toxic, non-inflammatory properties and efficient pharmacokinetics for the delivery of therapeutics [20,21].

In this study, a combined gene therapy approach for angiogenesis and inflammation has been considered for the treatment of CLI. It was hypothesized that the dual release of eNOS and IL-10 using an ELP based delivery platform will modulate inflammation and increase the blood perfusion in the ischemic tissue. The overall goal of this study was to deliver therapeutic genes for eNOS and IL-10 to treat the ischemic environment and to characterize at a molecular level the role of eNOS and IL-10 and assess the effect on angiogenic and inflammatory pathways (Fig. 1). The specific objectives are: 1) fabrication of an ELP based dual delivery system, 2) determination of a therapeutic dose for eNOS and IL-10, and 3) delivery of eNOS and IL-10 in a hind limb ischemia mouse model to characterize at a molecular level the role of eNOS and IL-10 in angiogenic and inflammatory pathways (Fig. 1).

2. Results

2.1. Optimal cross-linking of ELP-based injectable scaffold

An injectable ELP scaffold was fabricated using microbial transglutaminase (mTGase) as a cross-linker (Fig. 2A). A 20% ELP scaffold and mTGase of 100 U/g of ELP concentration was found to be the optimal ratio of ELP and mTGase to fabricate the scaffold with a gelation time of 10 min at 37 °C. The successful cross-linking of the ELP scaffold was verified by TNBSA (2,4,6-trinitrobenzene sulfonic acid) assay and it was shown that the cross-linked ELP scaffold exhibited 30% ± 10% reduction in free primary amino groups after cross-linking with mTGase compared to ELP without mTGase (Fig. 2B). Cellular cytotoxicity of ELP scaffold was measured using human umbilical vein endothelial cells (HUVECs). Metabolic activity using MTT assay revealed that ELP scaffold cross-linked with mTGase is non-cytotoxic, similar to the control, on tissue culture plastic. The ELP scaffold cross-linked with GTA as a negative control showed higher cellular cytotoxicity than that of ELP/mTGase and the control (Supplementary Fig. 2).

2.2. Internalization behavior of cells with respect to various sizes of ELP hollow spheres

Various sizes of ELP hollow spheres ranging from 0.1 μm to 10 μm were screened based on optimal loading of pDNA and efficient cellular uptake to be used as a gene delivery depot. Flow cytometry was performed on HUVECS and THP-1 cells treated with FITC-labeled ELP hollow spheres of 0.1, 0.5, 1, and 10 μm sizes. The internalization of ELP hollow spheres into HUVECS did not show any defined pattern, as shown in the case of macrophages, ELP hollow spheres of 0.5, 0.1, and 10 μm size showed more uptake in the HUVECs compared to 1 μm size of hollow spheres (Fig. 2C). The activated and non-activated THP1 cells showed a higher uptake of 10 μm ELP HS compared to 0.1, 0.5, and 1 μm sized hollow spheres (Supplementary Fig. 1).

2.3. Injectable ELP system for dual pDNA delivery

A release study was performed using the ELP-in-ELP injectable system comprising both ELP hollow spheres and the ELP injectable scaffold as gene delivery depot (Fig. 2D). Two different sample groups were used for this study: 1) ELP injectable scaffold containing pCMV-GLuc and 2) ELP hollow spheres containing pCMV-GLuc. The release profile of these systems was monitored for 10 days and a cumulative release profile was calculated. The scaffold/polyplex sample group released 20% of *p*DNA by day 1 as compared to ELP hollow sphere/polyplex, which was near to 0%. The scaffold/polyplex group released around 40% of its *p*DNA by day 4 and nearly 90% by the end of 10 days. The ELP hollow sphere/polyplex released a significantly lower percentage of *p*DNA as compared to scaffold/polyplex. The release for ELP hollow sphere/polyplex was only 20% at day 4 and almost 50% by day 10.

2.4. Different doses of eNOS and IL-10 and their effect on angiogenesis and inflammation level

An *in vivo* subcutaneous study was performed in C57BL/6 mice to determine a combination of dose for eNOS and IL-10. Nine different treatment groups were tested (Table 1). This *in vivo* subcutaneous study was performed to characterize the degradation profile of the injectable ELP system and also to elucidate an appropriate therapeutic dose to induce angiogenesis and reduce inflammation *in vivo*. The degradation of the ELP scaffold is shown in Supplementary Fig. 3. H & E sections of the tissue revealed a 40–50% higher degradation of the scaffold from day 7 to day 14.

2.4.1. Angiogenesis and inflammation analysis of subcutaneous mouse model

Surface and length density of blood vessels were measured from the H & E sections (Images not shown) of the subcutaneous implants of different treatment groups and control (Fig. 3A and Supplementary Fig. 4). Two different time points, days 7 and 14, were analyzed for this study. On day 7 the control and IL-10 treatment alone groups showed similar levels of blood vessel density of around 30 mm^2, whereas the eNOS treatment groups showed blood vessel density around 50 mm^2. A trend towards an increase in the blood vessel surface density was found in the eNOS treatment groups. On day 14 the blood vessel density increased up to 139 mm^2 in the eNOS group and was significantly higher than that seen in control groups and IL-10 treatment alone groups (40 up to 55 mm^2). The eNOS doses of both 10 and 20 μg showed significantly enhanced surface density of blood vessels at day 14. The sample treatment group IL-10 (20 μg)/eNOS (20 μg) showed 62% less blood vessel density, while, IL-10 (10 μg)/eNOS (20 μg) showed 30% less blood vessel surface density than eNOS 20 μg