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Co-delivery of a growth factor and a tissue-protective molecule using elastin biopolymers accelerates wound healing in diabetic mice

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ABSTRACT

Growth factor therapy is a promising approach for chronic diabetic wounds, but strategies to efficiently and cost-effectively deliver active molecules to the highly proteolytic wound environment remain as major obstacles. Here, we re-engineered keratinocyte growth factor (KGF) and the cellular protective peptide ARA290 into a protein polymer suspension with the purpose of increasing their proteolytic resistance, thus their activity *in vivo*. KGF and ARA290 were fused with elastin-like peptide (ELP), a protein polymer derived from tropoelastin, that confers the ability to separate into a colloidal suspension of liquid-like coacervates. ELP fusion did not diminish peptides activities as demonstrated by ability of KGF-ELP to accelerate keratinocyte proliferation and migration, and ARA290-ELP to protect cells from apoptosis. We examined the healing effect of ARA290-ELP and KGF-ELP alone or in combination, in a full-thickness diabetic wound model. In this model, ARA290-ELP was found to accelerate healing, notably by increasing angiogenesis in the wound bed. We further showed that co-delivery of ARA290 and KGF, with the 1:4 KGF-ELP to ARA290-ELP ratio, was the most effective wound treatment with the fastest healing rate, the thicker granulation tissue and regenerated epidermis after 28 days. Overall, this study shows that ARA290-ELP and KGF-ELP constitute promising new therapeutics for treatment of chronic wounds.

1. Introduction

The number of patients with diabetes is rapidly increasing around the world with an estimated 439 million adults affected by 2030 [1]. Around 25% of this population can be expected to develop diabetic ulcers, which could lead to amputation due to progression of the disease for more than 14% of these patients. Annual chronic wound management costs exceed $20 billion in the United States alone [2] and already severely burden the US healthcare system [3]. Clinical practice guidelines recommend the treatment of diabetic foot ulcers with surgical debridement, infection control, redistribution of pressure off the wound, and a selection of dressings that allow for a moist wound environment and control excess exudation [4]. Despite good wound care, non-healing ulcers remain a leading cause of non-traumatic amputation in the US [5] with an increased incidence of death among these patients [6], highlighting the need for new viable treatments.

The wound healing response is a complex and dynamic process that relies on a coordinated effort from different cell types along with protein and chemical mediators to restore skin function. The healing process is divided into several overlapping, interdependent phases: hemostasis, inflammation, cell proliferation, migration, angiogenesis, reepithelialization and remodeling of the extracellular matrix [7]. Hyperglycemia caused by diabetes often interferes with the initiation, regulation, and/or termination of the healing stages leading to an impaired wound healing response. Diabetic ulcers are non-healing wounds characterized by a chronically inflamed wound bed due to numerous factors including neuropathy, improper oxygenation, insufficient vascular supply to the extremities, and bacterial infection [4].

Growth factors and cytokines are essential in the organization of the molecular processes involved in making cutaneous wound healing efficient [8]. Specifically, growth factors have been shown

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to influence and modulate epidermal and dermal regeneration, angiogenesis and granulation formation [9]; all these processes are known to be inhibited in chronic wounds [7,10]. While many growth factor-based approaches have been explored to treat chronic wounds such as platelet derived growth factor (Regranex), epidermal growth factor or basic fibroblast growth factor delivery [11], none of them are widely used in clinical settings. Purified peptide growth factors are short-lived in the highly proteolytic wound environment requiring repeated topical application. The use of large quantities of growth factor make these wound healing therapies expensive, and their positive impacts remain mostly insufficient to justify the additional cost.

The repetitive elastin-like polypeptide (ELP) is an engineered biopolymer originally derived from an amino acid motif found in the hydrophobic domain of human tropoelastin. ELPs are non-immunogenic, biologically compatible peptides, typically composed of the sequence $[VPGXG]_n$, where X can represent any of the common amino acids except proline and n indicates the number of repeating pentapeptides. These biopolymers reversibly separate into a colloidal suspension of liquid-like coacervates above a specific transition temperature. The number of pentapeptide repeats and guest residue composition have been identified as tunable design parameters to tailor ELP properties including transition temperature. In addition, ELPs can be genetically fused to biologically active proteins and peptides which has led to chimeric fusion proteins that retain the function of each component. Thus, stimulus-responsive phase transition inherent to ELPs has been used to purify engineered fusion proteins after protein expression in microbial hosts, leading to cost-effective production of functional chimeric proteins. ELPs have promising clinical application as systems for drug loading, targeting and delivery in numerous fields such as cancer therapy [12], regenerative medicine [13,14] or diagnostics [15]. Recently, we have demonstrated that ELP-based fusion proteins shield an appended biologically active peptide from proteolytic degradation by forming colloidal suspension of liquid-like coacervates [16], making this delivery system particularly well adapted for wound care treatment [17,18].

In this work, we demonstrated the ability of ELPs to deliver keratinocyte growth factor (KGF) and ARA290, a cellular protective peptide, in a full-thickness diabetic wound mouse model. KGF is a member of the fibroblast growth factor (FGF) family [19] and is mainly synthesized in mesenchymal cells, such as fibroblasts, microvascular endothelial cells and smooth muscle cells [20]. KGF exerts its effects mainly in a paracrine fashion and has been shown to be essential for skin morphogenesis [21]. Studies have demonstrated that KGF stimulates epithelial cell differentiation, proliferation, migration and exerts an anti-apoptotic effect on epithelial cells [22–24]. While the local application of KGF was shown ineffective [7], the electroporation [25] and liposome delivery [26] of KGF DNA was found to accelerate wound closure and improved dermal and epidermal regeneration *in vivo*, demonstrating the importance of the delivery approach. The ARA290 peptide was originally derived from the tertiary structure of erythropoietin (EPO). While EPO is a well-known stimulator of erythrocyte production, recent studies have also demonstrated its role in protecting cells from a variety of stresses including ischemic stroke, peripheral nerve trauma and thermal injury [27,28]. By interacting specifically with the heteroreceptor composed of the EPO receptor (EPO-R) monomeric subunit and CD131 [29], ARA290 confers the tissue protective activity without the use-limiting side effects of EPO, that converts the vasculature into a prothrombic state at high doses, potentially leading to dangerous thromboses [30]. A number of studies have reported significant activity of EPO in promoting the healing of ischemic skin wounds [31], incisional wounds [32], colonic anastomosis [33] and skin thermal burns [28], strongly suggesting that ARA290 would be beneficial in diabetic wound healing process.

In this study, we hypothesized that delivering a combination of the two bioactive molecules KGF and ARA290 using ELP-based approach would be more effective in promoting healing of diabetic wounds. In this light, we examined the effect of KGF-ELP and ARA290-ELP alone or in combination (1:4 KGF-ELP to ARA290-ELP molar ratio) on wound healing rate, granulation tissue formation, vascularization as well as epithelial and dermal thickness after skin regeneration in a full-thickness diabetic wound model.

2. Materials and methods

2.1. Cloning expression cassettes

An empty pET24a(+) plasmid was modified to incorporate BseRI and AcuI endonuclease restriction sites as well as short leader and trailer sequences, as previously reported by McDaniel et al. [34]. A short cassette encoding five pentapeptide ELP repeats, $(VPGVG)_5$, was generated by annealing two overlapping oligonucleotides with appropriate overhangs for ligation into the modified pET24a(+) vector linearized with BseRI. Valine was chosen for the guest residue position to create hydrophobic biopolymers that undergo phase transition at lower temperatures. As previously described by Dooley et al., ELP cassettes were concatenated by recursive directional ligation by plasmid reconstruction (PRe-RDL) until the desired lengths of 120 pentapeptide repeats were achieved and fused with the ARA290 (QEQLERALNSS) peptide [16]. ARA290 was generated by annealing two short, overlapping oligonucleotides with appropriate overhangs for ligation into the modified pET24a(+) vector linearized with BseRI. The peptide gene was appended to the N-terminus of the ELP cassettes using the same PRe-RDL technique. The gene encoding KGF was appended to the N-terminus of the ELP cassette using the same BseRI restriction site and was originally amplified from the pUC19 vector described by Koria et al. [18]. Sequencing of the final cloned products was performed to ensure accuracy of the constructs. The resulting plasmids were transformed into BL21(DE3) *E. coli* for expression.

2.2. Expression & purification of ELP constructs

All ELP and ELP fusion proteins were expressed identically in terrific broth (TB) media supplemented with Kanamycin (100 μg/mL) and purified by inverse transition cycling (ITC), as previously described [16]. Briefly, after carrying out expression at 37 °C and 220 RPM, cells were harvested by centrifugation and lysed via microtip sonication. Cell debris was separated from the soluble protein by centrifugation and polyethyleneimine (0.7% w/v) was used to precipitate and eliminate the nucleic acid contaminants. The ELP and ELP fusion proteins were further purified by performing three rounds of ITC. ELP samples were heated to 45 °C to induce their precipitation. The precipitated proteins were separated from host cell contaminants by centrifugation at 10,000 g for 15 min in a warmed centrifuge. The soluble fraction was discarded and the precipitated pellet was resuspended in ice cold PBS. The samples were then centrifuged at 4 °C at 15,000 g for 15 min to remove any insoluble contamination, thus completing one round of ITC. Purity was verified by SDS-PAGE after completion of 3 cycles (Fig. 1).

2.3. ELP characterization

ELP and ELP fusion transition temperatures (T_t) were calculated using a temperature controlled Bio Rad Benchmark Plus microplate spectrophotometer. Varying ELP concentrations (1–100 μM) were