Journal of Controlled Release 250 (2017) 48–61

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Perlecan and vascular endothelial growth factor-encoding DNA-loaded chitosan scaffolds promote angiogenesis and wound healing

Megan S. Lord [a,*], April L. Ellis [b], Brooke L. Farrugia [a], John M. Whitelock [a], Hernan Grenett [b], Chuanyu Li [b], Robert L. O'Grady [a], Arthur A. DeCarlo [b,**]

[a] Graduate School of Biomedical Engineering, University of New South Wales, Sydney, NSW 2052, Australia
[b] Agenta Biotechnologies, Inc., P.O. Box 430021, Cahaba Heights, AL 35243, USA

ARTICLE INFO

Article history:
Received 14 October 2016
Accepted 7 February 2017
Available online 9 February 2017

Keywords:
Perlecan
Proteoglycan
Vascular endothelial growth factor
Chitosan
Wound healing
Skin tissue engineering

ABSTRACT

The repair of dermal wounds, particularly in the diabetic population, poses a significant healthcare burden. The impaired wound healing of diabetic wounds is attributed to low levels of endogenous growth factors, including vascular endothelial growth factor (VEGF), that normally stimulate multiple phases of wound healing. In this study, chitosan scaffolds were prepared *via* freeze drying and loaded with plasmid DNA encoding perlecan domain I and VEGF189 and analyzed *in vivo* for their ability to promote dermal wound healing. The plasmid DNA encoding perlecan domain I and VEGF189 loaded scaffolds promoted dermal wound healing in normal and diabetic rats. This treatment resulted in an increase in the number of blood vessels and sub-epithelial connective tissue matrix components within the wound beds compared to wounds treated with chitosan scaffolds containing control DNA or wounded controls. These results suggest that chitosan scaffolds containing plasmid DNA encoding VEGF189 and perlecan domain I have the potential to induce angiogenesis and wound healing.

1. Introduction

In the USA, it has been estimated that 1 to 2% of the population will experience a chronic wound during their lifetime [1] affecting approximately 6.5 million people [2]. That number is growing rapidly due to an aging population and an increase in the incidence of diabetes and obesity. The standard of care for these types of wounds is gauze bandages that have no inherent wound healing properties.

Re-epithelialization and angiogenesis of the wound site are critical events in the proliferative stage of dermal wound healing while the remodeling of dermal wounds includes changes in the expression of extracellular collagens and proteoglycans. In diabetes, dermal wound healing is characterized by prolonged inflammation, impaired neovascularization [3] and delayed re-epithelialization [4,5].

Growth factors play an important role in wound healing through promoting both angiogenesis and re-epithelialization. Growth factors including members of the fibroblast growth factor (FGF) and transforming growth factor β (TGFβ) families promote wound healing. Members of the vascular endothelial growth factor (VEGF) family, including VEGF 121, 165, 189 and 205, contribute to multiple stages of wound healing by promoting angiogenesis, epithelialization and collagen deposition [6,7]. Due to the multi-faceted contribution of VEGF to wound healing, it was of interest to explore its potential for regenerative medicine applications. VEGF165 has been investigated for dermal wound healing as the levels of this growth factor are reduced in wounds. Their single dose application to wounds alone has had limited success due to the short half-life of growth factors [3]. Repeated topical delivery of VEGF165 promoted wound healing in diabetic mice including rapid re-epithelialization and enhanced angiogenesis compared to untreated controls [3], demonstrating the utility of growth factors for dermal repair *in vivo*. While VEGF is known to stimulate angiogenesis, it is also involved in modulating oxidative damage that is associated with diabetic wounds [8]. Plasmid DNA encoding VEGF165 loaded into collagen/chitosan scaffolds promoted blood vessel formation and faster resorption of the scaffolds [9,10].

Plasmid DNA and growth factor delivery strategies to date have focused on the use of VEGF165, however the longer isoform of VEGF, VEGF189, binds more avidly to extracellular matrix (ECM) heparan sulfate proteoglycans, such as perlecan, than the VEGF165 isoform [11]. Both VEGF165 and VEGF189 stimulate vascularization; however VEGF189 must be released from the ECM to exert its mitogenic activity thus giving the advantage of a local distribution of VEGF in the wound site where the plasmid DNA was delivered. In contrast, VEGF165 is present both bound to the ECM and in a soluble form, giving rise to the possibility of this form acting on more distant tissues with unwanted side effects [12,13].

* Correspondence to: M. S. Lord, Graduate School of Biomedical Engineering, The University of New South Wales, Sydney, NSW 2052, Australia.
** Corresponding author.
E-mail addresses: m.lord@unsw.edu.au (M.S. Lord), DeCarlo@agentabiotechnologies.com (A.A. DeCarlo).

http://dx.doi.org/10.1016/j.jconrel.2017.02.009
0168-3659/

The ECM also provides many of the signals required for wound healing. Perlecan is an extracellular heparan sulfate proteoglycan expressed in the basement membrane of the dermoepidermal junction of normal skin fibroblasts [14], inflammatory cells and endothelial cells [15,16] and interacts with other basement membrane proteins, including fibronectin, laminin and type IV collagen, facilitating assembly and integrity of the basement membrane [17–19]. Additionally, perlecan heparan sulfate is required for dermal-epidermal homeostasis [20]. Mice lacking heparan sulfate chains attached to the N-terminal domain I of perlecan (Pln.DI) have delayed wound healing and impaired angiogenesis demonstrating the utility of this ECM molecule in wound healing [21]. Chronic venous ulcers have reduced expression of perlecan compared to normal skin [14]. Perlecan contributes to wound healing by acting as a reservoir for growth factors in the ECM and by binding and protecting them from proteolytic degradation *via* its heparan sulfate chains [22–26]. Perlecan heparan sulfate can regulate cell function by serving as a co-factor, or co-receptor, in growth factor interactions with their receptors and is necessary to induce signaling and growth factor activity [27–30]. Importantly, it has been shown that binding of heparin or heparan sulfate by VEGF165 is required for full activation of the VEGF receptor-2 [31]. However, the exogenous addition or over-expression of perlecan has not been explored in the context of cutaneous wound healing. As domain I of perlecan contains three glycosaminoglycan attachment sites that are most frequently decorated with heparan sulfate [32], this region of perlecan was explored in this study.

Many materials have been explored to promote cutaneous wound healing including synthetic hydrogels of poly(methacrylates) and polyvinylpyrrolidine that maintain a hydrated wound environment [33]. Natural polymers, including alginate and collagen, have also been explored alone, or in combination with, other polymers [33,34]. Collagen, a major component of the dermal ECM, has been processed into scaffolds and sponges with reported improvements in wound closure [35,36]. Chitosan is being widely explored to accelerate skin wound healing due to its hemostatic, anti-microbial and cyto-compatible properties [37,38]. This material also promoted granulation tissue formation, collagen expression and ECM organisation as well as inflammatory cell infiltration [39], which are all important hallmarks of wound healing. Chitosan scaffolds loaded with FGF2 promoted wound closure and granulation tissue formation to a greater extent than chitosan alone in a diabetic mouse model of wound healing [40].

The delivery of plasmid DNA encoding growth factors involved in wound healing is an alternative approach for the sustained expression of growth factors in the wound site. Transient delivery of DNA plasmid has low immunogenicity [41] with no evidence to date of associated neoplastic disease. Delivery of plasmid DNA alone *in vivo* is limited by its rapid degradation and clearance [42]. Due to the cationic nature of chitosan it has the ability to bind plasmid DNA enabling sustained delivery *in vivo* [9,43,44]. The delivery of genes encoding growth factors and their natural binding partners that provide protection against proteolytic degradation as well as potentiating their activity is the novel approach taken in this study. Plasmid DNA encoding VEGF189 was explored for the first time in this study as it binds more avidly to ECM heparan sulfates, such as those that decorate perlecan domain I enabling localized expression and delivery of this multi-faceted growth factor for epithelial wound healing. The aims of this study were to fabricate a chitosan scaffold loaded with plasmid DNA encoding perlecan domain I and VEGF189 and to investigate its ability to promote dermal wound healing in an *in vivo* full thickness wound healing model in normal and diabetic rats.

2. Materials and methods

2.1. Preparation of chitosan scaffold

Chitosan (Primex, 580 Siglufjordur, Iceland) with a deacetylation degree of 79–90% and viscosity of 400 MPa·s was solubilized in 1% (v/v) acetic acid to a final concentration of 4% (w/v). The chitosan solution was poured into 15.2 × 15.2 mm silicone molds at 0.5 g cm^{-2}, using 116 g of stock, and vibrated using a Whipmix professional vibrator until smooth. The chitosan was frozen at −80 °C for 1 h, removed from the mold and placed into 2 M NaOH. The scaffold was incubated for 16 h at room temperature with gentle agitation. The chitosan scaffolds were rinsed in MilliQ water with several changes until the pH of the solution was neutral (typically 16 h). The chitosan scaffolds were frozen at −80 °C for 1 h and then freeze dried (Labconco, Kansas City, MO, USA) at −40 °C and 110 psi for 48 to 72 h. The scaffolds were dissected in two for further experimentation.

2.2. Scanning electron microscopy (SEM)

Chitosan scaffolds were coated with gold-palladium and examined by SEM (FEI Quant FEG 650) with secondary electron imaging and 10 kV accelerating voltage.

2.3. Compression strength

The mechanical properties of the scaffold are a consideration given the skin environment that is subjected to a range of biomechanical forces. In this study the compression strength of the scaffolds was performed as tensile testing was not possible due to the delicate nature of the scaffolds once hydrated, particularly the commercial materials that became sponges. The compression strength of the chitosan scaffold (15.2 × 7.6 mm) was compared with commercially available collagen (Resorbable Collagen Plug, Tissue Specialists, Little Rock, AK, USA) and chitosan (HemCon Dental Dressing, HemCon Medical Technologies Inc., Portland, OR, USA) sponges. Compression strength was determined using a strain rate of 0.5 mm min^{-1} and a 500 N load cell on an Instron Universal Tester 5565 with Bluehill 2 software. Measurements were taken in duplicate in both dry and wet conditions and presented as the load at 30% compression. For dry conditions, materials were placed in a desiccator for 24 h prior to analysis and analyzed immediately after removal from the desiccator. For wet conditions, the sponges were removed from the desiccator that had been prepared in the same way as the dry samples and then soaked in MilliQ water for 5 min before analysis.

2.4. Scaffold degradation rate

Degradation of chitosan scaffolds was measured *in vitro* by incubation of the scaffolds (8 mm diameter discs with a height of 3 mm) in 200 μL of either 15 mg/L lysozyme or 100 μg/mL bovine serum albumin (BSA) at 37 °C for up to 72 h. Prior to exposure to the protein solutions, the dry mass of the scaffolds was determined. Scaffolds were imaged each day using a phase contrast microscope after exposure to the protein solutions. Scaffolds were then dried and the remaining mass recorded and compared to the initial dry mass of the scaffolds.

2.5. Construction of perlecan-VEGF transgene

The mammalian expression plasmid, pBI-CMV1-Kan (Clonetech Laboratories Inc., Mountain View, CA, USA), is a bidirectional expression vector enabling the simultaneous, yet separate expression of two proteins. This vector was used to express both the N-terminal region of human perlecan (amino acids 1–248) and VEGF189 (including the signal peptide). Base modifications were made to the cDNA inserted into the vector to enable efficient translation, to eliminate the possibility of specific recombination events *in vivo*, and to allow specific *in situ* recognition. The *Hspg2* sequence for the N-terminal region of perlecan included bases 81–855 in NM_005529.6 with modifications [45]. The VEGF189 sequence included bases 1039–1676 in NM_001171624.1 with modifications and the inclusion of *Eco*RI/Bg1II cloning sites.