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The effect of dextrin–rhEGF on the healing of full-thickness, excisional wounds in the (db/db) diabetic mouse

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ABSTRACT

Chronic wounds, such as ulceration of the lower limb, represent a significant clinical challenge in today's ageing society. With the aim of identifying improved therapeutics, we have previously described a bioresponsive, dextrin–recombinant human epidermal growth factor conjugate (dextrin–rhEGF), that (i) protects rhEGF against proteolytic degradation by human chronic wound fluid; and (ii) mediates rhEGF release by α-amylase, capable of stimulating increased proliferation/migration in normal dermal and chronic wound fibroblasts; and keratinocytes, *in vitro*. The aim of this study was to extend these findings, by investigating the effects of dextrin–rhEGF on wound healing in the (db/db) diabetic mouse, a widely used *in vivo* model of delayed wound healing. Standardised, full-thickness excisional wounds, created in the dorsal flank skin, were treated topically with succinoylated dextrin (50 μg/mL), rhEGF (10 μg/mL) or dextrin–rhEGF (1 or 10 μg/mL). Treatments were applied immediately after injury and subsequently on post-wounding, days 3 and 8. Wound healing was assessed macroscopically, in terms of initiation of neo-dermal tissue deposition and wound closure (including wound contraction and re-epithelialisation), over a 16 day period. Wound healing was assessed histologically, in terms of granulation tissue formation/maturity; cranio-caudal wound contraction and wound angiogenesis (CD31 immuno-staining), using tissues harvested at day 16. Blood samples were also analysed for α-amylase and rhEGF concentrations. In this established impaired wound healing model, the topically-applied dextrin–rhEGF significantly accelerated wound closure and neo-dermal tissue formation at the macroscopic level; and significantly increased granulation tissue deposition and angiogenesis at the histological level ($p<0.05$), relative to untreated, succinoylated dextrin and rhEGF alone controls. Overall, these findings support the further development of bioresponsive polymer conjugates, for tissue repair.

1. Introduction

Chronic ulceration of the lower limb presents a major clinical challenge, with incidence rising as a result of the ageing population and the increase in risk factors, such as smoking, obesity and diabetes [1]. Whilst the aetiology of these wounds is multi-factorial, these are characterised by a persistence of inflammatory cells, disordered extracellular matrix (ECM) synthesis and remodelling; and failed re-epithelialisation [1,2]. Mechanisms of wound healing have been systematically studied in both acute and chronic wounds. Whilst it has been established that a number of autocrine and paracrine growth factors act synergistically to direct normal dermal wound healing [3–5], impaired chronic wound healing is associated with reduced endogenous growth factor levels/activity; due to proteolytic degradation and/or denaturation by reactive oxygen species (ROS) in the wound environment [6,7]. Although often viewed as an opportunity to improve healing, the topical application of growth factors has met with limited success [3,8]. Regranex® (Becaplermin), a carboxymethylcellulose gel containing recombinant human platelet-derived growth factor (rhPDGF) is the only FDA approved growth factor therapy for chronic wounds [9], with its use limited to the treatment of deep, neuropathic diabetic foot ulcers. A recent retrospective study comparing cancer incidence and mortality in 1622 patients showed that although Regranex® did not cause an increase in cancer incidence, patients treated with 3 or more tubes of Regranex® appeared to have a five-fold increased risk of cancer mortality [10]. From these and other *in vivo* observations involving growth factor applications [11–15], it is clear that delivery must be carefully optimised in terms of dose, rate of delivery and local action, to achieve maximum therapeutic benefit, with minimal patient risk.

With this in mind, we have proposed a novel polymer therapeutic approach for growth factor delivery, based on Polymer-masking–UnMasking-Protein Therapy (PUMPT) [16,17]. Polymer conjugation is used to 'mask' the growth factor bioactivity and protect the protein from premature inactivation in the wound environment. Subsequent, locally

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triggered polymer degradation allows time-dependant protein 'unmasking', resulting in the controlled reinstatement of bioactivity. This hypothesis is discussed and explored further in Ref. [17–19]. A dextrin–recombinant human epidermal growth factor (rhEGF) conjugate was synthesised for first proof of concept studies *in vitro* [17]. Dextrin conjugation masked rhEGF bioactivity and protected against neutrophil elastase proteolysis [17]. In the presence of α-amylase, however, liberated rhEGF induced EGF receptor (EGFR) phosphorylation, resulting in a time-dependant increase in proliferation of high EGFR-expressing, HEp2 cells; and also promoted HaCaT keratinocyte proliferation and migration [17]. Moreover, we have recently confirmed that human chronic wound fluid contains sufficient α-amylase activity to activate dextrin–rhEGF [18]; and that the rhEGF released can induce the proliferation and migration of chronic wound fibroblasts, which are distinct from normal dermal fibroblasts, in terms of phenotype, reduced proliferative life-span, early onset of senescence and decreased resistance to oxidative stress [7]. Using an *ex vivo* whole-eye organ model, we have also shown that the dextrin–rhEGF conjugate stimulates corneal re-epithelialisation, post-wounding [19].

The aim of this study was to further investigate whether the conjugate/PUMPT concept would be effective in the more complex *in vivo* setting. Although it is widely acknowledged that no animal model fully reproduces the patho-physiologies of venous or diabetic ulcers, the genetically diabetic (db/db) mouse, delayed healing model was selected, based on its widespread use and acceptance in wound healing research [11,13,14,20–22]. These animals display significantly delayed dermal healing (i.e. wound closure, neo-dermal tissue formation and angiogenesis), compared to their non-diabetic littermates; similar to diabetic patients. Previous studies have used this model to study the ability of various growth factors and other agents, to promote wound healing [11,13,14,21]. Here, experiments were undertaken to determine the effects of a dextrin–rhEGF conjugate on wound healing in the diabetic (db/db) mouse model. The dextrin–rhEGF conjugate was formulated at concentrations equivalent to the presence of rhEGF at 1 or 10 μg/mL concentrations (1 or 10 μg/mL rhEGF equiv.). Unconjugated rhEGF was applied to controls at a maximum dose of 10 μg/mL, as this concentration has previously been demonstrated to be ineffective in promoting wound closure in the diabetic (db/db) mouse model [13]. Similarly, 10 μg/mL rhEGF concentrations have been shown to be ineffective for venous leg ulcer re-epithelialisation, during clinical trials [12]. Consequently, the dextrin–rhEGF conjugate was topically applied at both 10 μg/mL and 1 μg/mL rhEGF equiv. (100 or 25μL doses, as below); to evaluate the possibility of increased rhEGF efficacy at equivalent and lower conjugate doses, as evident *in vitro*/*ex vivo* [17–19]. The effects of the conjugate were compared to its components succinoylated dextrin (50 μg/mL) and unconjugated rhEGF (10 μg/mL). As dextrin–rhEGF conjugates typically contain 11–24 wt.% rhEGF [17–19], the remaining 80–90 wt.% would be comprised of succinoylated dextrin. Thus, succinoylated dextrin was applied at 50 μg/mL concentrations, based on the approximate 1:5 ratio of rhEGF-succinoylated dextrin, within the conjugate at 10 μg/mL rhEGF equiv. concentrations. The impact of these respective treatments on various wound healing parameters was examined over 16 days, using established gross and histological wound healing methodologies [11,13,14,20–23].

2. Materials and methods

2.1. Materials

rhEGF was obtained from Prospec-Tany Technogene Ltd, (Rehovot, Israel) and dextrin (Mw ~ 42,000 g/mol) from ML Laboratories (Liverpool, UK). Dextrin was first succinoylated, as previously described [17,24], to approximately 21 mol%. This intermediate was used to synthesise the dextrin–rhEGF conjugate, as described [17]; and also used as a reference control herein. The conjugate used had a MW of approximately 140,000 g/mol and a rhEGF content of approximately 16.5 wt.%. Free rhEGF content was <1%. CD-31 antibody (anti-mouse, polyclonal IgG, raised in rabbit) was from Abcam (Cambridge, UK). Male diabetic mice (BKS.Cg-m a/a +/+ Lepr^{db}/J db/db), aged 12–13 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, USA). The rhEGF ELISA kit was from R&D Systems (Abingdon, UK).

2.2. Methods

2.2.1. Wounding, treatment and gross assessment of healing

The protocol used for this study is summarised in Table 1. All animal procedures were performed, in accordance with UK Home Office Licences, and the health of animals was monitored on a daily basis, throughout the study. A total of 70 male diabetic mice were used, as per Table 1. The procedures used to create single, full-thickness wounds (10 × 10 mm) in each animal; are described in the Supplementary Information (SI), Section S1. Animals were randomly assigned to each experimental group and treatments (100 μL at days 0 and 3, and 25 μL at day 8) were administered topically to wounds (prior to film dressing) at day 0; and through the Bioclusive® dressing using a 27-gauge needle [11], at days 3 and 8 post-wounding. Dextrin–rhEGF (1 and 10 μg/mL), succinoylated dextrin (50 μg/mL) and free rhEGF (10 μg/mL); were administered solubilised in phosphate buffered saline (PBS). The methodologies employed to assess gross wound healing events are described in SI, Section S2. Animals were also weighed (post-wounding

Table 1
Study protocol, image analysis of wound closure and parameters evaluated. Treatments were administered on days 0, 3 and 11 (doses and volumes shown).

Study group	n	Time (days)
		0 5 10 15
Group 1. Succinoylated dextrin (50 μg/mL)	10	DAY 0 1. Animals weighed 2. Animals wounded 3. Digital photography 4. Bioclusive® dressing applied 5. Animals assigned to Groups 6. Treatment applied (100 μL)
Group 2. rhEGF (10 μg/mL)	10	
Group 3. Dextrin–rhEGF (1 μg/mL)	10	
Group 4. Dextrin–rhEGF (10 μg/mL)	20	DAY 3 1. Dressing removed 2. Digital photography 3. Bioclusive® dressing applied 4. Treatment applied (100 μL)
Group 5. Film dressing alone	20	DAY 8 1. Animals weighed 2. Dressing removed 3. Digital photography 4. Bioclusive® dressing applied 5. Treatment applied (25 μL)
		DAY 16 1. Animals sacrificed 2. Animals weighed 3. Dressing removed 4. Digital photography 5. Blood collected 6. Wound tissue harvested