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A synthetic connexin 43 mimetic peptide augments corneal wound healing

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ABSTRACT

The ability to safely and quickly close wounds and lacerations is an area of need in regenerative medicine, with implications toward healing a wide range of tissues and wounds. Using an in vivo corneal injury model, our study applied a newly developed peptide capable of promotion of wound healing and epithelial regeneration. The alpha-carboxy terminus 1 (αCT1) peptide is a 25 amino acid peptide from the C-terminus of connexin 43 (Cx43), modified to promote cellular uptake. Previous studies applying αCT1 to excisional skin wounds in porcine models produced tissues having an overall reduced level of scar tissue and decreased healing time. Rapid metabolism of αCT1 in previous work led to the investigation of extended release on wound healing rate used in this study. Here we delivered αCT1 both directly, in a concentrated pluronic solution, and in a sustained system, using polymeric alginate-poly-l-ornithine (A-PLO) microcapsules. Cell toxicity analysis showed minimal cell-loss with microcapsule treatment. Measurement of wound healing using histology and fluorescence microscopy indicated significant reduction in healing time of αCT1 microcapsule treated rat corneas compared with controls (88% vs. 38%). RT-PCR analysis showed an initial up regulation followed by down regulation of the gene keratin-19 (Krt19). Zonula occludens 1 (ZO-1) showed an opposite down regulation followed by an up regulation whereas Cx43 showed a biphasic response. Inflammatory indexes demonstrated a reduction in the inflammation of corneas treated with αCT1 microcapsules when compared with pluronic gel vehicle. These results suggest αCT1, when applied in a sustained release system, acts as a beneficial wound healing treatment.

1. Introduction

The ability to heal native corneal tissue would alleviate side effects such as infection and keratoplasty as well as the need for donor tissue (Aomatsu et al., 2012; Fukuda and Sasaki, 2012; Grupcheva et al., 2012; Karamichos et al., 2011; Lu et al., 2012; Shi et al., 2012; Shimmura et al., 2005; Trinkaus-Randall and Nugent, 1998; Yao et al., 2012). The cornea, with its avascular structure, follows a much different healing process than that seen in the skin. In the cornea a series of overlapping processes occur in a rapid fashion, beginning immediately after injury. Following epithelial injury, a cascade of cytokines (including IL-1 and PDGF) initiate keratocyte apoptosis (Wilson et al., 2001). Growth factor cytokines are then released by the lacrimal glands, which trigger keratocyte proliferation and migration from the stroma (Zeiske et al., 2001). This is followed by myofibroblast proliferation and migration to the wound site. Additional cytokines, such as TGFβ are released, triggering collagen production and remodeling (Netto et al., 2005). Inflammatory cells migrate to the wounded area and stromal remodeling occurs (Ye et al., 2000). As the epithelial layer completes closure the inflammatory cells undergo apoptosis and

Abbreviations: αCT1, alpha carboxy terminus 1; Cx43, connexin 43; Krt19, keratin-19; ZO-1, zonula occludens 1; $CaCl_2$, calcium chloride.

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the keratocytes return to their original state (Wilson et al., 2001). The limbus of the corneal epithelium is a source of stem cells capable of transmigrational proliferation, but is not known to be necessary for reepithelialization after wounding (Dua et al., 2010). While the outline of the healing process is known, many of the details of the process and associated factors are still under investigation.

As a means of investigating the potential in vivo actions of αCT1 in corneal healing the Epithelial Mesenchymal transition (EMT) pathway was investigated here. EMT is a biological method of cellular rearrangement and repair of damaged tissue where immobile cells used for structural integrity and boundary formation may be mobilized to an area of need (Lee et al., 2006). Once the EMT process is complete the mesenchymal cells convert back to epithelial cells in the process called MET, or mesenchymal–epithelial transition. Typically the EMT process is tightly regulated by the body, as in embryogenesis (Radisky, 2005). The EMT process is not as well understood in the eye, with differing opinions on the extent of the relationship with wound healing. However, both Kawakita et al., 2012 and Aomatsu et al., 2012 were able to show the prevalence of EMT in the eye using cornea epithelial cells in relation to TGFβ and Slug signaling in recent studies. Similar results summarizing the occurrence of endothelial mesenchymal transitions in the cornea have been published as well (Lee et al., 2012), leading to the belief that may EMT play a role in the corneal healing.

The αCT1 peptide is a biotinylated 25 amino acid sequence, comprised of a 16 amino acid antennapedia domain, connected to a 9 amino acid (RPRPDDLEI) sequence from the C-terminus of Cx43 (Rhett et al., 2008, 2011). The cytoplasmic tight junction protein zonula occludens 1 (ZO-1) binds at its PDZ-2 domain with the DDLEI sequence of the Cx43 C-terminus end (Duffy et al., 2002). Previously Barker et al. (2002) found that intact ventricular myocardium exhibited a low level of ZO-1-Cx43 interaction. This interaction may be disrupted, leading to changes in protein–protein interactions which affect formation of gap junctions. Connexins play a key role as mediators of both cell growth and death and function in immune response, hematopoiesis, and development of progenitor cells (Herve et al., 2004; Oviedo-Orta and Evans, 2004; Vinkin et al., 2011). Binding of the C-terminus of Cx43 to ZO-1 is believed to affect cellular communication and gap junction remodeling in wound healing (Soder et al., 2009). αCT1 competitively inhibits this binding (Hunter et al., 2005; Soder et al., 2009), increases the rate of wound healing, and when applied to in vivo models reduced scar tissue formation in multiple wound healing models including cardiovascular injury, biomedical device implantation, and excisional skin wound models (Ghatnekar et al., 2009; O'Quinn et al., 2011; Soder et al., 2009). Barker et al. 2002 also developed a control peptide with the active C-terminus 9 amino acid sequence reversed while the inactive 16 amino acid antennapedia portion was left unchanged. While these results are promising, αCT1 showed a rapid metabolism rate in previous studies (<2 h). As a result we synthesized microcapsules capable of extending release of αCT1 over 48 h to determine therapeutic potential.

Previous studies from our laboratory using alginate poly-l-ornithine and alginate poly-L-lysine microcapsules loaded with 200 μM concentrations of αCT1 detailed the release profiles of the microcapsules (Moore et al., 2013). Release characteristics were adjusted using different polymer coatings and reduction in pore size by adjusting pH to 4.3 from a biologically neutral 7.4. Electrospray microencapsulation uses a voltage differential between a positively charged syringe attached to a high voltage generator and a grounded material, such as a crosslinking gelling bath. A device to control flow rate, such as a syringe pump, passes a polymeric material through the voltage field, which overcomes the surface tension force to produce droplets of a specific size (Chakraborty et al., 2009; Enayati et al., 2011). Here we apply identically synthesized peptide loaded capsules to a corneal wound model.

2. Materials and methods

2.1. Surgical creation of corneal wounds and post surgical treatment

Male Sprague–Dawley rats (Harlan Laboratories) (~240–260 g) were given food and water ad libitum. All experiments were carried out in compliance with the University of South Carolina Animal Resources Facility guidelines and Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences. Prior to surgery, rats were placed under anesthesia using 60 mg/kg Ketamine, 7.5 mg/kg Xylazine, and 1 mg/kg Acepromazine. Two drops of a topical anesthetic, Alcaine (Alcon Canada, Mississauga, Canada) were applied to each eye, then wicked with a sterile ophthalmic sponge (Merocel, Beaver-Visitec International). A 5 mm trephine was placed on the surface of the cornea and two drops of 20% isopropyl alcohol were placed on the cornea surface for exactly 30 s. Care was taken to wound only the central cornea, excluding damage to the surrounding limbus. Exposure to the alcohol solution created a loosened 5 mm area of corneal epithelium, which was removed by gentle scraping under a dissecting microscope, leaving the exposed stromal layer. The cornea was then rinsed with 1% saline and wiped with an ophthalmic sponge to remove any remaining loose epithelium.

Post surgery, rats were placed into one of five time point groups spanning a one month period; 1 day, 3 days, 10 days, 21 days or 30 days. These five time points were repeated for four separate treatments with a total of 100 rats used in the study (25 per treatment/5 per time point). At each time point a total of 10 eyes were used with identical treatment in both eyes of each rat to eliminate eye to eye cross contamination through the tear ducts. To ensure sustained delivery of peptide in the cornea, a pluronic gel carrier (Pluronic F-127, Sigma) was used at a concentration of 25% w/v. The pluronic solution remains liquid at 4 °C, but gels once in contact with the warm eye. Treatment groups were comprised of two controls (10 μl 25% pluronic gel or 10 μl of 150 μM reverse peptide/25% pluronic gel) and two treatments with αCT1 peptide (10 μl 150 μM αCT1/25% pluronic gel per eye or 150 μM αCT1 A-PLO microcapsules/10 μl 25% pluronic gel per eye). Each treatment was applied immediately after surgery (0 h), 24 h, and 72 h. All rats were ethically sacrificed at the designated endpoints.

2.2. Synthesis and release of αCT1 from A-PLO microcapsules

Sterile sodium alginate (Sigma–Aldrich catalog#A0682, high maluronic acid content, low viscosity) poly-l-ornithine (Alfa Aesar L-ornithine hydrochloride 99%) microcapsules were synthesized and release of αCT1 was measured according to a previously published protocol (Moore et al., 2013). All microcapsules were synthesized with a 2% alginate/0.5% PLO concentration and gelled in 0.15 M calcium chloride ($CaCl_2$) (Sigma–Aldrich) solution 12 min. Synthesis parameters were constant at a needle to working bath distance of 7 mm, a voltage of 6.0 kV, and a flow rate of 60 mm/h. Microcapsule solutions were buffered to pH 4.3 using 0.1 M HEPES and 0.1 M hydrochloric acid (HCL) to control pore size and release rate. A-PLO microcapsules were loaded with αCT1 according to previous studies (Moore et al., 2013), with an initial concentration of 200 μM. To determine the peptide release profile, synthesized capsules were rinsed three times in deionized water, incubated at 37 °C, and the released peptide concentration determined with a microBCA Assay kit (Thermo Scientific).