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Sustained release of stromal cell derived factor-1 from an antioxidant thermoresponsive hydrogel enhances dermal wound healing in diabetes

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ABSTRACT

Diabetic foot ulcers (DFUs) are a severe complication of diabetes mellitus. Altered cell migration due to microcirculatory deficiencies as well as excessive and prolonged reactive oxygen species production are implicated in the delayed healing of DFUs. The goal of this research was to assess whether sustained release of SDF-1, a chemokine that promotes endothelial progenitor cell homing and angiogenesis, from a citrate-based antioxidant thermoresponsive polymer would significantly improve impaired dermal wound healing in diabetes. Poly (polyethylene glycol citrate-*co*-*N*-isopropylacrylamide) (PPCN) was synthesized *via* sequential polycondensation and free radical polymerization reactions. SDF-1 was entrapped *via* gelation of the PPCN + SDF-1 solution above its lower critical solution temperature (LCST) and its release and bioactivity was measured. The effect of sustained release of SDF-1 from PPCN (PPCN + SDF-1) *versus* a bolus application of SDF-1 in phosphate buffered saline (PBS) on wound healing was evaluated in a diabetic murine splinted excisional dermal wound model using gross observation, histology, immunohistochemistry, and optical coherence tomography microangiography. Increasing PPCN concentration decreased SDF-1 release rate. The time to 50% wound closure was 11 days, 16 days, 14 days, and 17 days for wounds treated with PPCN + SDF-1, SDF-1 only, PPCN only, and PBS, respectively. Wounds treated with PPCN + SDF-1 had the shortest time for complete healing (24 days) and exhibited accelerated granulation tissue production, epithelial maturation, and the highest density of perfused blood vessels. In conclusion, sustained release of SDF-1 from PPCN is a promising and easy to use therapeutic strategy to improve the treatment of chronic non-healing DFUs.

1. Introduction

Diabetic foot ulcers (DFUs) are a severe complication of diabetes mellitus with 15% of patients diagnosed with diabetes developing foot ulcers during their lifetime [1]. Chronic non-healing DFUs are a major cause of nontrauma-related lower limb amputations worldwide causing significant morbidity and a negative economic impact on society [2,3]. DFUs can be caused by neuropathy, ischemia, deformity, and edema at the distal part of the body and can quickly progress to tissue necrosis due to impaired healing and the propensity to infection if not treated properly. Patients with diabetes are more prone to have microcirculatory deficiencies at the distal part of their body due to impaired angiogenesis, reduced capillary size, and thickened basement membrane [4,5]. Impaired angiogenesis has been shown to be correlated with abnormal EPC (endothelial progenitor cells) migration, proliferation, and tubularization in these patients [6–10]. Furthermore, the inflammation phase of the overall healing process is often prolonged in such wounds [11]. Impaired angiogenesis and persistent inflammation may also be due to the overproduction of reactive oxygen species (ROS), a consequence of intracellular hyperglycemia in diabetic chronic wounds [12,13]. Furthermore, delayed re-epithelialization due to hyperproliferative keratinocytes with impaired differentiation and migration has also been shown to contribute to chronic non-healing DFU [14].

The fundamentals of good clinical care for these chronic wounds include adequate off-loading, frequent debridement, moist wound care, and anti-infection treatments. However, even when properly managed, some wounds remain open and difficult to heal [15]. Therapies involving biologically active components have been introduced recently to

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increase the probability of complete closure in difficult-to-heal DFUs. These include the use of recombinant platelet-derived growth factor (PDGF) (trade name Regranex®) and allogeneic cell-based dermal substitutes such as Apligraf® [16,17]. These products, although initially promising, are very costly and can have significant side effects. For example, the repeat use of high concentration of PDGF is associated with increased cancer risk due to the involvement of PDGF in melanoma progression [18,19]. As for cell-based products, limited graft survival rate, increased scarring, and host immune rejections have been reported [20,21].

Given the importance of angiogenesis in wound healing and our understanding of the vascular regenerative potential of endothelial progenitor cells (EPCs), strategies that promote the recruitment of this cell population could potentially lead to the development of better therapeutics for treating chronic wounds [22]. In this regard, endogenous secretion of SDF-1 has been shown to recruit circulating bone marrow-derived EPCs to wounds and improve angiogenesis as well as keratinocyte proliferation [23,24]. In the diabetic condition, the down-regulation of SDF-1 contributes to the delayed healing at the wound site by negatively impacting angiogenesis and keratinocytes differentiation and proliferation [23,25]. Obtaining a desirable biological healing response in the diabetic wound due to SDF-1 signaling will likely require sustained release of the chemokine from the wound site to create a chemotactic gradient and overcome its short half-life [26–28]. Furthermore, unlike multiple applications of high concentrations of SDF-1, slow release of significantly lower amounts of SDF-1 may avoid carcinogenesis [29].

Herein, we investigate the use of poly(polyethylene glycol citrate-*co*-*N*-isopropylacrylamide) (PPCN), a thermoresponsive biodegradable polymer that undergoes rapid and reversible phase transition from liquid to solid under physiologically relevant conditions [30], as a vehicle to slowly release SDF-1 at the wound site to treat dermal wounds in diabetes. We hypothesize that localized sustained release of SDF-1 from PPCN will result in an accelerated wound healing rate that is associated with an increase in vascularization at the wound site. PPCN has been shown to efficiently entrap and slowly release proteins and when injected in subcutaneous tissue it is completely absorbed within 30 days [30]. Also, PPCN has intrinsic antioxidant properties due to the diol-citrate esters in its backbone that may reduce oxidative stress at the wound. These properties make it a potentially interesting candidate biomaterial for an advanced wound dressing that would facilitate tissue regeneration and increase wound healing rate. In this study, we demonstrate that SDF-1 slowly delivered *via* PPCN accelerates wound closure, granulation tissue production, epithelial maturation and local angiogenesis. We also, for the first time, demonstrate the use of a non-invasive visible light optical coherence tomography-based imaging technique for monitoring functional microvascular structures at the wound site.

2. Materials and methods

2.1. Materials

Citric acid, polyethylene glycol (PEG), glycerol 1,3-diglycerolate diacrylate *N*-isopropylacrylamide monomer (NIPAAm), 2,2-Azobisisobutyronitrile (AIBN), phosphate buffered saline (PBS), sodium hydroxide and diethyl ether were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant Human and Mouse SDF-1 Protein and all the primary antibodies used for histological studies were purchased from Abcam (Cambridge, MA). SDF-1 Duo Set ELISA kit was from R&D Systems (Minneapolis, MN). Primary human dermal fibroblasts (HDF) were purchased from Life Technologies (Grand Island, NY), human umbilical vein endothelial cells (HUVECs), and human epithelial keratinocytes (HEKa) and all cell culture media were from Lonza (Walkersville, MD). All cells, between passage 2 to 4, were cultured in their corresponding optimized culture media, in a humidified incubator equilibrated with 5% CO_2 at 37 °C. Tissue culture supplies were ordered from Corning Inc. (Corning, NY). Animals used in the study were ordered from Jackson Laboratories (Bar Harbor, ME). Acrylate tape, TegaDerm™ and Vetbond were purchased from The 3M Company (St. Paul, MN), 6-0 nylon sutures from Ethicon (Cincinnati, OH), and 6 mm and 10 mm skin biopsy punches from Acuderm Inc. (Ft. Lauderdale, FL).

2.2. Synthesis of PPCN

PPCN, also referred to as Nanonets™, was prepared as reported previously [30]. Briefly, the poly (polyethylene glycol citrate) acrylate prepolymer (PPCac) was synthesized with citric acid, PEG and glycerol 1,3-diglycerolate diacrylate through a polycondensation reaction. The resulting solution was then reacted with pre-purified NIPAAm in a 1:1 weight ratio. The free radical initiator AIBN was used to initiate the polymerization reaction, which was conducted for 12 h. PPCN was purified by diethyl ether precipitation. The final product was dissolved in water, neutralized to PH 7.4 with sodium hydroxide, lyophilized and sterilized *via* ethylene oxide gas sterilization. ^{1}H NMR analysis was used to verify the successful synthesis of PPCN.

2.3. SDF-1 entrapment and release from PPCN

SDF-1 was labeled with I-125 using the chloramine T/sodium iodine, and quantified by enzyme-linked immunosorbent assay (ELISA) after labeling. SDF-1 doped with 1:100 iodinated SDF-1 was added to various concentrations of PPCN solution (70, 100 and 130 mg/ml, pH 7.4 in PBS) to achieve a final concentration of 500 ng of SDF-1 per milliliter. Three different concentrations were used to investigate the effect of PPCN concentration on the release kinetics of the SDF-1. Samples were allowed to solidify at 37 °C for 30 min, gently rinsed with pre-warmed PBS, and subsequently incubated in PBS at 37 °C. Loading efficiency was assessed by measuring SDF-1 labeled with I-125 remaining in the gel and comparing it to the initial amount of I-125-labeled SDF-1 added to the PPCN solution. At predetermined intervals, the PBS supernatant from the gels was sampled and replaced with fresh warm PBS. The released protein was quantified using a gamma counter (PerkinElmer, Waltham, MA) with a standard curve of iodinated SDF-1 protein at each time point. The study was carried out for 21 days.

2.4. Bioactivity of SDF-1 released from PPCN

The bioactivity of SDF-1 released from PPCN was evaluated using a modified cell transmigration assay (6.5 mm diameter transwell inserts with 8.0 μm pore size) (Fig. 1B). PPCN + SDF-1 was prepared in solution form by adding SDF-1/PBS solution to a PPCN/PBS solution (100 mg/ml) at room temperature to obtain a final SDF-1 concentration of 500 ng/ml. 100 μl of the resulting PPCN + SDF-1 solution was applied to the lower chamber of the transwell as a liquid and solidified at 37 °C to achieve the entrapment of the protein. On top of the solidified PPCN, 400 μl of pre-warmed basal cell culture media were added to serve as the releasing media. Cell culture media (Ctrl) and PPCN only were used as negative controls and a 500 ng/ml SDF-1 solution was used as a positive control. The upper compartment of the transwell was seeded with HUVECs immediately prior to the start of the assay ($10x10^3$ cells in 200 μl cell growth media). Cells were allowed to migrate for 10 h at 37 °C, fixed with 4% paraformaldehyde, and cells that crossed the transwell membrane were visualized *via* Hoechst staining and imaged using fluorescence microscopy.

To obtain percent migrating cells, instead of fixing the cells after 10 h, cells from both sides of the transwell membrane were detached using Accutase (Life Technologies, Carlsbad, *CA*), re-plated separately into new wells, and stained with CyQUANT (Life technologies). The fluorescence intensity (FI) of each well was measured with a plate reader