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In vitro and in vivo epidermal growth factor gene therapy for diabetic ulcers with electrospun fibrous meshes

Hye Sung Kim [a], Hyuk Sang Yoo [a,b,*]

[a] Department of Biomedical Materials Engineering, School of Bioscience and Bioengineering, Kangwon National University, Chuncheon 200-701, Republic of Korea
[b] Institute of Bioscience and Bioengineering, Kangwon National University, Republic of Korea

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ABSTRACT

Human epidermal growth factor (hEGF) gene therapy was achieved with an electrospun nanofibrous mesh with matrix metalloproteinase (MMP) responsiveness to control release of plasmid human epidermal growth factor (phEGF) in diabetic ulcers. For MMP responsiveness, linear poly(ethyleneimine) (LPEI) was immobilized on the surface of the nanofiber via an MMP-cleavable linker. phEGF was electrostatically incorporated into LPEI-immobilized nanofibrous meshes with various charge ratios and phEGF incorporation efficiency was increased with increasing charge ratios. The release of both phEGF and LPEI was significantly increased in the presence of MMP-2 due to the enzymatic digestion of the MMP-cleavable linkage between the matrix and LPEI. Human dermal fibroblasts with the released fraction showed a higher expression level of hEGF compared to naked phEGF or phEGF/LPEI complexes. Diabetic wounds treated with phEGF-incorporated nanofibrous meshes showed high hEGF expression level and accelerated wound recovery rates without wound contractions for 14 days. Neocollagen and cytokeratin accumulation were significantly increased as well as the expression of the keratinocyte-specific markers at the re-epithelized tissue treated with phEGF nanofibrous meshes, which clearly indicates that EGF gene was transfected to dermal cells and this consequently assisted wound recovery without phenotypic changes of the re-epithelized tissues. Thus, phEGF-incorporated nanofibrous mesh is expected to accelerate the wound-healing process as well as reduce wound contraction during recovery from diabetic ulcers.

1. Introduction

Diabetic impairment of the cutaneous wound-healing process is a major contributor of surgical amputations in patients with diabetes mellitus symptoms [1–6]. Aberrant cellular infiltration, insufficient macrophage activation and poor angiogenesis are considered to attenuate deposition of new extracellular matrix (ECM) and subsequently retard re-epithelialization of wound sites. Thus, in order to accelerate wound healing efficacy in the diabetic wounds, wound-dressing materials containing various growth factors and antibiotics have been widely prepared and clinically employed to enhance neo-angiogenesis and re-epithelialization in the affected individuals. Among these, wound-dressing materials containing epidermal growth factor (EGF) were shown to accelerate migration and proliferation of dermal cells in the wound sites and result in partial and complete healing [7–9]. Gelatin–hyaluronate sponges with antibiotic and EGF showed fast epithelium regeneration, resulting in good wound-healing performances during the whole healing period [10]. EGF-incorporated collagen sponges with an acid-soluble fraction of pig skin increased the fibroblast cell proliferation rate and showed well-developed collagen bundles in the wound site, which enhanced the formation of dermal matrix and improved the wound's mechanical strength [11]. Recombinant human EGF (hEGF) was immobilized on the electrospun nanofibers for wound healing in diabetic ulcers and the wound sites with the fibrous meshes showed higher expression of keratinocyte-specific genes and superior in vivo wound healing activities compared to no treatment or EGF solutions [12]. However, EGF-incorporated medical products showed only limited success for diabetic foot ulcers because of their short half-life and the necessity for repeated administration of such EGF pharmaceuticals. Both the toxicity associated with the repeated doses as well as the stability of EGF in diabetic ulcers need to be improved for further application of EGF for wound dressing.

Abnormally elevated levels of matrix metalloproteinases (MMPs) at wound sites is considered to be a major cause of diabetic wound impairments because ECM remodeling rates are significantly retarded. Unlike normal wounds, where MMPs promote infiltration of dermal cells to accelerate the wound-heal-

* Corresponding author at: Department of Biomedical Materials Engineering, School of Bioscience and Bioengineering, Kangwon National University, Chuncheon 200-701, Republic of Korea. Tel.: +82 33 250 6563; fax: +82 33 253 6560.
E-mail address: hsyoo@kangwon.ac.kr (H.S. Yoo).

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ing process, the unusually high activity of MMPs at diabetic wound beds causes the demolition of ECM proteins such as collagen and elastin because the disappearance rates of ECM surpass the neo-ECM formation rates. In terms of accelerating wound healing rates in diabetic ulcers, administration of EGF can effectively decrease the destruction of ECM by enhancing the proliferation of dermal cells. However, the bioavailability of EGF was rapidly reduced in the harsh conditions of the wound sites, where hydrolytic enzymes show robust activity [13]. One approach to potentially bypass the stability issue of EGF is to promote the proliferation of dermal cells by transferring and expressing the correct genes for the desired growth factors at wound sites. Although the number of gene therapies studied for wound healing is limited, transgene expression of growth factors in dermal cells was confirmed to improve wound recovery by a single dose of plasmid DNA. Plasmid DNA encoding vascular endothelial growth factor (VEGF) was combined with an arginine-grafted cationic dendrimer and injected subcutaneously into diabetic skin ulcers [14]. After 6 days, the skin in most of the wounds in diabetic mice was regenerated and displayed a well-ordered dermal structure, with a high level of VEGF expression. Keratinocyte growth factor (KGF) plasmid DNA was administered to the wound via electroporation [15]. Within 12 days, over 90% of wounds were healed in the presence of KGF and electroporation as opposed to 40% in the untreated group, and the recovered wounds showed intact and mature epithelial structures.

Electrospun nanofibrous meshes (NFs) have recently received much attention as potential candidates for wound-dressing materials in combination with various drugs. Their architecture, resembling ECM, has been shown to enhance cellular proliferation because the nanofibrous structures can render similar topological cues as collagen fibrils to the surrounding cells. Furthermore, the high surface-to-volume ratio and the nanoporous structure of the NFs are advantageous in terms of the immobilization of bioactive molecules and the antibacterial functions for wound repairs. Due to the high porosity and the nanoscaled pores, wound exudates and oxygen can be effectively transported and absorbed through the mesh while bacterial infiltration is strictly excluded [16–18]. Additionally, NFs with fine fibrous structures are very flexible and can easily match the 3-D contours of the skin surface, suggesting that they can provide excellent conformability, better coverage and good protection for the wounds. Thus, current evidence suggests that NF combined with a growth factor or therapeutic gene is a powerful tool for wound healing. Coaxial electrospun NF encapsulating basic fibroblast growth factor (bFGF) in the core with EGF simultaneously decorating the fiber surface was administered to the wounds of diabetic models. The biphasic release of bFGF and EGF promoted tissue recovery, with the accumulation of both collagen and a cemented matrix of keratin [19]. Polyplexes of basic fibroblast growth factor-encoding plasmid (pbFGF) with poly(ethyleneimine) (PEI) were incorporated into electrospun nanofiber made of poly(DL-lactide)–poly(ethylene glycol) and poly(ethylene glycol) (PEG). pbFGF-loaded fibrous mats significantly increased the recovery rate of dorsal wounds in diabetic rats, with improved vascularization, enhanced collagen deposition and maturation, and complete re-epithelialization [20].

In this study, we prepared MMP-responsive NF and incorporated plasmid DNA encoding human EGF (phEGF) to improve wound healing in diabetic ulcer. We previously reported that nucleic acids such as plasmid DNA encoding green fluorescence protein and small interfering RNA against matrix metalloproteinase-2 (MMP-2 siRNA) were successfully incorporated into MMP-responsive NF by electrostatic interaction with linear poly(ethyleneimine) (LPEI) tethered on the nanofibrous surface, and nucleic acid/LPEI complexes were significantly liberated from matrix by enzymatic attack of MMP-2 in diabetic ulcers [21,22]. Thus, we hypothesized that MMP-responsive NF incorporating phEGF can effectively transfer phEGF to the wound site and produce hEGF, which can promote the wound-healing process synergistically with the physical assistance of the NF.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) (PCL; Mw 70,000–100,000) was purchased from Wako Chemicals (Osaka, Japan). Bifunctional PEG (PEG-$(amine)_2$; Mw 2,000) was purchased from SunBio Co. (Anyang, South Korea). An MMP-cleavable peptide (DGPLGVC) was customized by Anygen Co. (Gwangju, South Korea). LPEI (Mw 25,000) was purchased from Polysciences Inc. (Warrington, PA). Plasmid DNA encoding human epidermal growth factor (phEGF) was a kind gift from Prof. Jeong Hyun Park at Inje College of Medicine. Purified MMP-2 was purchased from Calbiochem (San Diego, CA). Micro BCA™ Protein Assay Kit and N-succinimidyl-3(2-pyridyldithio) propionate (SPDP) were purchased from Thermo Fisher Scientific (Waltham, MA). Pyridine was obtained from Daejung Chemical Co. (Cheongwon, South Korea). Fluorescamine, *p*-nitrophenylchloroformate (p-NPC), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and Masson's Trichrome Staining Kit were purchased from Sigma–Aldrich (St. Louis, MO). Advanced Protein Assay (APA) reagent was purchased from Cytoskeleton (Denver, CO). The following were purchased from Invitrogen (Carlsbad, CA): Quant-iT™ PicoGreen® dsDNA Reagent and Kits, primary human dermal fibroblasts (HDFs), low-serum growth supplement (LSGS), Dulbecco's modified Eagle's medium (DMEM), streptomycin/penicillin and trypsin/ethylenediaminetetraacetic acid (EDTA). TRI Reagent® was purchased from Molecular Research Center, Inc (Cincinnati, OH). DuoSet® ELISA Development System was purchased from R&D system, Inc. (Minneapolis, MN). C57BL/6 female mice were supplied by Daehan Biolink (Eumseong, South Korea). Streptozotocin was purchased from Enzo Life Science (Farmingdale, NY). VECTASTAIN® Elite ABS Kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). Anti-keratin primary antibody (mouse polyclonal IgG) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5× Reverse Transcription Premix and HiPi Plus 5× PCR PreMix were purchased from Elpis Biotech (Daejeon, South Korea). All primers for reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

2.2. Synthesis of PCL–PEG diblock copolymer

Amine-terminated PCL–PEG diblock copolymer was synthesized as in a previous study [21]. Briefly, PCL (12 g) was activated with pyridine (45 mg) and p-NPC (60 mg) in dichloromethane (60 ml) at 0 °C and the mixture was reacted at room temperature for 3 h. The reaction mixture was then precipitated in ice-cold diethyl ether and dried under a vacuum. The activated PCL (10 g) in dichloromethane was added drop-by-drop to bis-amine PEG (1.2 g) in dichloromethane with vigorous stirring and incubation at room temperature for 23 h (molar ratio of activated PCL:bis-amine PEG = 1:5). The mixture was then precipitated in ice-cold ethanol (95%) and completely dried under a vacuum. The PCL–PEG conjugation was confirmed by ^{1}H-nuclear magnetic resonance spectroscopy in $CDCl_3$ at the central laboratory of Kangwon National University (Bruker, DPX 400 MHz). We confirmed that PCL was conjugated to PEG in a 1:1 M ratio, suggesting that diblock copolymer was successfully synthesized (data not shown).

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