Evaluation of a Highly Skin Permeable Low-Molecular-Weight Protamine Conjugated Epidermal Growth Factor for Novel Burn Wound Healing Therapy

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ABSTRACT: We evaluated the laser induced burn wound healing efficacy of a recombinant low-molecular-weight protamine conjugated epidermal growth factor (rLMWP–EGF). rLMWP–EGF was prepared by genetically combining LMWP with the N-terminal sequence of EGF; we obtained a homogeneous modified EGF without reduced biological activity. Because of the protein transduction domain of LMWP, rLMWP–EGF showed enhanced drug penetration across artificial skin constructs and excised mouse skin layers versus EGF and showed significantly improved burn wound healing efficacy, with accelerated wound closure and minimized eschar and scar formation, compared with EGF or no treatment. Histological examination also revealed that rLMWP–EGF permeated through the intact skin around the wound and facilitated residual epithelial cell proliferation in an integrated manner to reform an intact epidermis. Radiofrequency microwound formation was effective for reducing large hypertrophic scars formed after severe laser burning by collagen remodeling but rLMWP–EGF did not show a meaningful synergistic effect in burn scar reduction. However, rLMWP–EGF was helpful for forming skin with a more normal appearance and texture. Thus, rLMWP–EGF demonstrated therapeutic potential as a novel topical burn wound healing drug with no obvious toxic effect. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

The healing of wounds is one of the most complex and interactive biological processes, involving inflammation, proliferation, angiogenesis, reepithelialization, tissue regeneration, and remodeling. In particular, burns are special types of wounds that require specialized management protocols and are classified according to the depth of injury. Burns to the skin or other tissues produce a markedly different healing response because of their effects on the viability of cells and tissues.[1,2] Thermal burns, in particular, create an extensive zone of frank necrosis that includes dead cells and denatured or even charred connective tissue. Beyond the area of total destruction, a zone of coagulation necrosis exists, in which denaturation of plasma and cellular proteins leads to the obstruction of blood vessels and lymphatics. This effect, in turn, induces nutrient starvation in the involved tissue.[3]

Numerous cells release pro-inflammatory and anti-inflammatory cytokines, and various growth factors modulate the wound healing process and accelerate or slow tissue repair.[4,5] Among them, growth factors act as critical extracellular signals that orchestrate the wound healing process by binding to specific high affinity receptors on the cell surface to stimulate cell proliferation and synthesis of new extracellular matrix (ECM).[6–8]

To date, various growth factors have been examined for their potential as wound therapeutics, such as transforming growth factor-β and vascular endothelial growth factor,[9–11] which accelerate the healing of wounds in animal models. Platelet-derived growth factor has also been shown to accelerate the healing of human diabetic foot ulcers, and basic fibroblast growth factor has been approved as a treatment for wounds in Japan.[12,13] Several other growth factors, including granulocyte-macrophage colony-stimulating factor and epidermal growth factor (EGF), have shown benefits in clinical trials.[14,15] Some reports regarding growth factors in burn wound healing have suggested that they may play an important role in the healing process.[16–18]

Of the various growth factors described above, EGF is a polypeptide composed of 53 amino acids that accelerates epidermal and mesenchymal regeneration, cell motility, and cellular proliferation. EGF acts by binding to the EGF receptor, a tyrosine kinase, thereby initiating a series of events that regulates cell proliferation.[19,20] The role of EGF in wound healing in human and animals has been investigated extensively. In addition, topical administration of EGF accelerates dermal regeneration of partial-thickness or second-degree burn wounds.[21–24] Currently, at least three companies worldwide are marketing EGF-based formulations to treat burns.

However, most products are applied topically to the wound; thus, the efficacy of EGF products is critically limited by its poor transdermal permeability and biological stability. Many studies

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have suggested that the local concentration of EGF needs to be sufficiently high and prolonged for effective wound healing.[25–27] Thus, many delivery systems have been developed to obtain high concentrations and extended release of EGF, including hydrogels, sponges, polymeric pellets, eletrospun nanofibers, microspheres, a coacervate delivery system with heparin, and a biomimetic delivery system that incorporates EGF with biocompatible ECM components such as hyaluronic acid, collagen, and vitronectin.[28–34] However, these systems still have limitations, such as poor loading efficacy and penetration through the skin, and reductions in bioactivity during preparation and after application.[35–37]

Here, we introduce a powerful percutaneous delivery system that enhances skin penetration, which is otherwise a strong barrier to drug efficacy, using a genetically designed conjugation of a protein transduction domain (PTD) to EGF. We can fully preserve EGF's original biological activity during preparation and facilitate rapid and highly localized delivery of EGF around the wound area. In a recent study, we genetically conjugated a highly positively charged low-molecular-weight protamine (LMWP), a nontoxic, arginine-rich cell translocating sequence (VSRRRRRRGGRRRR) in protamine, to the N-terminal of EGF (rLMWP–EGF), and demonstrated its markedly improved skin permeability *in vitro*. In addition, topically applied rLMWP–EGF was significantly effective for accelerating wound healing in full thickness and diabetic wound models.[38]

In the present study, we prepared a poloxamer gel formulation of rLMWP–EGF and demonstrated its enhanced skin penetration and efficacy for the treatment of laser-induced burn wounds in mice. We also evaluated the synergistic effect of radiofrequency (RF)-induced microwound formation with topical administration of rLMWP–EGF in reestablishing postburn scarring by percutaneous collagen induction. Finally, we conducted dermal irritation experiments in rabbits and skin sensitization tests in mice using rLMWP–EGF in compliance with Organization for Economic Cooperation and Development (OECD) guidelines to evaluate any toxicological effects because of its increased skin penetration.

MATERIALS AND METHODS

Materials

Recombinant human EGF (rEGF) and LMWP were supplied by BIO FD&C (Whasun, Jeonnam, South Korea). A polyethylene polyoxypropylene block copolymer (poloxamer 188) was obtained from BASF for the topical gel formulation (Ludwigshafen, Germany).

Animal Care

The animals were acclimatized for 1 week in an animal facility under controlled conditions of temperature (23 ± 3°C), relative humidity (55 ± 10%), and light (12/12 h light/dark, with no ultraviolet exposure). The animals had free access to a laboratory diet (Purina Company, St. Louis, Missouri) and ion-sterilized tap water. All experiments were performed in accordance with the Pacific Pharma Institutional Animal Care and Use Committee and the OECD "Guide for the Care and Use of Laboratory Animals."

Methods

Preparation of rLMWP–EGF.

rEGF carrying LMWP at its N-terminus was prepared with an expression plasmid that would overproduce a protein containing LMWP–EGF in *Escherichia coli*, as described previously. Briefly, cDNA for LMWP–EGF was constructed by serial polymerase chain reaction (PCR) of the successive addition of amino acids of LMWP to the EGF gene (GenBank Accession No. P01133) as the first template. Then, the LMWP–EGF gene fragment was amplified by PCR to clone the cDNA for LMWP–EGF between *Nde*I and *Xho*I restriction sites. The forward primer was 5′-CATATGGTGAGCCGTAGACGTAGACG-3′ and the reverse primer was 5′-CTCGAGTCATTAGCGCAGTTCCCACCACTTCAGGTCTCGGT-3′. The resulting PCR product was sub-cloned into the TOPO vector. After plasmid purification and double digestion with *Nde*I/*Xho*I, the insert DNA fragment was cloned into a pET-41b(+) expression vector. Then, the correct expression vector, pET-41b(+)-LMWP–EGF, was transformed into *E. coli* competent cells (BL21-DE3). BL21-DE3 cells harboring pET-41b(+)-LMWP–EGF were grown in 2.0 L LB medium supplemented with 100 μg/mL ampicillin at 37°C until the optical density of the culture at 600 nm was 0.6–0.8. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 M, IPTG) and the culture was incubated overnight at 100 rpm and 25°C. After cell lysis, the supernatant containing rLMWP–EGF was purified by application to an ion-exchange column packed with Q-cellulose and a Sephadex G-100 column.

The purity of rLMWP–EGF was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining and the protein concentration was determined using the Micro BCA Protein Assay kit with bovine serum albumin as the standard (Pierce, Rockford, Illinois). The N-terminal amino-acid sequence of the purified rLMWP–EGF was analyzed using an Applied Biosystems Procise 491 protein sequencer (Courtaboeuf, France) to confirm the LMWP conjugation.

Biological Activity of rLMWP–EGF.

In vitro biological activity of rLMWP–EGF was evaluated using a cell proliferation assay in a mouse fibroblast cell line (NIH3T3) seeded at a density of 5×10^3 cells per well in 100 μL Dulbecco's modified Eagle's medium (DMEM; Lonza, Zurich, Switzerland) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, New York) and 1% penicillin/streptomycin (Gibco). Then, the culture was incubated at 37°C for 24 h. The serum concentration in the medium was lowered to 0.05%, and the cells were incubated for a further 24 h. The cells were stimulated with 1, 10, 100, and 1000 nM rEGF or rLMWP–EGF in low-serum medium (0.5% FBS) for 24 h. To determine cell proliferation activity, the WST-1 assay (Roche Diagnostics, Mannheim, Germany) was performed according to the instruction manual. Diluted WST-1 solution [5 mg/mL in phosphate-buffered saline (PBS)] was added and incubated for 2 h. The absorbance was measured with a spectrophotometer (Molecular Devices, Sunnyvale, California). Cell viability was calculated compared with the control.

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