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Liposomal Gene Transfer of Keratinocyte Growth Factor Improves Wound Healing by Altering Growth Factor and Collagen Expression

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Background. **Growth factors affect the complex cascade of wound healing; however, interaction between different growth factors during dermal and epidermal regeneration are still not entirely defined. In the present study, we thought to determine the interaction between keratinocyte growth factor (KGF) administered as liposomal cDNA with other dermal and epidermal growth factors and collagen synthesis in an acute wound.**

Materials and methods. **Rats received an acute wound and were divided into two groups to receive weekly subcutaneous injections of liposomes plus the Lac-Z gene (0.22 μg, vehicle), or liposomes plus the KGF cDNA (2.2 μg) and Lac-Z gene (0.22 μg). Histological and immunohistochemical techniques were used to determine growth factor, collagen expression, and dermal and epidermal structure.**

Results. **KGF cDNA increased insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-3 (IGFBP-3), and fibroblast growth factor (FGF), decreased transforming growth factor-beta (TGF-β), while it had no effect on platelet-derived growth factor (PDGF) levels in the wound. KGF cDNA significantly increased collagen Type IV at both the wound edge as well as the wound bed, while it had no effect on collagen Type I and III. KGF cDNA increased re-epithelialization, improved dermal regeneration, and increased neovascularization.**

Conclusions. **Exogenous administered KGF cDNA causes increases in IGF-I, IGF-BP3, FGF, and collagen IV and decreases TGF-β concentration. KGF gene transfer accelerates wound healing without causing an increase in collagen I or III.**

Key Words: **gene transfer; KGF; wound healing; growth factors; gene therapy; skin.**

INTRODUCTION

Skin serves as a protective barrier against the environment. Loss of integrity of the skin can lead to major disability or even death [1]. Impaired healing is a major cause of morbidity for many patients. Delayed healing, as seen in the elderly and the immunocompromised patient, and impaired function due to excessive scarring as seen after a burn injury, are two examples [2]. Cutaneous wound healing is a dynamic process involving blood cells, parenchymal cells, extracellular matrix, and growth factors [3]. The primary goals of the treatment of wounds are rapid wound closure and a functional and esthetically satisfactory scar [2]. The understanding of cellular and molecular mechanisms of normal and impaired wound healing is important to develop therapies to effectively treat impaired healing. Growth factors are known to directly regulate many of the processes crucial for normal wound healing. Keratinocyte growth factor (KGF), a 26- to 28-kDa protein, is a member of the fibroblast growth factor (FGF) family and is mainly synthesized in fibroblasts, endothelial cells, and vascular smooth muscle cells [4–6]. KGF is not expressed in epithelial cells but stimulates epithelial cell proliferation and differentiation in a paracrine fashion. Its effects on other growth factors, mesenchymal cells, and the extracellular matrix are poorly understood [7].

We have previously shown that KGF cDNA gene therapy is feasible in an acute wound-healing model [8]. Liposomes containing the KGF cDNA construct effectively improved epidermal regeneration by 170% by increasing epithelial proliferation and decreasing apoptosis. Dermal morphology and neovascularization was improved with concomitant increases in vascular epithelial growth factor (VEGF) concentrations [8]. In a porcine model of epidermal wound healing, KGF

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stimulated the rate of re-epithelialization and increased the deposition of collagen fibers in the superficial dermis [9]. Since increasing the deposition of collagen fibers in the wound has an inherent risk of leading to hypertrophic scarring, we were interested in quantifying and identifying the type of collagen fiber expression that KGF induced. Furthermore, this study answered our primary questions but generated questions regarding the interaction of KGF with other growth factors and the extracellular matrix and mesenchymal cells. This study was designed to elucidate these interactions and improve our understanding of the indirect effects of KGF on acute cutaneous wound healing.

MATERIALS AND METHODS

Twenty-two adult male Sprague Dawley rats (350–375 g) were housed in a temperature-controlled room with a 12-h light–dark cycle. Rats were acclimatized to their environment for 7 days before the blinded study. All animals received similar amounts of the Fresubin liquid diet (Fresenius Medical Care, Bad Homburg, Germany) and water *ad libitum* throughout the study. Each rat received a 30% total body surface area (TBSA) full-thickness scald burn under general anesthesia (pentobarbital 50 mg/kg body weight) and analgesia (Buprenorphin 1 mg/kg body weight) following a modified procedure as previously described [10]. Rats were anesthetized, shaved, and placed in a mole, and received a 30% TBSA scald burn (99°C hot water contact 10 s to the back). After the scald, rats were immediately resuscitated by intraperitoneal injection of Ringer's lactate (50 mL/kg body weight). Thermally injured rats were then randomly divided into the two following groups to receive either weekly subcutaneous injection:

Group A: liposomes (10 μL liposomes in 180 μL saline) containing 0.22 μg of the reporter gene for β-galactosidase Lac-Z cDNA construct, vehicle, $n = 11$ or

Group B: liposomes (10 μL liposomes in 180 μL saline) containing 2.2 μg of a KGF cDNA construct, plus 0.22 μg of the reporter gene for β-galactosidase Lac-Z cDNA construct, $n = 11$.

These studies were reviewed and approved by the Animal Care and Use Committee of the Regierung der Oberpfalz, Bayern, Germany, assuring that all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. The animals were visited twice daily by the group and daily by the ACUC to ensure that animals were not suffering or in pain. Animals were treated humanely, and pain medication, special nutrition, and fluid substitution according to human burn treatment were given.

In previous experiments we found that animals receiving liposomes containing the Lac-Z gene represented a better control compared to animals receiving saline, in having better wound-healing rates and an attenuated acute phase response [11]. Therefore, we chose in the present study to use liposomes with Lac-Z as the control group. The rat KGF cDNA construct consisted of a cytomegalovirus-driven KGF cDNA plasmid prepared at the UTMB Sealy Center for Molecular Science Recombinant DNA Core Facility, Galveston, Texas. The liposomes were formulated from 1:1 (M/M) DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyl ethyl ammonium bromide) and cholesterol suspended in membrane-filtered water (Life Technologies, Inc., Rockville, MD). This reagent interacts spontaneously with KGF cDNA to form the lipid cDNA complex. The dose of 2.2 μg cDNA transfer was defined in a dose–response study [12].

Immediately after the thermal injury, each rat received 0.2 mL of the lipoplexes subcutaneously injected at two sites opposite from each other at the wound edge in healthy unburned skin. The injection occurs at the wound edge, which was designed this way as most of the cellular and metabolic activity occurs at the edge, and we wanted to transfect as many cells as possible. This was repeated each week for 4 weeks with freshly prepared mixtures shortly before injections. Animals were humanely sacrificed by decapitation 5 days after the last injection. Skin samples from the back were harvested, fixated in 4% paraformaldehyde, 1% glutaraldehyde, or snap-frozen in liquid nitrogen and stored at −73°C for analysis.

Transfection

Transfection was determined in skin samples taken 33 days after burn and 5 days after the last injection by measuring the presence of β-galactosidase. The presence of the β-galactosidase protein was detected by histochemical staining with halogenated indolyl-β-D-galactoside (Life Technologies, Inc.) for β-galactosidase in the skin. Linear skin biopsies 4 mm thick in width and extended from the center of the burn wound well into surrounding normal skin were taken at the end of the experiment and processed as previously published [8, 13].

Growth Factor and Collagen Expression (Immunohistochemistry)

Growth factor concentration in the dermis and epidermis was determined using the peroxidase immunohistochemical technique. Primary antibodies (and their respective dilutions and secondary antibody) used for detecting insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-3 (IGFBP-3), KGF, transforming growth factor-β (TGF-β), FGF, and platelet derived growth factor (PDGF) are summarized in Table 1.

Briefly, paraffin-embedded samples were cut 4 μm in thickness and rehydrated in increasing alcohol concentrations and finally in PBS. Protease K (100 μg/mL) was applied at 37°C for 30 min. After washing with PBS, endogenous peroxidases were blocked using methanol–H_2O_2 for 15 min. The samples were washed again. Samples were incubated with blocking serum for 20 min. After washing, primary antibody was applied to the samples and incubated at 4°C overnight. After washing, biotinylated secondary antibody was incubated for 1 h at 37°C. The samples were then washed and incubated with Streptavidin (1:300; DAKO P0397, Dako, Glostrup, Denmark) for 1 h at 37°C. The samples were thoroughly washed and diaminobenzidine–hydrogen peroxidase was applied for color development. Counterstaining was performed using hematoxylin. Sections were then dehydrated in increasing alcohol concentrations and 100% xylene and mounted. Growth factor concentration was determined in normal skin, wound edge, and wound bed, by the number of positive cells per high power field (HPF) and per μm^2.

Three observers blinded to treatment counted each sample at three different sites for positive cells. Collagen I, III, and IV staining followed a similar protocol with the respective primary antibody (Table 1). Three observers blinded for treatment counted each sample at three different sites (i.e., normal skin, wound edge, wound bed) for collagen I, III, and IV staining intensity. Intensity scoring ranged from 1 (weak) to 4 (strong).

To ensure objectivity, all observers were blinded to treatment and looked at the samples and graded the staining intensity at the same time. We had a central microscope that connected to two others with the same sharpness and picture quality. All samples were stained within the same batch, avoiding differences in the staining procedure.

Re-epithelialization

Re-epithelialization was determined by planimetry and by histology. The wound eschar was left intact for the first 28 days and then