Growth Factors in Porcine Full and Partial Thickness Burn Repair

Differing Targets and Effects of Keratinocyte Growth Factor, Platelet-Derived Growth Factor-BB, Epidermal Growth Factor, and neu Differentiation Factor

Dimitry M. Danilenko,* Brian D. Ring,* John E. Tarpley,* Barry Morris,* Gwyneth Y. Van,* Andrew Morawiecki,† William Callahan,† Merrill Goldenberg,† Susan Hershenson,† and Glenn F. Pierce*

From the Departments of Experimental Pathology* and Pharmaceutics,† Amgen Incorporated, Thousand Oaks, California

The topical application of recombinant growth factors such as epidermal growth factor, platelet-derived growth factor-BB bomodimer (rPDGF-BB), keratinocyte growth factor (rKGF), and neu differentiation factor has resulted in significant acceleration of healing in several animal models of wound repair. In this study, we established highly reproducible and quantifiable full and deep partial thickness porcine burn models in which burns were escharectomized 4 or 5 days postburn and covered with an occlusive dressing to replicate the standard treatment in human burn patients. We then applied these growth factors to assess their efficacy on several parameters of wound repair: extracellular matrix and granulation tissue production, percent reepithelialization, and new epithelial area. In full thickness burns, only rPDGF-BB and the combination of rPDGF-BB and rKGF induced significant changes in burn repair. rPDGF-BB induced marked extracellular matrix and granulation tissue production (P = 0.013) such that the burn defect was filled within several days of escharectomy, but had no effect on new epithelial area or reepithelialization. The combination of rPDGF-BB and rKGF in full thickness burns resulted in a highly significant increase in extracellular matrix and granulation tissue area (P = 0.0009) and a significant increase in new epithelial area (P = 0.007), but had no effect on reepithelialization. In deep partial thickness burns, rKGF induced the most consistent changes. Daily application of rKGF induced a highly significant increase in new epithelial area (P < 0.0001) but induced only a modest increase in reepithelialization (83.7% rKGF-treated versus 70.2% control; P = 0.016) 12 days postburn. rKGF also doubled the number of fully reepithelialized burns (P = 0.02) at 13 days postburn, at least partially because of marked stimulation of both epidermal and follicular proliferation as assessed by proliferating cell nuclear antigen expression. In situ hybridization for KGF in porcine burns revealed strong expression of KGF on hair follicles and basal epidermis, confirming direct rKGF action on follicular as well as epidermal keratinocytes. Although the epidermal proliferation induced by rKGF resulted in marked neopidermal psoriasiform hyperplasia with exaggerated rete ridges and neopidermal and follicular maturation as assessed by expression of cytokeratin 10, a marker of keratinocyte terminal differentiation was not delayed and appeared to be accelerated in some rKGF-treated

Accepted for publication July 17, 1995.

Address reprint requests to Dimitry M Danilenko, DVM, PhD, Department of Experimental Pathology, MS 15-2-A-226, Amgen, Inc., 1840 DeHaviland Drive, Thousand Oaks, CA 91320-1789.

Glenn F Pierce's current address is Department of Preclinical Sciences, PRIZM Pharmaceuticals, 11035 Roselle St., San Diego, CA 92121.

A portion of this work was published in abstract form (Wound Rep Reg 1995, 3:100).
burns. Recombinant epidermal growth factor induced a trend toward increased new epithelial area in deep partial thickness burns, but bad no effect on reapithelialization. The recombinant neu differentiation factor-α isoforrm bad no significant biological effects in either full or deep partial thickness burns. These results suggest that although topical application of rKGF and rPDGF-BB to burns results in highly significant increases in new epithelial area and extracellular matrix/granulation tissue production, respectively, that under the rigorous experimental parameters of our two models, these growth factors only marginally accelerate reapithelialization, the parameter most clinically relevant to burn repair. (Am J Pathol 1995, 147:1261-1277)

The application of recombinant growth factors such as epidermal growth factor (rEGF),1-3 transforming growth factor-α (rTGF-α),4 platelet-derived growth factor-BB homodimer (rPDGF-BB),3,5 basic fibroblast growth factor,3,5,6 and more recently keratinocyte growth factor (rKGF),7,8 and neu differentiation factor (rNDF)9 has resulted in significant acceleration of healing in several animal models of wound repair. Although all of these recombinant growth factors have accelerated excisional wound repair in one or more animal models, only rEGF and rTGF-α have been applied to more rigorous partial thickness burn repair models, where they moderately accelerated (both rEGF and rTGF-α)1-4 or had no effect (rEGF)10 on wound repair.

EGF is a 53 amino acid polypeptide that was originally isolated from murine submaxillary salivary gland extracts based on its ability to stimulate premature eyelid opening and incisor eruption in neonatal mice.11 EGF is a potent mitogen for many cell types, including keratinocytes.12 rEGF has stimulated wound reapithelialization in several animal models of excisional wound repair,1-3 in a porcine model of partial thickness burn repair,1 and in donor graft sites in one human clinical trial.13 In a rat model of deep partial thickness burn repair, however, rEGF was unable to significantly accelerate reapithelialization.10

PDGF was originally isolated from human platelets as a potent serum mitogen for cells of mesenchymal origin.14,15 PDGF stimulates proliferation and induces chemotaxis of multiple mesenchymal cell types, including fibroblasts, microvascular endothelial cells, smooth muscle cells, monocytes, and neutrophils.14-19 PDGF is stored in platelet α-granules, and is released from platelets at sites of tissue damage.20,21 PDGF is also synthesized by macrophages, fibroblasts, endothelial cells, and smooth muscle cells, all of which are important in wound repair.22-24 Naturally occurring PDGF from human platelets is a ~30 kd heterodimer composed of two peptide chains, designated A and B, which share ~60% homology at the amino acid level.24-27 PDGF-B chain is the product of the c-sis proto-oncogene,28 and shares ~93% homology with the product of the v-sis oncogene, a component of the acute transforming simian sarcoma virus.29,30 rPDGF-BB homodimer has stimulated dermal cellular proliferation, granulation tissue production, and collagen deposition in several animal models of wound repair,31-36 and has enhanced wound reapithelialization in several studies as well.3,5

KGF is a recently identified 28 kd member of the FGF family (alternative designation FGF7),37 which specifically binds to the KGF receptor (KGFR), a splice variant of FGF receptor 2 (FGF2).38 We and others have identified KGF as a paracrine mediator of proliferation in a wide variety of epithelial cells, including hepatocytes and gastrointestinal epithelial cells,39 type II pneumocytes,40,41 mammary epithelial cells,42,43 and keratinocytes. S,42,44 In the skin, rKGF stimulated not only epithelial keratinocytes, but also stimulated keratinocytes within hair follicles and sebaceous glands.9 This effect contrasted with the follicular inhibition induced by recombinant basic fibroblast growth factor and rEGF.45,46 KGF has significantly increased reapithelialization in two different animal models, porcine and lapine, of partial thickness excisional wound repair.7,8 In addition, KGF mRNA expression was found to be increased by 160-fold in mouse excisional wounds,47 a finding that suggests that KGF is an important endogenous mediator of wound repair.

NDF is a 44 kd glycoprotein that was isolated from ras-transformed rat fibroblasts,48,49 and indirectly induces tyrosine phosphorylation of the HER-2/neu receptor via binding to either the HER-3 or HER-4 receptor.50-53 NDF contains a receptor-binding EGF-like domain, is a member of the EGF family,48,49 and exists as multiple different isoforms which arise by alternative splicing of a single gene.54 In a rabbit ear dermal excisional wound model, we found that the NDF-α2 isoform accelerated reapithelialization and upregulated epidermal integrin expression.9

Because rEGF, rPDGF-BB, rKGF, and rNDF-α2 have all significantly accelerated one or more aspects of excisional wound repair, we sought to investigate whether these growth factors would have similar effects in porcine models of full and partial thickness wound repair, models that we felt were
much more rigorous and clinically relevant than the excisional and partial thickness burn wound models in which these growth factors had been previously assessed. In this study, we first carefully developed our porcine burn models to obtain standardized burns of reproducible depth, escharectomized the burns 4 or 5 days postburn to replicate the standard treatment in human burn patients (detailed in Materials and Methods), and then applied these four recombinant growth factors (rEGF, rPDGF-BB, rKGF, and the rNDF-α2 isoform), both singly and in limited combinations, to assess their efficacy in both full and partial thickness burn repair.

Materials and Methods

Recombinant Growth Factors

rEGF, rPDGF-B chain, rKGF, and rNDF-α2 were each produced in Escherichia coli, refolded and/or dimerized (for rPDGF-BB) and purified to homogeneity by conventional chromatography techniques, and tested endotoxin-free. Each growth factor was determined to have bioactivity by specific in vitro assays: proliferation of normal rat kidney fibroblasts by rEGF with half maximal activity at 0.24 ng/ml,55 proliferation of normal rat kidney fibroblasts by PDGF-BB with half maximal activity at 0.5 ng/ml,33 proliferation of BALB/MK cells by rKGF with half maximal activity at 19 ng/ml,56 and ability to induce tyrosine phosphorylation of p185neu in MDA-MB453 human breast carcinoma cells by rNDF-α2 at concentrations as low as 1 ng/ml (22 pmol/L).48 Each recombinant growth factor was formulated into either 2.6% carboxymethylcellulose or hydroxyethylcellulose gel to allow for accurate placement of the growth factor into the burn bed with no leakage. Each growth factor gel was shown to retain full bioactivity in its specific bioassay (described above) before in vivo application.

Porcine Burn Models

All procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Amgen, Inc. (Thousand Oaks, CA). Young, adult, female Yucatan micropigs (40 to 60 kg; Charles River Laboratories, Wilmington, MA) were used for all experiments because they are considered the best animal model for assessment of wound repair because of their size at adulthood and the similarity between their skin and human skin.56-58 Pigs were suspended in a Panepinto sling (Charles River Laboratories) for the burn procedure, escharectomy, and all bandage change/growth factor application procedures, and anesthetized using isoflurane delivered via a 2:1 oxygen:nitrous oxide mixture. Pigs were clipped and surgically scrubbed, and then had burns created on both sides of their dorsal midline, under aseptic conditions, by placing heated 2.5 cm diameter custom-made brass bars (Venture Welding, Oak View, CA), on the dorsum of each animal for defined times and for defined pressures as measured by a pressure gauge (Shimpo America Corp., Lincolnwood, IL). Times, temperatures, and pressures necessary to attain reproducible full and deep partial thickness burns were determined by creating burns using various combinations of these three parameters on Yucatan micropigs, taking biopsies of the burns 2, 3, and 4 days postburn, and assessing the burn depth by determining the depth of collagen denaturation via Masson's trichrome stain as described by Chvapil et al59 and determining the depth of cell viability via immunohistochemical staining for proliferating cell nuclear antigen (PCNA; see below for detailed methods; Figure 1). Using the results of these range-finding experiments, full thickness burns were created by heating the bars to 160°C in a convection oven (Baxter Scientific, McGaw Park, IL) and placing them on the pig for 25 seconds at 2000 × g of pressure. For deep partial thickness burns, the bars were heated to 100°C in boiling water and placed on the pig for 20 seconds at 1500 × g of pressure. Ten burns were created per pig on either side of the dorsal midline, for a total of 20 burns per pig. Immediately after the burn procedure, the blisters were removed and the burns packed with sterile, saline-soaked gauze. The burns were then covered with a semiocclusive dressing (Opsite, Smith and Nephew, Hull, UK) to provide for a moist environment and wrapped with a cohesive flexible bandage (ProFlex; Pro Vet, Leves Park, IL), to prevent possible self-trauma to the burns. To make our model more similar to the clinical situation in burn patients, we waited 4 or 5 days for an eschar to form, and then removed the dressing and bandages and aseptically escharectomized the burns. Full thickness burns were surgically escharectomized to the depth of necrotic tissue. Deep partial thickness burns were escharectomized with an air dermatome (Zimmer Patient Care, Dover, OH) to a depth of 750 μm.

Treatment Groups

Immediately after escharectomy on day 4 or 5 postburn, recombinant growth factor or vehicle control was applied to the burn areas. In the majority of studies, treatment group sites were rotated from one pig to the next to account for potential differences in healing based on location (Figure 2). In some studies
evaluating the effects of rKGF in partial thickness burns, 20 mirror-image burns were created on both sides of the dorsal midline; 20 μg/cm² rKGF was applied to burns on one side, and vehicle control gel was applied to burns on the other side. In studies where treatment groups were rotated from pig to pig, each treatment was applied every third day to five burns on each of two pigs, for a total of 10 burns per treatment. On subsequent treatment days, the burns were photographed and re-treated. After re-treatment, the burns were covered again as above. At the time of harvest, the pigs were euthanized with 10 ml sodium pentobarbital (Eutha-6, Western Medical Supply Co. Inc., Arcadia, CA). Full thickness burns were harvested on day 18 after five recombinant growth factor treatments, and partial thickness burns were harvested on day 12 or 13 after three recombinant growth factor treatments. In the mirror-image partial thickness burn studies, burns were treated daily for 7 consecutive days and harvested on day 12. Burns were harvested using a custom-made biopsy instrument (Amgen, Inc.) consisting of a Plexiglas handle holding two razor blades in parallel 8 mm apart. Burns were consistently excised perpendicularly to the dorsal midline at their centers. The biopsies were then bisected lengthwise, with one half fixed in zinc formalin (Anatech, Battle Creek, MI) for routine histological evaluation and immunohistochemical staining, and the other half embedded in OCT media (Miles, Inc., Elkhart, IN) and snap frozen in isopentane chilled to its freezing point in liquid nitrogen for integrin immunohistochemical staining.

Assessment of Reepithelialization and Burn Healing

All epithelial measurements were done via a calibrated Quantimet 520 image analyzer (Leica, Deerfield, IL) coupled to a Nikon FXA microscope. Three
μm thick, hematoxylin and eosin (H&E)-stained sections of each burn were assessed for percent reepithelialization, amount of new epithelium covering the burn bed, and amount of granulation tissue and extracellular matrix filling the burn bed using several different measurements. The epithelial gap (EG) is a linear measurement defined as any part of the burn bed not covered by new epithelium, and was calculated by adding all linear non-epithelialized regions within the burn bed. The burn length (BL) is the diameter measured between the burn margins. The new epithelium (NE) value was derived by subtracting EG from BL, thus standardizing any variability that might have been present in the burn diameter. Epithelial measurements were further standardized by dividing the NE by the BL for each burn to calculate percent reepithelialization. The new epithelial area (EA) is the total amount of new epithelium covering the burn bed, is roughly equivalent to epithelial thickness, and was derived by measuring all of the epithelium within the burn margins by image analysis in mm². The granulation tissue/extracellular matrix area (GTA), a measurement of all tissue within the burn bed tissue exclusive of epithelium, was also measured in mm² via a calibrated Quantimet 520 image analyzer (Leica) coupled to a Nikon SMZ-U stereomicroscope. Rete ridges per burn cross-section were quantified on a Nikon Optiphot microscope using the 4x objective. All burn measurements were sorted by dose, and each dose analyzed versus the vehicle control using one way analysis of variance (one-tailed) coupled with the Bonferroni/Dunn post-hoc test at a 5% significance level (Statview 4.02, Abacus Concepts). In studies evaluating the effects of rKGF in partial thickness mirror-image burns, rKGF-treated burns group by treatment were analyzed versus the vehicle control using an unpaired t-test (one-tailed) at a 5% significance level (Statview 4.02, Abacus Concepts Berkeley, CA), and each individual rKGF-treated burn was compared with its mirror-image control by a Wilcoxon signed rank test (one-tailed) at a 5% significance level (Statview 4.02, Abacus Concepts). Hair follicles were counted on gross photographs, and a regression analysis between hair follicles (independent variable) and percent reepithelialization (dependent variable), as a function of treatment group, was performed on the Statview 4.02 program (Abacus Concepts).

Immunohistochemical Staining

Zinc formalin-fixed, paraffin-embedded 3 μm thick sections were used for immunohistochemical analysis of filaggrin, involucrin, and cytokertatins 10 and 14. Before immunohistochemical staining, all sections were deparaffinized and digested for 10 minutes (except sections stained for cytokertatin 10 expression) using 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) in phosphate buffered saline (PBS). Filaggrin was detected using an anti-human filaggrin monoclonal antibody (MAb; Biomedical Technologies, Inc., Stoughton, MA), involucrin using rabbit anti-human involucrin antiserum (Biomedical Technologies, Inc.), cytokertatin 10 using an anti-human cytokertatin 10 MAb (clone DE-K10, Dako, Carpinteria, CA), and cytokertatin 14 using an anti-human cytokertatin 14 (clone LL002, Biogenex, San Ramon, CA). PCNA was detected using a mouse anti-human PCNA MAb (clone 19A2, Coulter Immunology, Hialeah, FL), and sections were placed in sodium citrate buffer (BioTek Solutions, Santa Barbara, CA) and heated in a microwave oven rather than digested for antigen retrieval. For immunohistochemi-
Analysis of integrin expression, 5 \( \mu \)m thick frozen sections of burns were used. The \( \alpha_2 \) integrin was detected with a mouse anti-human \( \alpha_2 \) integrin MAb (clone P1E6, GIBCO BRL, Grand Island, NY), \( \alpha_3 \) integrin was detected with a rabbit anti-human \( \alpha_3 \) integrin antibody (Chemicon International, Inc., Temecula, CA), \( \alpha_5 \) integrin was detected with a rabbit anti-human \( \alpha_5 \) integrin antibody (Chemicon International, Inc.), \( \alpha_6 \) integrin was detected with a rat anti-human \( \alpha_6 \) integrin MAb (clone GoH3, AMAC, Inc., Westbrook, ME), and \( \beta_1 \) integrin was detected with a rabbit anti-human \( \beta_1 \) integrin antibody (Chemicon International, Inc.) Immunohistochemical staining was done on an automated TechMate Immuno-stainer (BioTek Solutions) using a biotinylated anti-rabbit/anti-mouse secondary cocktail (BioTek Solutions) at BioTek's recommended dilution. All staining reactions other than PCNA used an avidin-biotin complex (ABC) tertiary linked to horseradish peroxidase (BioTek Solutions) at BioTek's recommended dilution, and diaminobenzidine (DAB, Sigma Chemical) as the chromogen. For PCNA immunostaining, an ABC linked to alkaline phosphatase (BioTek Solutions) was used as the tertiary at BioTek’s recommended dilution, and BioTek Red (BioTek Solutions) was used as the chromogen.

Assessment of Proliferation Using PCNA

PCNA counts were done using a calibrated Quantimet 520 image analyzer (Leica) coupled to a Nikon Optiphot microscope. At 20×X, four horizontal frames were measured starting at the tip of the migrating neopidermis back toward the burn margin. If the neopidermis was shorter than four horizontal frames, measurements stopped at the burn margin. Additional vertical frames were read as needed, depending on the epidermal thickness. The measurements for each frame consisted of 1) total PCNA-positive keratinocytes (PK), 2) epithelial length (EL), and 3) new epithelial area (EA). PK/EA and PK/EL were calculated, and burns treated with rKGF at 20 \( \mu \)g/cm\(^2\) were analyzed versus the vehicle control using an unpaired \( t \) test (one-tailed) at a 5% significance level (Statview 4.02, Abacus Concepts).

In Situ Hybridization for KGFR

Porcine full thickness burns with adjacent viable skin were harvested from female Yucatan micropigs (see above for details of the burn models). After harvest, porcine skin was immediately immersion-fixed in 4% paraformaldehyde buffered with PBS overnight, followed by cryoprotection by immersion in 30% sucrose until the burns sank. Burns were embedded in OCT media (Miles, Inc.) and then snap frozen in isopentane chilled to its freezing point in liquid nitrogen. Frozen sections of burns were cut at 9 \( \mu \)m on a Jung CM3000 cryostat (Leica), and placed on clean positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA).

For KGFR in situ hybridization, bases 1358 to 1487 of human KGFR cDNA (GenBank accession number M80634) were cloned into the pSP72 transcription vector (Promega, Madison, WI), linearized, and an antisense riboprobe specific for the human KGFR splice variant of FGFR2 was synthesized using T7 RNA polymerase and labeled with \(^{35}\)P-labeled re-combinant uridine triphosphate (New England Nuclear, Boston, MA). This antisense probe was specific for KGFR, did not cross-hybridize with FGFR2, and shared 98% base homology with the analogous region of porcine KGFR (R Blitz and CF Morris, unpublished data). Sense strands of the transcription vector were also transcribed and used as negative controls. Sections were hybridized and washed according to the protocol described by Yan et al. After the slides dried, they were dipped in Kodak NTB-2 (Rochester, NY) emulsion, exposed for 21 days, and then developed and counterstained with H&E.

Results

Effects of Growth Factors in Full Thickness Burns

rKGF Stimulates Keratinocyte Proliferation but Does Not Significantly Increase Reepithelialization or New Epithelial Area

rKGF, when applied at a dose of 20 \( \mu \)g/cm\(^2\) to full thickness burns every 3 days until harvest 18 days postburn (five treatments), did not have any significant effects on new epithelial area, percent reepithelialization, or granulation tissue/extracellular matrix production, although rKGF-treated burns did show a trend toward increased new epithelial area (epidermal thickness) versus control burns (Figure 3). rKGF-treated burns did, however, show a significant increase in the number of keratinocytes expressing PCNA per mm\(^2\) of neopidermis versus control burns \((P = 0.023, \text{Table 1})\). Full thickness burns treated with rKGF at a dose of 100 \( \mu \)g/cm\(^2\) also exhibited no significant enhancement of any healing parameter, and actually had lower values for percent reepithelialization, new epithelial area, and PCNA-positive
neopidermal keratinocytes per mm² than burns treated with rKGF at 20 μg/cm² (data not shown).

**rPDGF-BB Induces Production of Granulation Tissue/Extracellular Matrix**

rPDGF-BB, when applied at a dose of 28 μg/cm² to full thickness burns every 3 days until harvest 18 days postburn (five treatments), induced a marked increase in the production of granulation tissue and extracellular matrix (P = 0.013, Table 2) such that granulation tissue and extracellular matrix filled the burn defect within several days of escharectomy (not illustrated). rPDGF-BB did not induce a significant increase in either new epithelial area or percent reepithelialization (Figure 3).

**The Combination of rKGF and rPDGF-BB Induces Production of Both Granulation Tissue/Extracellular Matrix and New Epithelium**

Because rKGF induced a trend toward increased new epithelial area and rPDGF-BB induced a marked increase in granulation tissue area, we investigated the possibility of synergy between these two growth factors in full thickness burns. The combination of rKGF and rPDGF-BB, applied every 3 days for a total of five treatments at harvest 18 days postburn, induced a highly significant increase in granulation tissue and extracellular matrix area (P = 0.0009, Table 2) as well as a significant increase in new epithelial area (P = 0.007, Figure 3A), but had no effect on percent reepithelialization (Figure 3B). The increase in granulation tissue/extracellular matrix area and new epithelial area induced by the combination of rKGF and rPDGF-BB manifested grossly and histologically as multifocal fibropapillomatous hyperplasia, primarily at the burn margins (Figure 4).

**Effects of Growth Factors in Deep Partial Thickness Burns**

rKGF Markedly Increases New Epithelial Area and Significantly Increases Reepithelialization

Deep partial thickness burns treated every third day with rKGF and harvested 12 days postburn (three

---

**Table 2. Effect of rPDGF-BB, rKGF, and the Combination of rPDGF + rKGF on Granulation Tissue/Extracellular Matrix Production within Full Thickness Burns**

<table>
<thead>
<tr>
<th></th>
<th>Granulation tissue/extracellular matrix area (mm²)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.2 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>rKGF</td>
<td>71.4 ± 4.8</td>
<td>NS*</td>
</tr>
<tr>
<td>rPDGF-BB</td>
<td>85.3 ± 2.4</td>
<td>0.013</td>
</tr>
<tr>
<td>rPDGF-BB + rKGF</td>
<td>91.1 ± 4.2</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

The production of new granulation tissue/extracellular matrix within full thickness burns 18 days postburn was measured via a calibrated Quantimet 520 image analyzer (Leica) coupled to a Nikon SMZ-U stereomicroscope. The areas were outlined by an observer blinded to the treatment groups and the subsequent measurements were sorted by dose. Each growth factor or combination was analyzed versus the vehicle control using one way analysis of variance (one-tailed) with the Bonferroni/Dunn post-hoc test at a 5% significance level (Statview 4.02). Results are reported as mean granulation tissue/extracellular tissue area ± SEM.

*Not significant.

---

**Table 1. Effect of rKGF on Keratinocyte Proliferation in Full and Partial Thickness Burns**

<table>
<thead>
<tr>
<th>Thickness</th>
<th>PCNA positive keratinocytes/mm²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>neoeidermis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>Control</td>
<td>772.3 ± 114.7</td>
</tr>
<tr>
<td></td>
<td>rKGF</td>
<td>1282.2 ± 198.1</td>
</tr>
<tr>
<td>Partial</td>
<td>Control</td>
<td>758.1 ± 93.5</td>
</tr>
<tr>
<td></td>
<td>rKGF</td>
<td>1404.8 ± 104.7</td>
</tr>
</tbody>
</table>

PCNA counts were done on full thickness burns 18 days postburn and on partial thickness burns 12 days postburn using a calibrated Quantimet 520 image analyzer (Leica) coupled to a Nikon Optiphot microscope. At 20x, four horizontal frames were measured starting at the tip of the migrating neoeidermis back toward the burn margin. If the neoeidermis was shorter than four horizontal frames, measurements stopped at the burn margin. Additional vertical frames were read as needed, depending on the epidermal thickness. PCNA-positive keratinocytes (PK) and new epithelial area (EA) were quantified, and PK/EA was calculated. PK/EA values for burns treated with rKGF at 20 μg/cm² were analyzed versus PK/EA values for the vehicle control using an unpaired t-test (one-tailed) at a 5% significance level (Statview 4.02, Abacus Concepts). Results are reported as mean number PCNA-positive keratinocytes per mm² neoeidermis ± SEM.

---

**Figure 3. New epithelial area and percent reepithelialization of porcine full thickness burns 18 days postburn sorted by recombinant growth factor treatment and analyzed by one way analysis of variance coupled to the Bonferroni/Dunn post-hoc test (n = 10 except pooled controls, n = 20). rKGF and rPDGF-BB were both applied at 20 μg/cm² while rPDGF-BB was applied at 28 μg/cm². These doses were determined to be optimal based upon data from previous dose-response experiments (data not shown). There is a significant increase in new epithelial area in burns treated with the combination of rPDGF-BB and rKGF.**
applications) exhibited a dose-dependent increase in new epithelial area, which was highly significant (P < 0.0001) and nearly twice that of control burns at doses of both 20 and 60 μg/cm² (Figure 5A). rKGF-treated burns did not exhibit a significant increase in percent reepithelialization at any dose tested, but there was a trend toward a modest increase in reepithelialization in burns treated with 20 μg rKGF/cm² (89.2% rKGF-treated versus 79.9% control, P = 0.09, Figure 5B). Although rKGF did not significantly increase reepithelialization, it did double the number of fully reepithelialized burns 13 days postburn at doses of both 20 and 60 μg/cm² (P = 0.02 by χ² analysis, Table 3).

This rKGF-induced increase in reepithelialization, particularly at a dose of 20 μg/cm², prompted us to then investigate the effects of daily treatment with this dose of rKGF. At 12 days postburn, after 7 consecutive days of rKGF treatment, rKGF-treated deep partial thickness burns again exhibited a highly significant increase in new epithelial area (epidermal thickness), which was greater than twice that of control burns (P < 0.0001), as well as a moderate but significant increase in percent reepithelialization (83.7% rKGF versus 70.2% control, P = 0.016; Figure 6, A and B). In addition, comparison of each burn with its mirror image revealed that 100% of rKGF-treated burns contained greater new epithelial area than their corresponding control burn (P < 0.0001 by Wilcoxon signed rank test, Figure 6C), whereas 75% of rKGF-treated burns exhibited greater reepithelialization than their corresponding control burn (P = 0.01 by Wilcoxon signed rank test, Figure 6D).

**rKGF Induces Psoriasiform Epidermal Hyperplasia but Normal to Accelerated Differentiation in Deep Partial Thickness Burns**

The increase in new epithelial area in rKGF-treated deep partial thickness burns was primarily due to

**Table 3. Effect of rKGF on full reepithelialization in partial thickness burns 13 days postburn**

<table>
<thead>
<tr>
<th>rKGF (μg/cm²)</th>
<th>Percent burns fully reepithelialized</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>35</td>
<td>NS*</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>0.02</td>
</tr>
<tr>
<td>60</td>
<td>70</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The percent of partial thickness burns fully reepithelialized at 13 days postburn was measured via a calibrated Quansimet 520 image analyzer (Leica) coupled to a Nikon Optiphot microscope. The percent burns fully reepithelialized at each of the four different doses of rKGF (n = 20 per dose) were analyzed for significant variance from the percent burns expected to be fully reepithelialized per dose using χ² analysis.

*Not significant.
marked psoriasiform epidermal hyperplasia characterized by prominent elongation and thickening of rete ridges, particularly near hair follicles (Figure 7A and B). Quantification of rete ridges revealed a dose-dependent increase in the number of rete ridges in rKGF-treated burns, with a highly significant increase in rete ridges in burns treated with 20 μg rKGF/cm² ($P = 0.0004$, Table 4). Although rKGF-treated burns exhibited marked psoriasiform hyperplasia, immunostaining for cytokeratin 10 (a marker of keratinocyte terminal differentiation$^{61}$) revealed either normal (7/10 rKGF-treated burns) or accelerated (3/10 rKGF-treated burns) neoproliferative and follicular differentiation compared with control burns (0/10 control burns exhibited neoproliferative cytokeratin 10 staining; Figure 7, C and D). Immunostaining for filaggrin and involucrin, two other markers of keratinocyte terminal differentiation, revealed that filaggrin had an epidermal and follicular expression pattern similar to that of cytokeratin 10 but with less extensive and less intense expression (not illustrated), whereas the anti-invulucrin antibody stained porcine epidermis very inconsistently, and was therefore of no value in assessing the degree of epidermal differentiation.

The marked epidermal hyperplasia exhibited by rKGF-treated deep partial thickness burns corresponded to a marked increase in the number of PCNA-positive keratinocytes per mm² of neoepliphemis in rKGF-treated burns ($P < 0.0001$, Table 1) with an increase in the number of PCNA-positive keratinocytes in both the neoepliphemis and hair follicles of rKGF-treated burns (Figure 7 D and E). This increased follicular proliferation in rKGF-treated burns corresponded with a dose-dependent trend toward increased numbers of hair follicles per burn in rKGF-treated burns (data not shown). This trend, in turn, corresponded with a moderate positive correlation between the number of hair follicles per burn and percent reepithelialization ($r^2 = 0.0521, P = 0.018$; data not shown).

In Situ Hybridization for KGFR in Porcine Burns

In situ hybridization using an antisense riboprobe specific for the KGFR splice variant of FGFR2 re-
revealed that KGFR mRNA was strongly expressed by keratinocytes in the basal epidermis and hair follicles, particularly within follicular bulbs (Figure 8, A and C), findings identical to those we had previously described in murine skin. KGFR was also expressed by eccrine sweat gland epithelium (Figure 8C). These results confirmed that rKGF acted directly on hair follicles as well as the epidermis within partial thickness burns.

**rKGF Has No Effect on Epidermal Integrin Expression**

Because rKGF stimulated a modest increase in reepithelialization of partial thickness burns, we analyzed rKGF-treated burns for integrin expression to see if any integrins were upregulated by rKGF treatment. Ten control and 10 rKGF-treated partial thickness wounds were stained for expression...
Table 4. Effect of rKGF on rete ridges in partial thickness burns

<table>
<thead>
<tr>
<th>rKGF (μg/cm²)</th>
<th>Rete ridges per burn</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70.4 ± 9.3</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>84.3 ± 8.4</td>
<td>NS*</td>
</tr>
<tr>
<td>20</td>
<td>114.8 ± 6.8</td>
<td>0.0004</td>
</tr>
<tr>
<td>60</td>
<td>95.4 ± 6.7</td>
<td>0.036*</td>
</tr>
</tbody>
</table>

Quantification of rete ridges in partial thickness burns harvested 13 days postburn was done using a calibrated Quantimet 520 image analyzer (Leica) coupled to a Nikon Optiphot microscope. The number of rete ridges per burn cross section was sorted by dose (n = 20 per dose) and analyzed by one way analysis of variance (one-tailed) coupled with the Bonferroni/Dunn post-hoc test at a 5% significance level (Statview 4.02). Results are reported as mean rete ridges per burn cross section ± SEM. rKGF induced a highly significant increase in the number of rete ridges per burn at a dose of 20 μg/cm² and a nearly significant increase in rete ridges per burn at a dose of 60 μg/cm².

*Not significant.

Discussion

In this study, we have developed porcine full and deep partial thickness burn models in which burns are escharoectomized and covered with an occlusive dressing, and therefore represent realistic and clinically relevant models of human burn wounds. Using these porcine burn models, we have demonstrated that the application of recombinant growth factors to full and deep partial thickness burns can result in significant increases in production of granulation tissue and extracellular matrix as well as generation of new epithelium as measured by new epithelial area and percent reepithelialization. In full thickness burns, rPDGF-BB and the combination of rPDGF-BB and rKGF induced significant increases in granulation tissue/extracellular matrix production as well as generation of new epithelium (combination of rPDGF-BB and rKGF only), but had no effect on reepithelialization. In deep partial thickness burns, only rKGF induced significant changes in burn repair, namely a highly significant increase in new epithelial area, a modest increase in reepithelialization, and a doubling of the number of fully reepithelialized burns at 13 days postburn. The effects of rKGF on burn repair were at least partially due to marked stimulation of both epidermal and follicular proliferation that resulted in marked neoeipidermal psoriasiform hyperplasia with exaggerated rete ridges but normal to accelerated neoeipidermal and follicular maturation. rEGF and the rNDF-α2 isoform had no significant biological effects when applied to either full thickness (rNDF-α2 only, EGF not tested) or deep partial thickness burns (both growth factors tested).

Although rPDGF-BB and rKGF appeared to have synergistic effects in full thickness burns, neither the increase in production of granulation tissue/extracellular matrix nor new epithelial area induced by this combination of growth factors corresponded to any increase in rate of reepithelialization, suggesting that this combination induced a biologically significant effect that was of limited clinical relevance. The induction of fibropapillomatous hyperplasia by the combination of rPDGF-BB and rKGF further suggests that the combination of these growth factors...
induces marked epidermal and mesenchymal proliferation rather than epidermal migration over a maturing mesenchymal matrix. Although the stimulation of repair induced by the combination of rPDGF-BB and rKGF was of limited clinical relevance, these results indicate that it is possible to further stimulate...
wound repair by using optimal concentrations of exogenous growth factors, and encourage further investigation in this area.

rKGF enhanced both production of new epithelium as well as burn reepithelialization to a much greater extent in deep partial thickness burns than in full thickness burns, largely because of the stimulatory effect of rKGF on the hair follicles, which were present in deep partial thickness burns but absent in full thickness burns. This rKGF-induced enhancement of reepithelialization due to follicular hyperplasia was anticipated based upon our previous results, which demonstrated marked stimulation of hair follicles by rKGF in several animal model systems,\(^8\) and is largely in agreement with our previous results in modified partial excisional wounds in rabbit ears.\(^6\)

In addition to demonstrating that rKGF stimulates marked epidermal and follicular proliferation, we have also demonstrated that rKGF does not inhibit, and sometimes stimulates (3/10 rKGF-treated burns examined), epidermal and follicular maturation. These results are in agreement with those of Marchese et al.,\(^44\) who demonstrated enhanced differentiation in keratinocytes treated with rKGF in vitro; the results of Staiano-Coico et al.,\(^7\) who demonstrated normal epidermal maturation in porcine excisional wounds treated with rKGF; and our own results in nude mice, which demonstrated increased follicular keratin differentiation induced by rKGF.\(^64\)

Despite stimulating a modest increase in reepithelialization in deep partial thickness burns, rKGF had no apparent effect on the expression of epidermal integrins in our models. This result suggests that the increase in reepithelialization induced by rKGF in burns is predominantly secondary to the marked rKGF-induced increase in new epithelium, and not due to a significant increase in neoeipidermal migration. In contrast to rKGF, rNDF-\(\alpha_2\) appears to increase reepithelialization in partial thickness excisional wounds largely by stimulating epidermal migration as evidenced by increased neoeipidermal expression of both \(\alpha_5\) and \(\alpha_6\) integrins.\(^8\) Because rNDF-\(\alpha_2\) stimulates epidermal migration, its lack of effect in our models was somewhat unexpected, but may have been due to the absence of a proper extracellular matrix in burn beds versus the partial thickness excisional wounds where we observed the stimulation of neoeipidermal migration by rNDF-\(\alpha_2\). This hypothesis is supported in part by some preliminary observations in the cartilage intact rabbit ear excisional wound model,\(^3\) where rNDF-\(\alpha_2\) failed to stimulate an increase in neoeipidermal migration over the bare cartilage that forms the wound bed in this model (TA Mustoe and DM Danilenko, unpublished data).

The lack of a significant rEGF effect in deep partial thickness burns was also somewhat unanticipated given the extensive literature describing acceleration of partial thickness wound repair by rEGF.\(^1-3,13\) Because burns are more severe wounds and heal more slowly than excisional wounds,\(^65\) the lack of significant enhancement of burn repair by rEGF in our model was most likely due to the increased severity of our burn model compared with typical excisional wound models, a hypothesis that is supported by an earlier study, which demonstrated lack of significant enhancement of reepithelialization by rEGF in a rat deep partial thickness burn model.\(^10\) Another earlier study in a porcine partial thickness burn model did demonstrate modest enhancement of reepithelialization by rEGF, but used a burn model in which burns were neither covered by an occlusive dressing nor escharerectomized before rEGF application.\(^1\) A comparison of the results obtained in this previous study with those of our own suggests that allowing burns to be exposed to air and leaving the eschar in place slows the healing rate such that the addition of an exogenous recombinant growth factor can significantly accelerate wound repair, and treating burns in a manner similar to that in which burn surgeons clinically treat human burns (our model) optimizes the healing rate such that exogenous recombinant growth factors such as rEGF may not be able to significantly accelerate wound repair.

Although rEGF alone failed to significantly enhance reepithelialization in deep partial thickness burns, it did moderately increase new epithelial area, a result that suggested that the combination of rEGF and rKGF might improve upon the increase in new epithelial area and reepithelialization induced by rKGF alone. When rEGF and rKGF were combined, however, there was a slight decrease in both reepithelialization and production of new epithelial area compared with either growth factor alone. These results suggest that rEGF and rKGF at least partially antagonize each other in deep partial thickness burns, possibly via rEGF-induced inhibition of hair follicle proliferation,\(^45,46\) antagonizing some or all of the follicular proliferation induced by rKGF.

The enhancement of healing we have demonstrated in our porcine burn models, although statistically significant, was either modest (reepithelialization) or of limited clinical relevance (production of granulation tissue/extracellular matrix and new epithelial area). These findings are not altogether unexpected, given that most previously described models of excisional wound repair, such as porcine
excisional wound models\(^1\,^6\,^7\) and our rabbit ear dermal model\(^2\,^8\,^9\) are good models for the in vivo screening of recombinant growth factor biological activity, but are acute wound model systems and generally heal relatively rapidly on their own, making these models limited in terms of clinical relevance. Because burns are known to heal more slowly than excisional wounds due to extensive necrosis, increased inflammation with altered levels of inflammatory mediators, and high levels of endogenous proteases\(^65\,^66\) as compared with excisional wounds, our porcine burn repair models are more severe and rigorous than most previously described excisional wound healing models where recombinant growth factors have accelerated wound repair, and therefore provide much more clinically relevant data.

Similarly, previously described porcine burn models in which recombinant growth factors (rEGF and rTGF-α) have accelerated wound repair\(^1\,^4\) did not provide information that was highly clinically representative, because burns were neither covered with an occlusive dressing nor escharectomized before growth factor treatment. Therefore, in these previous studies, the porcine models used were dissimilar to standard human clinical burn treatment, and burns were relatively slow to heal because of the presence of eschar and exposure to air. Our burn models, in contrast, more closely mimicked standard human clinical burn care, and therefore allowed more rapid burn repair than these previously described models. Under the relatively rigorous but more clinically relevant parameters of our burn models, our results suggest that although topical application of rKGF and rPDGF-BB to escharectomized and occluded burns results in highly significant increases in new epithelial area (roughly equivalent to epidermal thickness) and extracellular matrix/granulation tissue production, respectively, that these growth factors only marginally accelerate reepithelialization, the parameter most clinically relevant to burn repair.

Our results, combined with the lack of significant clinical enhancement of reepithelialization by rEGF in burn patient donor graft sites,\(^13\) as well as the lack of significant clinical acceleration of chronic ulcer repair by a number of other recombinant growth factors,\(^68\) suggest that additional strategies using recombinant growth factors should be pursued to achieve clinically significant acceleration of wound repair. These strategies might include growth factors in conjunction with extracellular matrix components, growth factors in conjunction with protease inhibitors, growth factors added to exogenously cultured keratinocytes, and/or additional combinations of recombinant growth factors.

**References**


**Acknowledgments**

We thank Dr Lillian Nanney and Dr Eloi Eriksson for their assistance in the initial development of our porcine burn models; and Dr Regina M Housley, Cindy Gregory, Debbie Iverson, John Lu, Karen Rex, and Ruth Stage for their expert assistance with pig handling and anesthesia. We also thank Dr Marjory Nicolson for rEGF; Dr C Fred Morris and Rebecca Blitz for human KGFR cDNA; Sally Kutsunai for assistance with in situ hybridization for KGFR; Dr Joseph Caraher for the in vitro assay of rNDF-α on cultured porcine Schwann cells; Diane Duryea and Carol Burgh for excellent assistance in histological preparations; Jennifer Keyser, Danette Barron, and Kathryn Rubenstein for expert technical assistance with figures; and Dr Arlen Thomason for his helpful suggestions and critical review of the manuscript.

Pierce TF, Kawahara R, Mustoe TA, Pierce GF: Growth factors and wound healing: platelet-derived growth factor.


64. Danilenko DM, Ring BD, Yanagihara D, Benson W, Wi-