Biomimetic Delivery of Keratinocyte Growth Factor upon Cellular Demand for Accelerated Wound Healing *in Vitro* and *in Vivo*

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Exogenous keratinocyte growth factor (KGF) significantly enhances wound healing, but its use is hampered by a short biological half-life and lack of tissue selectivity. We used a biomimetic approach to achieve cell-controlled delivery of KGF by covalently attaching a fluorescent matrix-binding peptide that contained two domains: one recognized by factor XIII and the other by plasmin. Modified KGF was incorporated into the fibrin matrix at high concentration in a factor XIII-dependent manner. Cell-mediated activation of plasminogen to plasmin degraded the fibrin matrix and cleaved the peptides, releasing active KGF to the local microenvironment and enhancing epithelial cell proliferation and migration. To demonstrate *in vivo* effectiveness, we used a hybrid model of wound healing that involved transplanting human bioengineered skin onto athymic mice. At 6 weeks after grafting, the transplanted tissues underwent full thickness wounding and treatment with fibrin gels containing bound KGF. In contrast to topical KGF, fibrin-bound KGF persisted in the wounds for several days and was released gradually, resulting in significantly enhanced wound closure. A fibrinolytic inhibitor prevented this healing, indicating the requirement for cell-mediated fibrin degradation to release KGF. In conclusion, this biomimetic approach of localized, cell-controlled delivery of growth factors may accelerate healing of large full-thickness wounds and chronic wounds that are notoriously difficult to heal. (Am J Pathol 2005, 167:1575–1586)

Re-establishing an epithelial barrier in injured skin is a crucial wound-healing event that protects the body against

further water loss and exposure to external pathogens. Skin epithelial cells or keratinocytes begin migrating to repair the epithelium ~18 to 24 hours after injury¹ carefully degrading a fibrin-rich clot through the activation of plasminogen into plasmin.² Transient interactions with extracellular matrix proteins in the wound such as collagen and fibronectin during fibrinolysis of the clot guide the keratinocytes into the wound space. Degranulating platelets are embedded in the fibrin matrix and release growth factors and cytokines, which activate keratinocytes to proliferate and migrate until they heal the defect.

Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (FGF-7), plays a prominent role in epithelial morphogenesis and wound healing.^{3,4} Although initial studies restricted expression of KGF in cells of mesenchymal origin,^{5,6} a recent study documented expression of KGF by dendritic epidermal T cells of the skin immediately after wounding,⁷ suggesting that KGF may play an important role in epidermal immunity. The paracrine action of KGF on epithelial cells is mediated through the KGF receptor (KGFR or FGFR2IIIb), a splice variant of FGF-2 receptor encoded by the gene *fgfr-2*.^{8,9}

KGF is thought to be an important player in development and morphogenesis of epithelial tissues and a promoter of mesenchymal-epithelial interactions.^{10,11} Targeting KGF expression to the basal keratinocytes of developing mouse epidermis caused epidermal hyperthickening and altered the pattern of epidermal differentiation.¹² Similarly, development of tissue-engineered epidermis with genetically modified, KGF-expressing keratinocytes exhibited dramatic changes in three-dimensional organization including hyperthickening and flattening of the corrugations of the dermo-epidermal junction

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(rete-ridges). In addition to increased proliferation of basal cells, KGF induced proliferation in the normally quiescent suprabasal cell compartment and delayed differentiation.¹³

KGF is also important in protecting epithelial tissues from injury and apoptosis and promoting wound healing.¹⁴ For example, KGF was strongly up-regulated in the intestines of patients suffering from bowel inflammatory disease^{15,16} and protected the gut epithelium from colitis-induced inflammation.¹⁷ Similarly, several studies showed that KGF protected the lung epithelium from hyperoxic injury^{18–21} by promoting expression of surfactant proteins,²² secretion of surfactants,²³ and DNA repair.²⁴ More recently, KGF was found to protect intestinal, oral, and mucosal epithelia from chemotherapy- and radiation-induced injury and mortality.^{25–27} As a result, phase 2/3 clinical trials are in progress to examine the effects of KGF on mucositis (painful sores in the mouth), a common condition that affects cancer patients undergoing chemotherapy or radiation treatment (see <http://www.amgentrials.com/>).¹⁴

In skin, KGF is strongly up-regulated after injury, suggesting that KGF may be crucial in early stages of the healing process.²⁸ KGF knockout mice healed at normal rates and proliferation of keratinocytes at the wound edge was not altered,²⁹ possibly due to the compensatory action of other members of the FGF family, eg, FGF-10³⁰ or FGF-22.³¹ Despite these inherent biological redundancies, exogenous KGF significantly enhanced re-epithelialization in full and partial thickness wounds in porcine and rabbit ear wound models.^{32,33} In addition to epithelialization, KGF increased new granulation tissue formation in an ischemic rabbit ear wound model, possibly through the induction of indirect effects.³⁴ Interestingly, expression of KGF was suppressed in diabetic wounds,^{35,36} suggesting that exogenous KGF may promote faster closure of chronic wounds that are notoriously difficult to heal. Indeed, a recent study showed that a single injection of KGF DNA accelerated wound closure and reduced inflammation in a diabetic mouse model.³⁷

In general it has been difficult to maintain full bioactivity of the proteins applied in the wound space due to protein instability in the protease-rich environment of the wound.^{38,39} In addition, bolus administration does not keep the protein localized and necessitates large amounts of growth factors that may have dangerous side effects, such as vascularization of nontarget tissues or growth of tumors.⁴⁰ The short biological half-life, lack of tissue-selectivity, and potential risk for carcinogenesis demand temporal and spatial control of growth factor delivery.^{41,42} To this end, biomaterials can be used to achieve controlled and localized release and at the same time serve as scaffolds to promote tissue regeneration.

Many studies have used biomaterials to deliver growth factors or genes for regeneration of tissues including bone,⁴³ nerve,⁴⁴ skin,⁴⁵ and vasculature.^{46,47} In particular, it was recently demonstrated that bi-domain peptides could be used to covalently attach small cell adhesion peptides^{48,49} or heparin-binding growth factors, such as nerve growth factor or brain-derived neurotrophic factor, into fibrin gels.⁴⁴ For the latter, one domain of the peptide

was recognized by factor XIIIa and incorporated into fibrin during polymerization, while the heparin-binding domain immobilized heparin, which in turn interacted with the heparin-binding growth factor. This approach was later extended to engineer fusion proteins containing the factor XIIIa recognition domain at the N-terminus and was used successfully to deliver β -nerve growth factor and vascular endothelial growth factor, in a manner that was controlled by cellular activity.^{46,50}

We adopted this methodology to conjugate KGF into a fibrin matrix to achieve localized delivery that is in tune with cellular demand at the wound microenvironment. Instead of engineering a fusion protein, we covalently attached a peptide containing the α 2-plasmin inhibitor fibrin-binding site to the free amines on the surface of the KGF molecule. The peptide-KGF complex (P-KGF) was conjugated to fibrin during polymerization and the binding efficiency depended strongly on the concentration of factor XIII. Plasmin digestion of the fibrin gels yielded KGF that was devoid of large fibrinogen fragments, detectable by gel electrophoresis. The residual grafted linker and small peptide fragment were not sufficient to alter KGF activity, as determined by promotion of epithelial cell proliferation. Using a scratch wound assay that we developed previously,⁵¹ we found that release of KGF via cell-mediated degradation of fibrin increased the rate of wound closure significantly and that KGF-induced healing was delayed by inhibition of fibrinolysis. To evaluate this delivery system *in vivo* we used a model system that we developed in our laboratory.^{52,53} This system was based on transplantation of human skin equivalents onto full-thickness wound defects on the back of nude mice. In agreement with previous studies,⁵⁴ the transplanted tissues integrated well with the mouse skin and were infiltrated by dermal elements including fibroblasts and blood vessels to create a hybrid tissue comprising human epidermis and mouse dermis. At 6 weeks after transplantation, the humanized skin was subjected to a full thickness wound that was immediately treated with fibrin-bound KGF (Fb-P-KGF). Fluorescence imaging showed that Fb-P-KGF remained in the wound space for at least 7 days and enhanced re-epithelialization significantly. In agreement with our *in vitro* data, aprotinin delayed healing possibly by preventing fibrin degradation by the migrating cells. Taken together, our data shows that active KGF can be released from fibrin hydrogels in tune with cellular demand, providing localized treatment and enhancing tissue regeneration.

Materials and Methods

Conjugation of Peptide to KGF

Recombinant human KGF (6.25 mg/ml; Amgen, Thousand Oaks, CA) in 1× phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) was reacted with succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC) (5 mg/ml; Molecular Probes, Eugene, OR) in dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA) at a 1:10 (SMCC:KGF) molar ratio for 1 to 1.5 hours (Figure

A

B

Figure 1. Functional analysis of KGF for binding to fibrin gels and Western analysis of products. **A:** KGF was functionalized with 1.7-kD peptides by a two-step reaction. In the first reaction (1), KGF was derivatized with a heterobifunctional linker molecule (SMCC) at a 10:1 (SMCC:KGF) molecular ratio, yielding a reactive maleimido-KGF. After purification, a second reaction (2) involved the peptide conjugation to maleimide-derivatized KGF to form a stable thioether bond yielding P_N -KGF (N = molecular ratio of peptides to KGF molecules reacted). **B:** Western analysis was used to examine the KGF protein (18.9 kD) after modification and release from fibrin gels through the action of plasmin. **Lane 1,** stock KGF was used as control; **lane 2,** P_2 -KGF; **lane 3,** P_5 -KGF; **lane 4,** PL- P_2 -KGF; **lane 5,** PL- P_5 -KGF, after conjugation to fibrin, the gels were washed extensively and degraded with 0.25 U/ml of plasmin at 37°C for 3 hours; **lane 6,** soluble P_5 -KGF (not conjugated to fibrin) was treated with 0.25 U/ml plasmin at 37°C for 3 hours.

1A). Maleimido KGF was dialyzed against 500 ml of Tris-buffered saline (TBS; Invitrogen) at 4°C, using a 3500 molecular weight cutoff dialysis cassette (Slide-A-Lyzer; Pierce, Rockford, IL). TBS was changed every hour for a total of 3 hours. SMCC precipitate that formed during dialysis was removed by spinning the sample in centrifuge at $16,000 \times g$ for 5 minutes. A peptide having the sequence, (Flc)-LNQEQVSPRKKC [(Flc) = fluorescein], was synthesized (Sigma Genosys, The Woodlands, TX) and contained the $\alpha 2$ -plasmin inhibitor motif, NQEQVSP for binding to fibrin gels as described before.⁴⁸ The peptide (2.5 mg/ml in PBS) was then reacted with the derivatized KGF at the indicated molar ratio for 1 hour at room temperature then overnight at 4°C. Using a 10,000 molecular weight cutoff dialysis cassette (Pierce), KGF with bound peptide (P-KGF) was purified from unbound peptide by dialysis against 500 ml of TBS at 4°C. TBS was changed every hour for a total of 3 hours. Final concentration of P-KGF was determined enzymatically using an enzyme-linked immunosorbent assay (ELISA) as discussed below.

Western Blot

Different KGF unknowns were diluted with sample buffer [0.0625 mol/L Tris-HCl, 25% (v/v) glycerol, 2%

(w/v) sodium dodecyl sulfate, 0.01% bromophenol blue, and 5% (v/v) β -mercaptoethanol] and heated at 95°C for 5 minutes. The KGF samples were then placed on ice for 10 minutes before they were loaded onto a 14% denaturing gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and run for 45 minutes. The proteins were then transferred onto a polyvinylidene difluoride membrane (Immun-Blot PVDF; Bio-Rad Laboratories, Hercules, CA) for 1 hour using an electrophoretic transfer cell (Mini Trans-Blot, Bio-Rad Laboratories, Hercules, CA). The membrane was incubated in blocking agent [5% (w/v) nonfat milk in wash buffer (TBS/0.1% Tween 20)] overnight at 4°C. The next day, the membrane was incubated with goat polyclonal anti-KGF (R&D Systems, Minneapolis, MN) at a concentration of 0.1 μ g/ml in blocking agent. The membrane was washed five times with wash buffer and incubated with horseradish peroxidase-conjugated mouse anti-goat IgG polyclonal secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 0.2 μ g/ml in blocking agent for 1 hour at room temperature. The membrane was washed five times with wash buffer, and the bands were detected using chemiluminescence (LumiGLO; KPL, Gaithersburg, MD) according to the manufacturer's instructions.

Preparation of Fibrin Gels and Plasmin-Mediated Digestion for Recovery of Bound P-KGF

Fibrin gels were prepared by mixing two solutions: one containing fibrinogen (6.25 mg/ml; Sigma, St. Louis, MO) or highly purified fibrinogen (6.25 mg/ml; Enzyme Research Laboratories, South Bend, IN), P-KGF (100 to 500 μ g/ml) and factor XIII (1.25 to 12.5 PEU/ml, Enzyme Research Laboratories) and the other containing thrombin (12.5 U/ml, Sigma) and calcium chloride (12.5 mmol/L, Sigma) in $1 \times$ TBS. The two solutions were mixed in a 4:1 volumetric ratio to make a fibrin gel containing 5 mg/ml fibrinogen, 2.5 U/ml thrombin, 2.5 mmol/L calcium chloride, 1 to 10 PEU/ml factor XIII, and 100 to 500 μ g/ml of P-KGF. For some experiments, the plasmin inhibitor aprotinin (0 to 1000 kIU/ml, Sigma) was added to the thrombin-containing fraction at the indicated concentration.

To assess binding efficiency, gels were allowed to fully polymerize at 37°C for 30 minutes then submerged in wash buffer (TBS) at 4°C. The wash buffer was collected at indicated times after polymerization for measuring the release of unbound growth factor as a function of time and then replaced with fresh buffer. After 15 hours the gels were degraded by incubation with plasmin (0.25 U/ml in TBS; Calbiochem, San Diego, CA) for 3 hours at 37°C.

ELISA and Fluorescence Measurements of Degraded Gels

An ELISA was used to quantify the amount of KGF recovered from the fibrin gels. The wells of 96-well ELISA plates

were coated (100 μ l/well) with 1.0 μ g/ml of monoclonal mouse anti-human KGF antibody (R&D Systems) in PBS overnight at 4°C. The next day, the plates were washed three times with PBS/0.5% Tween-20 (Sigma) and nonspecific binding sites were blocked by incubation with PBS/10% horse serum (Invitrogen) for 1 hour at room temperature. A standard curve was generated with stock KGF (0.5 to 16 ng/ml) as recommended by the manufacturer (R&D Systems). Unknown KGF samples were serially diluted in PBS/1% bovine serum albumin and incubated (100 μ l/well) for 1 hour at room temperature. The plates were then washed three times with PBS/0.5% Tween-20 and polyclonal goat anti-human KGF (R&D Systems) was added (100 μ l/well) at a concentration of 1.0 μ g/ml in PBS/10% horse serum for 1 hour at room temperature. After three washes with PBS/0.5% Tween-20, horseradish peroxidase-conjugated mouse anti-goat IgG secondary antibody (Jackson ImmunoResearch Laboratories) was incubated (100 μ l/well) at a concentration of 1.0 μ g/ml for 1 hour at room temperature. Plates were washed three times with PBS/0.5% Tween-20 and substrate was added (100 μ l/well) (Sigma Fast o-phenylenediaminedihydrochloride tablet sets; Sigma). The reaction was allowed to proceed for 3 to 5 minutes before the addition of 50 μ l per well of 4 mol/L H₂SO₄. The OD₄₉₀ was measured with an absorbance microplate reader (SpectraMax 340; Molecular Devices, Menlo Park, CA).

To assess total recovered fluorescence, 100 μ l of each sample was removed and placed in a black 96-well plate. The fluorescence intensity (excitation, 490 nm; emission, 520 nm) was measured in a fluorescence microplate reader (SpectraMax Gemini, Molecular Devices). A standard curve was generated by serially diluting stock P-KGF. The intensity of degraded fibrin without P-KGF was subtracted as background.

Proliferation Assay

A growth assay was used to assess functionality of KGF after chemical modifications. Rhesus monkey bronchial lung epithelial cells (4MBr-5, CCL208; American Type Culture Collection, Manassas, VA) were chosen because they have been shown to be very sensitive to KGF and epidermal growth factor.⁵⁵ The cells were cultured in F-12K media (American Type Culture Collection) with 10% fetal bovine serum (FBS) (Invitrogen), 30 ng/ml epidermal growth factor (BD Biosciences, Bedford, MA), and 100 μ g/ml penicillin-streptomycin (Invitrogen). Media was changed every 3 to 4 days and cells were passed when they reached 70% confluence.

For the growth assay, nontissue culture-treated 96-well plates were coated (100 μ l/well) with 100 μ g/ml of collagen for 2 hours at room temperature. Before trypsinization of the cells, different KGF unknowns were serially diluted in assay media (50 μ l/well) that consisted of Ham's F-12 with 2.5% FBS and penicillin-streptomycin and placed in an incubator. Assay medium (2.5% FBS) and regular media (10% FBS) served as negative and positive controls, respectively. Cells were then trypsinized and resuspended in assay medium at a con-

centration of 2×10^5 cells/ml. Cells were plated (50 μ l/well) onto the KGF unknowns and placed back in the incubator. The number of cells at each indicated time point was quantified using a FluoReporter Blue fluorometric double-stranded DNA quantitation kit (Molecular Probes) as described previously.⁵¹

Wounding of 4MBr-5 Cell Monolayers

4MBr-5 cells were seeded in collagen-coated (20 μ g/ml overnight) 24-well plates (100,000 cells/well) and grown to confluence in media with 10% FBS and 30 ng/ml of epidermal growth factor. Two days later, the monolayers were scratch wounded with a plastic pipette tip creating a wound ~1.4 mm in width. Immediately after wounding, monolayers were washed with TBS to remove cell remnants, and either fibrin gels (300 μ l) or media (600 μ l) were applied, completely covering the wound and monolayer. Fibrin gels containing residual amounts of plasminogen and 150 ng of P-KGF gelled in 5 to 10 seconds. Immediately after gel formation, fresh assay media with 2.5% serum (300 μ l) was added to the top of each polymerized gel.

Skin Equivalents and Grafting to Athymic Mice

Human keratinocytes were isolated from neonatal foreskins and were propagated on feeder layers of 3T3-J2 mouse fibroblasts (American Type Culture Collection) as described previously.⁵² Keratinocytes used for seeding of skin equivalents were maintained within one to four passages. Production and grafting of tissue-engineered skin equivalents were performed as described previously.⁵¹

Wounding of Grafted Skin Equivalents

At 3 weeks after grafting dressings were removed and at 6 weeks the grafts were examined and wounded in the center with a 4-mm biopsy punch. The full thickness wound (including the panniculus carnosus) was then gently washed with saline. Gels were mixed as outline above and applied to the wound where they were allowed to polymerize for ~5 minutes. Then, a large piece of Tegaderm was placed over the wound and bandaged with tape. At 8 days, animals were sacrificed by an intraperitoneal injection of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI) as outlined in the approved Institutional Animal Care and Use Committee protocol. Harvested grafts were fixed in 10% buffered formalin (Fisher Scientific) or 4% paraformaldehyde solutions for paraffin or OCT embedding, respectively.

Live in Vivo Imaging of KGF-Loaded Gels

For visualization of the wounds using fluorescence microscopy, a molded piece of cured poly(dimethyl siloxane) (Silicone Elastomer kit; Dow Corning, Midland, MI) with a 1-cm² cut-out was placed over each wounded graft and glued into place with a tissue adhesive. The wound chambers were

then covered with glass coverslips to allow fluorescent imaging of the gels during live healing of the wounded skin equivalents. Using a Nikon FXA inverted fluorescent microscope, we imaged the wounds using a charge-coupled device camera in a small air-tight chamber that was connected to an anesthesia machine. Exposure times and settings were identical for each sample and image. All images were collected using a $\times 4$ objective.

Analysis of Re-Epithelialization in Vivo

Tissue morphology was assessed with standard hematoxylin and eosin (H&E) and Masson's trichrome staining of paraffin-embedded tissue sections. A montage of images of the wounds at various times after wounding were acquired at $\times 20$ magnification on an inverted microscope (Diaphot-TMD; Nikon Corp., Melville, NY) using a Retiga 1300 digital camera and QCapture2 software, version 1.1 (Quantitative Imaging Corporation, Burnaby, Canada). Digital images were analyzed using public domain ImageJ 1.28k software (National Institutes of Health, Bethesda, MD). Re-epithelialization of the wounds was assessed by the ratio of the migration distance to the length of the initial denuded area as determined from the cut marks in the stratum corneum as previously described.⁵¹

Statistical Analysis

Statistical analysis of the data were performed using a two-tailed Student's *t*-test ($\alpha = 0.05$) using Microsoft Excel (Microsoft, Redwood, CA).

Results

Conjugation of a Fibrin-Binding Peptide to KGF

We used fibrin gels to deliver KGF on cellular demand to promote wound healing *in vitro* and *in vivo*. To do this we conjugated KGF to the peptide (Flc)-LNQEQVSPRKCC, which contains the substrate for factor XIII (NQEQVSP) and can therefore be incorporated into fibrin gels during polymerization.^{48,49,56} The first leucine was conjugated to fluorescein to allow quantitation of the bound KGF via fluorescence measurements. The peptide was conjugated to KGF through a cysteine residue that was strategically placed at the end of a highly charged spacer region (RKK). In the first step, KGF was reacted with a heterobifunctional reagent, SMCC, yielding a maleimido-KGF derivative (Figure 1A). Then, the maleimido-KGF was reacted with the peptide creating a stable thioester bond between the cysteine residue of the peptide and the SMCC derivative on the KGF molecule. The entire modification procedure resulted in very little loss of functional growth factor as measured by ELISA using a KGF antibody.

Immunoblotting of the reaction products clearly demonstrated that the number of conjugated peptides per KGF molecule depended strongly on the initial molar ratio of peptide to KGF. Specifically, at molar ratio of 5:1 the reaction product contained a distribution of conjugated molecules with up to four peptides per KGF (Figure 1B,

lane 3), whereas at a 2:1 ratio the growth factor was only partially conjugated with a maximum of two peptides per KGF (Figure 1B, lane 2).

P-KGF was mixed with highly purified fibrinogen (devoid of plasminogen, von Willebrand factor, and fibronectin) and polymerized with thrombin in the presence of 10 PEU/ml of factor XIII. The gels were washed extensively and then degraded with plasmin to release KGF (denoted as PL-P-KGF) by mimicking the process of fibrin clot degradation during wound healing. Interestingly, Western blot analysis revealed that KGF derived from degraded gels contained no peptide (Figure 1B, lanes 4 and 5), suggesting that plasmin may cleave at the peptide-KGF junction. Indeed, treatment of soluble P-KGF (not conjugated to fibrin gel) with plasmin yielded a product with similar molecular weight as free KGF (Figure 1B, lane 6).

Binding Efficiency of P-KGF in Fibrin Gels

To measure the amount of P-KGF that was bound to fibrin, the gels were washed extensively to release unbound P-KGF and then degraded with plasmin. The binding efficiency was measured as the ratio of initial over recovered fluorescence intensity (Figure 2A). Using these measurements we found that the binding efficiency was dose-dependent on factor XIII and reached $>90\%$ at 10 PEU/ml (Figure 2A). Interestingly, when a different fibrinogen source was used that was plasminogen-free but contained residual blood components such as fibronectin and von Willebrand factor, the binding efficiency was greatly reduced at low concentrations of factor XIII. However, at a high concentration of factor XIII (10 PEU/ml), the binding efficiency attained similar levels as those seen with highly purified fibrinogen, suggesting that overall binding may be limited by the rate of factor XIII-mediated conjugation rather than the number of binding sites. Indeed, the binding efficiency remained unchanged even when the load of P-KGF increased by fivefold from 100 to 500 $\mu\text{g/ml}$ (Figure 2B), demonstrating the high binding capacity of the fibrin matrix.

Next, we examined the kinetics of KGF release from the fibrin gels using an enzymatic immunoassay (Figure 2C). In agreement with the fluorescence measurements, 10% or 40% of the growth factor was released from the gels containing 10 or 0 PEU/ml of factor XIII, respectively. In contrast, gels containing unmodified KGF (CTRL with no peptide) released $\sim 90\%$ of the growth factor within 24 hours. In all cases, $\sim 45\%$ of the unbound growth factor was released in less than 1 hour and $\sim 80\%$ of the unbound KGF was released within 6 hours.

P-KGF and Plasmin-Derived P-KGF Retain Biological Activity

The biological activity of modified KGF was tested by proliferation of bronchial lung epithelial cells (4MBR-5), which are known to be highly sensitive to epidermal growth factor and KGF.⁵⁵ P-KGF and PL-P-KGF increased proliferation of 4MBR-5 cells to a similar extent as unmodified KGF, suggesting that conjugation with peptides, binding to fibrin

Figure 3. Modified KGF promotes proliferation of epithelial cells. KGF samples (stock KGF, P₂-KGF, P₅-KGF, PL-P₂-KGF, and PL-P₅-KGF) were added to the wells of a 96-well plate at 500 ng/ml and 4Mbr-5 lung epithelial cells (10,000 per well) were then seeded in low-serum media (CTRL, 2.5% FBS). For comparison, cells were grown in regular, high-serum media (CTRL, 10% FBS) with 30 ng/ml of epidermal growth factor. At 2 and 6 days after seeding, plates were washed once and frozen for Hoechst DNA quantitation. Total DNA was measured at the indicated times using a fluorescence microplate reader. The number of cells was determined from fluorescence measurements using a standard curve. Values are the mean $\pm$ SD of triplicate samples in a representative experiment ($n = 3$). An **asterisk** indicates a significant decrease ($P = 0.0002$) in growth at 6 days as compared to 2 days. A **double dagger** indicates a significant increase ($P < 0.001$) in growth for all samples with unmodified and modified KGF as compared to CTRL (2.5%) at 6 days.

gels, and release through the action of plasmin had no adverse effect on biological activity of KGF (Figure 3). In contrast, the number of cells in control samples decreased throughout time likely due to cell death. In agreement with previous studies,^{15–21} these results suggest that in addition to promoting proliferation, KGF, P-KGF, and PL-P-KGF may also promote cell survival.

Cell-Controlled Delivery of KGF in Vitro

The effect of Fb-P-KGF on wound healing was first examined using a scratch wound assay that we reported previously.⁵¹ In this model, a wounded cell monolayer was sandwiched between collagen and fibrin to mimic the environment that epithelial cells encounter during wound healing *in vivo*, where they migrate between the collagen of

Figure 2. Fibrin hydrogels have high binding capacity and the binding efficiency is dose-dependent on factor XIII. **A:** P-KGF was added to fibrin gels in the presence of factor XIII at the indicated concentrations (0.01 to 10 PEU/ml). Impure and purified fibrinogen preparations were used for comparison. Gels were thoroughly washed in buffer and then degraded with plasmin at 37°C for 3 hours. Total fluorescence measurements of the gel components before gel formation and after plasmin degradation were used to calculate the binding efficiency. **B:** The amount of bound P-KGF in fibrin matrix increases linearly with the amount added up to 500 µg/ml. **C:** Kinetics of KGF release from Fb-KGF and Fb-P-KGF formulations that were polymerized in the presence of 0 or 10 PEU/ml of factor XIII. Values are the mean $\pm$ SD of triplicate samples in a representative experiment ($n = 3$).

Figure 4. Cell-mediated release of KGF from fibrin gels promotes wound closure *in vitro*. Lung epithelial 4MBr-5 cells were grown on collagen-coated plates. At confluence the monolayers were wounded and overlaid with 300 μ l per well of Fb or Fb-P-KGF (150 ng of P-KGF) in the presence of 0, 10, or 100 kIU/ml aprotinin. The solutions polymerized within 10 to 20 seconds after addition in the wells. The rate of healing was determined by measuring the denuded area in the wound at various times after wounding and dividing by the initial area. All values are the mean $\pm$ SD of triplicate samples in a representative experiment ($n = 2$).

the dermis and the fibrin clot in the wound space.⁵⁷ To this end, 4MBr-5 cells were cultured on collagen-coated 24-well plates. When they reached confluence, the monolayers were wounded and overlaid with Fb-P-KGF (300 μ l/well), which gelled within 10 to 20 seconds after application to the cell monolayer. The fibrin gels contained 10 PEU/ml factor XIII and different concentrations of aprotinin as indicated.

We found that Fb-P-KGF accelerated wound healing significantly. Specifically, cell monolayers healed by 90% after 96 hours in the presence of Fb-P-KGF as compared to only 30% with fibrin (Fb) alone (Figure 4; compare Fb-P-KGF and Fb at 0 kIU/ml aprotinin). Interestingly, aprotinin retarded degradation of the fibrin gels and wound healing in a dose-dependent manner. In the absence of aprotinin fibrin gels degraded very fast (1 to 2 hours) and healing response was maximum. At 10 kIU/ml of aprotinin the fibrin gels degraded within $\sim$ 48 hours and the rate of healing decreased. Finally, 100 kIU/ml of aprotinin prevented breakdown of fibrin gels and impaired wound healing. These results demonstrate that proteolytic degradation of fibrin by the migrating cells may be necessary to release KGF and accelerate wound healing.

Cell-Controlled Delivery of KGF Enhances Re-Epithelialization *in Vivo*

To assess the efficacy of Fb-P-KGF *in vivo*, we used an *in vivo* wound model that we developed recently in our laboratory.⁵³ This model was based on bioengineered human epidermis that was transplanted onto athymic mice. By 6 weeks after grafting the implanted tissues were infiltrated with mouse fibroblasts and blood vessels and integrated

very well with the surrounding mouse skin to generate a hybrid skin tissue with human epidermis and mouse dermis. When the implants were subjected to full-thickness excisional wounds they healed with similar kinetics as human rather than mouse epidermis, suggesting that this may be a realistic model of human epidermal regeneration.⁵³

To evaluate the efficacy of Fb-P-KGF in promoting wound healing, transplanted skin equivalents were wounded at 6 weeks after grafting, and the wounds were immediately treated with a single application of Fb-P-KGF (20 μ g, $n = 6$), unmodified KGF mixed with fibrin gels (denoted Fb-KGF, 20 μ g; $n = 8$), or fibrin-vehicle only (Fb; $n = 7$). Using fluorescence microscopy, we found Fb-P-KGF was present at the wound site at 2 and 7 days after wounding (Figure 5, A and B). At 7 days, there was clearly less fluorescence in the wound, indicating release of the growth factor from the matrix into the wound area (Figure 5B). In contrast, topically applied P-KGF without fibrin gel disappeared from the wound at 1 day (Figure 5C). Histological examination and fluorescence imaging of the tissues at 7 days after wounding showed epidermal cells migrating on and degrading the fibrin gel loaded with fluorescent P-KGF (Figure 5D).

Examination of the wounded tissues macroscopically revealed significantly faster healing of the wounds treated with Fb-KGF and Fb-P-KGF (Figure 6, B and C), as compared to Fb controls (Figure 6A). H&E staining of tissue sections revealed faster re-epithelialization and more advanced formation of granulation tissue in the wounds treated with Fb-KGF and Fb-P-KGF (Figure 7, B and C) as compared to Fb controls (Figure 7A).

Quantitative analysis of the migrating epidermis revealed that wounds treated with Fb-KGF or Fb-P-KGF healed completely within 8 days, while Fb controls healed by only 50% (Figure 8A). To examine if fibrin degradation was necessary for KGF delivery, we increased the aprotinin concentration by 10-fold to 1000 kIU/ml to prevent fibrinolysis. Aprotinin had no effect in Fb-KGF-treated wounds, suggesting that there was no need for fibrin degradation when KGF was released by diffusion from the hydrogel (Figure 8B). In contrast, at high concentration of aprotinin, healing of Fb-P-KGF-treated wounds was reduced to the levels of Fb controls, demonstrating that release of KGF from Fb-P-KGF requires fibrin degradation by the wound-infiltrating cells. Furthermore, these results indicate that soluble inhibitors of fibrinolysis may control the rate of wound healing *in vivo*.

Discussion

In this study we used a growth factor delivery system that ensures long-term, local delivery of KGF on cellular demand to promote wound healing. To this end, we cross-linked the growth factor to fibrin hydrogels by linking fibrin-binding peptides to the free amines of the protein. *In vitro*, we thoroughly examined the capacity of modified KGF to bind fibrin, the kinetics of release and the biological activity after plasmin digestion of fibrin gels. We further explored the efficacy of this drug-release system *in vivo* using a novel model of wound healing based on transplanted human

Figure 5. Fluorescence imaging of wounds *in vivo*. Skin equivalents were grafted to athymic mice and allowed to take for 6 weeks before subjected to a full-thickness 4.0-mm excisional wound. An inverted fluorescence microscope was used to image the Fb-P-KGF or P-KGF in the wound (W) and periphery (P). Wounds were treated with Fb-P-KGF and imaged at 2 days (A) and 7 days (B) after wounding. Other wounds were treated by topical application of P-KGF (without fibrin gel) and imaged at 1 day (C). D: Tissues treated with Fb-P-KGF were excised at 8 days after wounding and processed for histology. Overlaid fluorescent and bright-field images of the wound show migrating cells as they degrade the fibrin matrix (green). Arrows represent wound edges and an asterisk denotes tip of migrating cells. Original magnifications: $\times 4$ (A–C); $\times 10$ (D).

bioengineered skin. Fb-P-KGF remained in the wound for several days, while P-KGF disappeared in 1 day demonstrating the importance of the fibrin matrix for retaining the growth factor at the wound site. As cells migrated into the wound space, they degraded the fibrin gel, releasing KGF locally. This cell-mediated release resulted in enhanced re-epithelialization and improved granulation tissue formation. Finally, aprotinin could modulate the release of bound but not free KGF, suggesting that plasmin-mediated degradation of Fb-P-KGF may be necessary for enhanced healing response.

Conjugation of fibrin-binding peptides to KGF was performed using a simple reaction scheme that allowed control of the number of peptides per KGF molecule. KGF contains several lysine residues that could potentially react with SMCC to provide a conduit for secondary reaction with the

peptide. As such, several peptides were incorporated to the surface of the protein, thus increasing the likelihood that at least one peptide might be accessible to factor XIII. Because KGF does not have any amine groups in its active site, peptide conjugation did not affect the biological activity of the protein, as shown by the growth and migration experiments (Figure 3 and 4). For growth factors or enzymes with side amine groups in their active site, genetic engineering of peptide-protein chimeras may be more appropriate to ensure that the peptide does not destroy protein activity. Fusion proteins containing a growth factor with the peptide linked at the N-terminus have been successfully delivered from fibrin gels in a cell-controlled manner.^{46,47,50} However, the activity of the fusion protein may also be affected if the binding site is localized near the N- or C-terminus, as is the case with interleukin-1 α and IGF-II.^{58,59} Therefore, the

Figure 6. Macroscopic evaluation of wounded tissues. Skin equivalents were grafted to athymic mice and allowed to take for 6 weeks before subjected to a full-thickness 4.0-mm excisional wound. At 8 days after wounding, the excised tissues were placed on a stereomicroscope and the wounds were photographed with a digital camera. Wounds were treated with Fb (A), Fb-KGF (B), or Fb-P-KGF (C). The grafted human skin equivalent is dark because the cells were harvested from a dark-skin donor. **Solid lines** represent the original wound edges. **Dotted lines** indicate portion of the tissue that did not heal.

method of peptide incorporation should be decided based on structural information about the presence of amino acids with side amine groups and the topology of the binding site.

Large amounts of P-KGF, in excess of 500 $\mu\text{g/ml}$, could be incorporated into fibrin gels, even in the presence of other proteins (eg, fibronectin) that may compete for incorporation into the fibrinogen A α -chain.⁶⁰ A high concentration of factor XIII was required to ensure fast incorporation of P-KGF before fibrin gel formation, which might retard molecular diffusion or conceal binding sites. Interestingly, the degree of conjugation could be modulated by the amount of factor XIII, generating formulations with partially conjugated growth factor. These formulations would exhibit a short-term burst release of free growth factor to initiate a cellular re-

Figure 7. Histological evaluation of wounded tissues. Skin equivalents were grafted to athymic mice and allowed to take for 6 weeks before subjected to a full-thickness 4-mm excisional wound. At 8 days after wounding, tissues were excised and processed for H&E staining. Wounds treated with Fb (A), Fb-KGF (B), or Fb-P-KGF (C). A montage of images was created to facilitate visualization of the whole wound and evaluation of healing. **Short arrows** demarcate original wound edges, **long arrows** represent the tip of the migrating epithelium, and **asterisks** denote granulation tissue. Morphological features are indicated by E, epidermis; D, dermis; and Fb, fibrin hydrogel. Original magnifications, $\times 4$.

sponse, followed by sustained release of the conjugated factor on fibrin degradation by the infiltrating cells.

Plasmin is known to specifically cleave a peptide bond adjacent to exposed arginine or lysine residues (Arg-X or Lys-X).² Plasmin-sensitive sequences such as LIKMKQ⁵⁰ or LIKMKP⁶¹ have been inserted into the peptide motif and shown to be effective for cell-demand release applications. In our experiments, Western blots demonstrated that treatment with plasmin cleaves the peptide, suggesting that the highly charged linker, RKK, may also be conducive to plasmin-mediated cleavage. It is important to note that although the cysteine residues likely remained linked to the surface coupling sites, the activity of plasmin-released KGF was not affected.

Indeed, proliferation and migration studies with lung epithelial cells showed that chemical conjugation of the peptide and treatment with plasmin did not affect the biological activity of KGF. In addition, KGF that was released from Fb-P-KGF by treatment with plasmin also retained its biological activity, suggesting that this technology may be applicable *in vivo*. Our data support this hypothesis as Fb-P-KGF accelerated healing of human skin equivalents that were transplanted onto athymic mice. Notably, this model system recapitulates several of the characteristics of human wounds, including the rate of re-epithelialization and the

Figure 8. Cell-mediated release of KGF from fibrin gels accelerates wound healing *in vivo*. Skin equivalents were grafted to athymic mice and allowed to take for 6 weeks before subjected to a full-thickness 4-mm excisional wound. The wounds were immediately treated with Fb ($n = 7$), Fb-KGF ($n = 8$), or Fb-P-KGF ($n = 6$). At 8 days after wounding, tissues were excised and processed for H&E staining. **A:** Re-epithelialization of the tissues was assessed by measuring the length of the migratory epithelium in the wound and dividing by the total wound size (% healing). **B:** Aprotinin was added at 100 or 1000 KIU/ml in Fb-KGF or Fb-P-KGF ($n = 2$). Results were averaged from histological measurements in two independent experiments. An **asterisk** indicates significant difference between Fb-KGF ($P = 0.00047$) or Fb-P-KGF ($P = 0.013$) as compared to Fb controls.

pattern of neo-epidermal differentiation,⁵³ suggesting that the effects of Fb-P-KGF formulations may be pertinent to human wound healing.

High concentrations of aprotinin inhibited degradation of fibrin gels and impaired wound healing *in vitro* and *in vivo*, suggesting that fibrin degradation may be necessary for release of P-KGF and wound healing. The rate of fibrin degradation determines the rate and duration of growth factor release, ultimately affecting the rate of wound healing.

More studies are needed to determine whether healing is optimum at intermediate concentrations of aprotinin. The results are likely to depend on the type of wound (acute versus chronic) because chronic wounds display higher proteolytic activity^{62–64} and may require higher concentrations of aprotinin for optimum fibrin degradation.

At the concentration of KGF used in this study (20 μ g/ml), both Fb-KGF and Fb-P-KGF result in complete wound healing in 1 week. It is possible that P-KGF may increase the rate of healing or may be effective at much lower concentrations than free KGF. Besides, conjugated KGF offers the advantage of localized delivery with no adverse effects on efficacy, thus providing a formulation with potential for use in clinical treatment of wounds. In contrast to current modes of passive release that rely on molecular diffusion, release of KGF from fibrin is in tune with cellular demand and is restricted to the local microenvironment of the migrating cell. Consequently, KGF remains in the wound until migrating cells degrade the fibrin matrix as we demonstrated by fluorescence imaging of the gels *in vivo*. Localized action is likely to obviate the need for long diffusion distances thus decreasing the chances of proteolytic degradation and minimizing the effective dose of KGF, ultimately limiting unwanted effects on nontarget sites.^{65,66} This is very important in light of several studies that suggested that protein instability or rapid clearance from the target site has tapered the success of clinical trials despite large repetitive doses of growth factors.^{67–69}

Surprisingly, quantitative immunostaining for the nuclear antigen Ki67 showed that the fraction of basal or suprabasal proliferating cells was not significantly different between Fb and Fb-KGF- or Fb-P-KGF-treated wounds ($P > 0.05$; data not shown). Because Fb-KGF and Fb-P-KGF were not statistically different, this result cannot be explained by the different mode of KGF delivery. A more likely explanation may be that at 8 days after wounding cell proliferation may have already subsided in Fb-KGF and Fb-P-KGF wounds because they were completely healed. In contrast, Fb-treated wounds had only re-epithelialized by $\sim 50\%$ and therefore, keratinocytes were still actively proliferating and migrating to close the wound. Because KGF has been implicated in the early phases of the healing process,²⁸ it is possible that Fb-KGF and Fb-P-KGF might have affected basal and suprabasal proliferation at earlier time points. A kinetic study would be required to assess the effects of Fb-KGF and Fb-P-KGF on basal and suprabasal epidermal proliferation.

The success of Fb-P-KGF in our *in vivo* model suggests that this formulation may be used successfully for treatment of surgical wounds, which are currently treated with fibrin glue.^{70–72} Local delivery to internal sites in the body could also be feasible with minimal invasion using catheter-based devices to mix and polymerize the two aqueous formulations (fibrinogen/factor XIII/P-KGF and thrombin) *in situ*. This method of delivery may also be effective for the treatment of chronic wounds such as diabetic ulcers, in which KGF may increase not only epithelialization but neovascularization as well, possibly through up-regulation of vascular endothelial growth factor.⁷³ Because expression of KGF is known to be suppressed in the wounds of diabetic animals,^{35,36} cell-controlled deliv-

ery might prove to be more effective in diabetic as compared to acute wounds. Chronic wounds are known to have high proteolytic activity, suggesting that aprotinin could be used to modulate the release of P-KGF or other growth factors for optimal delivery. This would not be possible with unconjugated KGF because diffusion from the matrix does not depend on fibrinolysis.

The chemistry that we used is general and could be used to conjugate any growth factor into the polymerizing fibrin gels. The large capacity of fibrin gels would also allow combinations of growth factors that could affect multiple steps of the healing cascade. Potential candidates include vascular endothelial growth factor and hepatocyte growth factor, which have been under recent investigation for treatments of chronic diabetic wounds.^{74–77} Finally, the same reaction scheme could be used for delivery of plasmid DNA, oligonucleotides, or siRNA to achieve localized gene transfer into the wound space. The natural propensity of wound-infiltrating cells to divide may facilitate gene transfer, thus obviating the need for cell-damaging methods such as electroporation.³⁷ Transplantation of bioengineered human skin onto nude mice provides a humanized model system that can be used to test these approaches in a realistic *in vivo* setting.

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