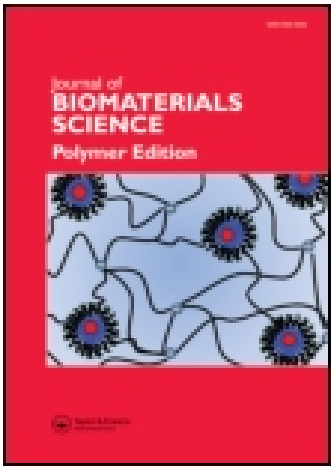

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Modulation of the Keratinocyte-Fibroblast Paracrine Relationship with Gelatin-Based Semi-interpenetrating Networks Containing Bioactive Factors for Wound Repair

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Modulation of the Keratinocyte–Fibroblast Paracrine Relationship with Gelatin-Based Semi-interpenetrating Networks Containing Bioactive Factors for Wound Repair

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Abstract

Gelatin-based semi-interpenetrating networks (sIPNs) containing soluble and covalently-linked bioactive factors have been shown to aid in wound healing; however, the biological responses elicited by the introduction of sIPN biomaterials remain unclear. In the current study, modulation of the re-epithelialization phase of wound healing by sIPNs grafted with PEGylated fibronectin-derived peptides and utilized as platforms for the delivery of exogenous keratinocyte growth factor (KGF) was evaluated. Following wounding, keratinocyte migration, proliferation and protein secretion is largely controlled by diffusible factors, such as KGF, released by the underlying fibroblasts. The impact of sIPNs and exogenous KGF upon the latter keratinocyte–fibroblast paracrine relationship and keratinocyte behavior was explored by monitoring keratinocyte adhesion and cytokine (IL-1α, IL-1β, IL-6, KGF, GM-CSF and TGF-α) release. Results were generally similar for keratinocyte monoculture and keratinocyte–fibroblast co-culture systems. Although keratinocyte adhesion increased over time for positive control surfaces, adhesion to the sIPNs remained low

Abbreviations: bis-COOH-PEG, bis-carboxylate-PEG; bis-NHS-PEG, bis-N-hydroxysuccini- mide-PEG; bis-tBuOAc-PEG, bis-t-butylacetate-PEG; COOH-mPEG, carboxylate monomethoxy PEG; Gelatin-sIPN, sIPN generated from unmodified gelatin and PEGdA; GGG-gelatin, gelatin grafted with PEGylated GGG; GGG-sIPN, sIPN generated from GGG-gelatin and PEGdA; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-6, interleukin-6; KGF, keratinocyte growth factor; mPEG-gelatin, gelatin grafted with monomethoxy PEG; mPEG-sIPN, sIPN generated from mPEG-gelatin and PEGdA; NHS-mPEG, N-hydroxysuccinimide monomethoxy PEG; PC, polycarbonate; PDGF-BB, platelet-derived growth factor BB homo-dimer; PTHrP, parathyroid-hormone-related protein; PEG, poly(ethylene glycol); PEGdA, PEG diacrylate; PHSRN-gelatin, gelatin grafted with PEGylated PHSRN; PHSRN-sIPN, sIPN generated from PHSRN-gelatin and PEGdA; RGD-gelatin, gelatin grafted with PEGylated RGD; RGD-sIPN, sIPN generated from RGD-gelatin and PEGdA; tBuOAC-mPEG, t-butylacetate PEG monomethyl ether; TCPS, tissue-culture polystyrene; TGF-α, transforming growth factor α; TGF-β, transforming growth factor β.

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throughout the course of the study. Release of IL-1α and GM-CSF was increased by exogenous KGF. The effects were more noticeable on the positive control surfaces relative to the sIPN surfaces. Regulation of the release of TGF-α was surface dependent, while IL-6 release was dependent upon surface type, the inclusion of exogenous KGF and the presence of fibroblasts. The findings indicate that during re-epithelialization, sIPNs containing soluble bioactive factors aid in wound healing primarily by serving as conduits for KGF, which induces the release of other key cytokines involved in tissue repair.

Keywords
Keratinocyte, paracrine, co-culture, semi-interpenetrating network, KGF

1. Introduction

The migration and proliferation of keratinocytes during the re-epithelialization phase of wound healing is predominantly mediated by soluble, diffusible factors produced by cells in the underlying dermis [1, 2]. Although fibroblast-derived keratinocyte growth factor (KGF/FGF-7) has gained notoriety as being crucial for epithelial cell proliferation [3–7], expression of additional growth factors and cytokines is also regulated by the interplay between the epidermis and dermis. As illustrated in Fig. 1, a complex autocrine/paracrine relationship exists between keratinocytes and fibroblasts. 1L-1α and IL-1β from keratinocytes controls expression of KGF/FGF-7 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by fibroblasts through c-Jun and Jun-B transcription factors [8–12]. KGF expression is also regulated by IL-6 [13–15], parathyroid-hormone-related

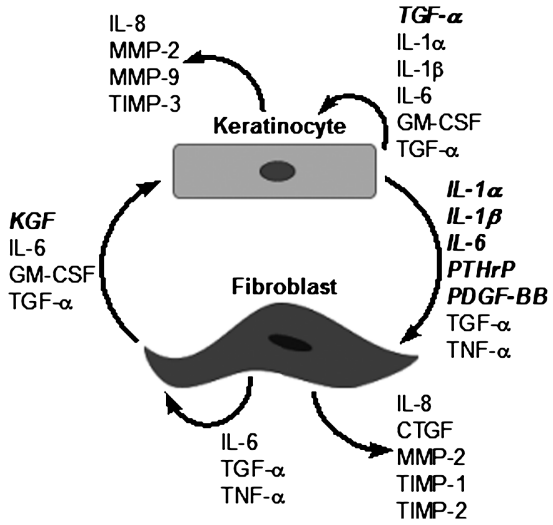

Figure 1. Schematic illustration of the keratinocyte–fibroblast autocrine/paracrine relationship. Bold and italicized factors are associated with the release of KGF by fibroblasts. Keratinocyte-derived IL-1α and IL-1β stimulate fibroblasts to express KGF. IL-6, PTHrP and PDGF-BB further regulate KGF expression. KGF, in turn, enhances keratinocyte proliferation and induces release of TGF-α.

protein (PTHrP) [16] and platelet-derived growth factor BB (PDGF-BB) [13–15]. In addition to directly enhancing cell proliferation, KGF promotes the expression of TGF-α, which acts by an autocrine mechanism to further increase cell proliferation [17]. IL-1α and IL-1β also operate in an autocrine and paracrine manner to stimulate IL-6 and IL-8 expression in keratinocytes and fibroblasts, respectively [12]. Keratinocyte-derived tumor necrosis factor-α (TNF-α) regulates collagen synthesis by fibroblasts through direct interference with transforming growth factor-β (TGF-β) and suppression of connective tissue growth factor (CTGF) activation [18]. IL-1α additionally down-regulates CTGF expression [19]. In contrast, TGF-β functions in the up-regulation of CTGF [20]. Expression of specific matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) is enhanced in both keratinocytes and fibroblasts [21]. Cross-talk between the dermis and epidermis is imperative in maintaining homeostasis during tissue repair.

Biomaterials are currently being explored to promote the wound healing process. Natural and synthetic materials that contain components of extracellular matrix molecules increase the rate of healing by providing sites of attachment for cell migration [22–25]. Upon epidermal injury, keratinocytes near the wound edge withdraw from terminal differentiation and surface exposure of the fibronectin receptors $\alpha_5\beta_1$ and $\alpha_V\beta_6$ is increased [26], thereby facilitating migration over the fibronectin-containing fibrin clot [27]. In addition to promoting keratinocyte proliferation, KGF aids in re-epithelialization by inducing integrin avidity [4]. A recent study demonstrated that significantly enhanced wound closure could be achieved by controlled delivery of KGF from a fibrin matrix [3]. Biomaterials that contain fibronectin-derived peptides and that can be used to deliver KGF will likely promote re-epithelialization by mediating keratinocyte adhesion, migration and proliferation. The potential impact of the matrix and exogenous KGF on the complex keratinocyte–fibroblast interaction needs to be better understood so as to provide an improved material design paradigm.

Our lab developed a photopolymerizable system that has been shown *in vivo* to aid in wound healing and tissue repair. Poly(ethylene glycol) diacrylate (PEGdA) is cross-linked *via* radical polymerization in the presence of gelatin to yield semi-interpenetrating networks (sIPNs) that are biodegradable and mechanically stable [28–30]. Functional, fibronectin-derived peptides can be grafted onto the gelatin *via* poly(ethylene glycol) (PEG) linkers prior to photopolymerization (Fig. 2). RGD and its synergy sequence, PHSRN, have been shown to have a significant impact upon adhesion, migration and cytokine expression of various cell types involved in wound healing [31–33]. When applied to full thickness wounds in rats, sIPNs grafted with PEGylated RGD and loaded with KGF gave rise to regenerated tissue that possessed higher cellularity, larger occurrences of neovascularization, and a more organized extracellular matrix compared to conventional dressings and sIPNs that did not incorporate bioactive factors. Tissue regenerated in the presence of sIPNs that included PEGylated RGD and exogenous KGF also possessed macrophage and fibroblast densities comparable to undamaged tissue [32]. The lat-

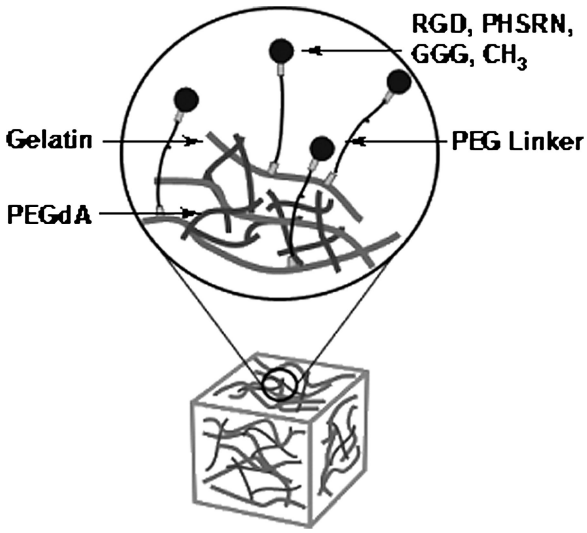

Figure 2. Illustration of the semi-interpenetrating network. PEGdA was cross-linked *via* radical polymerization in the presence of unmodified gelatin, mPEG-gelatin and gelatin conjugated to peptides *via* a PEG linker to yield sIPNs.

ter response indicates that sIPNs modified with fibronectin-derived peptides and loaded with growth factors accelerate wound healing.

Current research is aimed at obtaining a better understanding of the underlying biological response induced by sIPNs with bioactive factors that leads to quicker tissue repair. The impact of sIPNs grafted with PEGylated peptides and loaded with exogenous KGF upon the re-epithelialization phase of wound healing is unknown. Although previous research has shown that keratinocytes do not adhere to unmodified sIPNs [34], the influence of bioactive factors on adhesion and cytokine expression and on the keratinocyte–fibroblast paracrine relationship remains unclear. Our hypothesis is that sIPNs modified by covalently attached fibronectin-derived peptides and/or soluble, exogenous keratinocyte growth factor provide matrices for cell migration and alter the release of cytokines in a manner that accelerates wound healing.

In this study, the impact of semi-interpenetrating gelatin-based networks grafted with PEGylated, fibronectin-derived peptides upon adhesion and cytokine expression of keratinocytes was explored. A new method for the synthesis of activated poly(ethylene glycol) for attachment to peptides and gelatin was developed. A two-chamber co-culture system was used to replicate the re-epithelialization phase of wound healing and to investigate the effect of the modified sIPNs upon the autocrine/paracrine relationship between fibroblasts and keratinocytes (Fig. 3). The sIPNs were also utilized as vehicles for delivery of keratinocyte growth factor, and the effect of exogenous KGF on keratinocyte monoculture and keratinocyte–fibroblast co-culture was assessed. The research presented herein is intended to focus only upon soluble, diffusible factors, which are

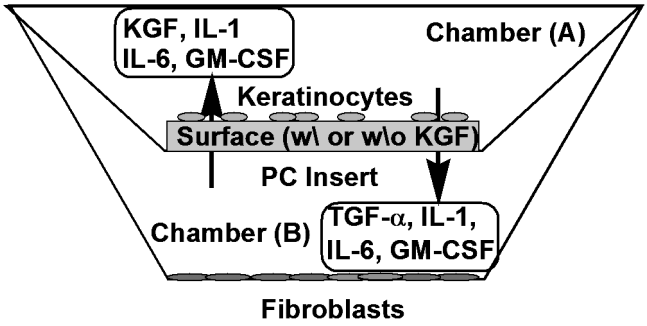

Figure 3. Illustration of the co-culture setup. Neonatal human epidermal keratinocytes (NHEK) were co-cultured with confluent human dermal fibroblasts (HDF) on sIPN and PC surfaces with and without exogenous KGF. The upper (A) and lower (B) chambers of the assembly were assayed for KGF, IL-1α, IL-1β, IL-6, GM-CSF and TGF-α. As controls, keratinocytes and fibroblasts were grown in monoculture on PC and TCPS, respectively.

the primary mediators of the epidermal–dermal interactions observed during re-epithelialization. In order to truly understand the complex interplay between keratinocytes and fibroblasts, several significant proteins involved in the autocrine/paracrine pathway were assayed, namely KGF, IL-1α, IL-1β, IL-6, GM-CSF and TGF-α.

2. Materials and Methods

2.1. Reagents and Cells

Anhydrous methylene chloride, tetrahydrofuran and N,N-dimethylformamide (DMF) were obtained from a dry solvent system. Poly(ethylene glycol) was received from Fluka (Buchs, Switzerland) with a molecular mass range of 1900–2200 Da. Type A gelatin from porcine skin with a bloom strength of approx. 300 g was ordered from Aldrich (Milwaukee, WI, USA). Gelatin molecular mass was determined to be approx. 76 kDa by GPC, as described below. The experimentally determined value correlated with literature values. G_3 was purchased from Bachem Bioscience (King of Prussia, PA, USA). Rink amide resin and Fmoc protected amino acids used in solid phase peptide synthesis were ordered from EMD Biosciences (San Diego, CA, USA). All other commercial reagents were purchased from Aldrich and used without further purification. Normal human epidermal keratinocytes from neonatal foreskin (NHEK; Lonza, Allendale, NJ, USA) were cultured in keratinocyte growth medium (KGM; Lonza) supplemented with 5% fetal bovine serum (FBS; Atlantic Biologics, Miami, FL, USA), while human dermal fibroblasts (HDF; Lonza) were cultured in fibroblast growth media (FGM; Lonza) plus 2% FBS. Both cell lines were maintained at 37°C and 5% CO_2 and were used after passage 2 or 3. Cells were passaged at approximately 80% confluence using trypsin/EDTA (Lonza).

2.2. Analytical Data

NMR was used to confirm product formation. ^{1}H- and ^{13}C-NMR spectra were recorded using a Varian 400 (400 MHz). Proton (^{1}H) and carbon (^{13}C) chemical shifts, reported in parts per million (ppm), were referenced using internal $CDCl_3$. The following abbreviations for multiplicities are used: singlet, s; multiplet, m. Gel-permeation chromatography (GPC) performed with a Waters® Breeze™ System was used to verify the predicted molecular weights of modified gelatins. Separation was carried out with a Waters® Ultrahydrogel™ column, using a Waters® 2410 differential refractometer as detector. Millipore filtered 1 × phosphate-buffered saline (PBS) was used as the eluent at a flow rate of 1 ml/min. Molecular weights were estimated from a Universal calibration curve ($\log M(\eta)$ *vs.* elution volume) constructed from poly(ethylene oxide) standards. The intrinsic viscosities (η) of the samples and standards were measured in an Ubbelohde capillary viscometer. All samples for GPC analysis were prepared at a concentration of 5 μg/ml in 1 × PBS and were passed through a 0.45 μm filter prior to injection. The percentage of lysyl groups on gelatin modified with PEGylated peptides was estimated using the established trinitrobenzenesulfonic acid (TNBS) method [35, 36]. The results were used to calculate predicted molecular weights for modified gelatins, which were compared to those experimentally determined by GPC.

2.3. Preparation of Bis-N-hydroxysuccinimide-PEG (bis-NHS-PEG) and N-hydroxysuccinimide Monomethoxy PEG (NHS-mPEG)

N-hydroxysuccinimide activated esters were prepared from PEG as described below and as shown in Fig. 4. The method presented herein deviates from that given in previous works [31–33]. Bis-t-butylacetate-PEG was prepared by adding PEG (10 g, 5 mmol) to a round-bottom flask equipped with a magnetic stir bar. An inert atmosphere was obtained by sequentially evacuating and filling the flask with argon. THF (100 ml, 10%, w/v) was added and the suspension was heated until all PEG was dissolved. Sodium hydride (0.48 g, 20 mmol) was added slowly and the reaction mixture was allowed to stir at room temperature for 30 min. t-Butyl bromoacetate (3.90 g, 20 mmol) was added *via* a syringe, and stirring was continued for 24 h. Salts were removed by filtration through cellite, and the filtrate was added drop-wise to cold hexanes to give a white power. The powder was isolated by filtration, dissolved in CH_2Cl_2 and extracted with 1 M NaOH, followed by DI water. The organic layer was dried over $MgSO_4$ and concentrated to give 7.6 g (67%) of bis-tBuOAC-PEG as off-white crystals. ^{1}H-NMR ($CDCl_3$): δ 4.02 (s, 2H), δ 3.63 (m, 90.6H) and δ 1.48 (s, 9H). ^{13}C-NMR ($CDCl_3$): δ 169.61, δ 81.43, δ 70.54, δ 69.00 and δ 28.10.

Bis-carboxylate-PEG (bis-COOH-PEG) was prepared by dissolving bis-tBuOAc-PEG (7.5 g, 3.37 mmol) in 26 ml CH_2Cl_2. HCl (34 ml, 4 M) in dioxane was added and the reaction mixture was stirred at room temperature overnight. The solvents were removed under reduced pressure to give 7.1 g (100%) of bis-

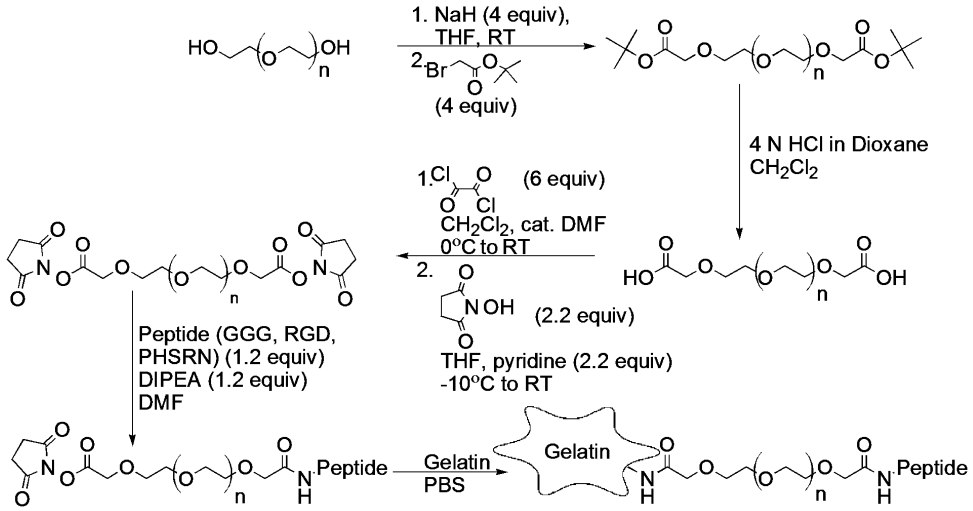

Figure 4. Preparation of gelatin grafted with PEGylated peptides. PEG was converted to bis-tBuOAc-PEG, which was hydrolyzed to give bis-COOH-PEG. Conjugation of the desired peptide to gelatin through a PEG linker was achieved by N-hydroxysuccinimide activation. In a similar fashion, monomethoxy PEG and gelatin were coupled *via* the N-hydroxysuccinimide-activated ester.

COOH-PEG as off-white crystals. ^{1}H-NMR ($CDCl_3$): δ 4.17 (s, 2H) and δ 3.63 (m, 90.6H). ^{13}C-NMR ($CDCl_3$): δ 170.39, δ 70.55 and δ 63.73.

Bis-N-hydroxysuccinimide-PEG (bis-NHS-PEG) was prepared by adding bis-COOH-PEG (5.5 g, 2.60 mmol) to a round-bottom flask equipped with a magnetic stir bar. An inert atmosphere was obtained by sequentially evacuating and filling the flask with argon. CH_2Cl_2 (19 ml) was added and the solution was cooled to 0°C. 7.8 ml of oxalyl chloride solution (2 M in CH_2Cl_2, 15.6 mmol) was added slowly *via* a syringe, followed by one drop of catalytic DMF, where catalytic DMF refers to one drop of DMF in 1 ml of CH_2Cl_2. The reaction mixture was warmed to room temperature and stirred for 1 h. The solvent was removed under reduced pressure and the resultant residue was re-dissolved in CH_2Cl_2 and re-concentrated two times. The flask was evacuated and filled with argon, THF (19 ml) was added and the solution was cooled to −10°C. N-hydroxysuccinimide (NHS; 0.66 g, 5.71 mmol) was added to a separate round bottom flask, which was also evacuated and filled with argon prior to the addition of 8.5 ml THF. The NHS solution was added slowly to the acid chloride solution, followed by pyridine (0.45 g, 5.71 mmol). The reaction mixture was warmed to room temperature and stirred for 2 h. Salts were removed *via* filtration through celite and the filtrate was added drop-wise to cold ether to give a white powder. The powder was collected by filtration and dried for 24–48 h *in vacuo* to give 6.0 g (76%) of bis-NHS-PEG. ^{1}H-NMR ($CDCl_3$): δ 4.50 (s, 2H), δ 3.63 (m, 90.6H) and δ 3.63 (s, 4H). ^{13}C-NMR ($CDCl_3$): δ 176.11, δ 171.93, δ 70.65, δ 66.64 and δ 25.53.

tBuOAc-mPEG was prepared from monomethoxy poly(ethylene glycol) (mPEG) as described above for bis-tBuOAc-mPEG. ^{1}H-NMR ($CDCl_3$): δ 4.00 (s, 2H) and

δ 3.63 (m, 181.2H), δ 3.36 (s, 3H) and δ 1.45 (s, 9H). ^{13}C-NMR ($CDCl_3$): δ 169.73, δ 81.54, δ 70.78, δ 59.09, δ 53.59 and δ 28.18.

COOH-mPEG was prepared from tBuOAc-mPEG as described above for bis-COOH-PEG. ^{1}H-NMR ($CDCl_3$): δ 4.14 (s, 2H), δ 3.63 (m, 181.2H) and δ 3.36 (s, 3H). ^{13}C-NMR ($CDCl_3$): δ 171.62, δ 70.56, δ 59.02 and δ 53.58.

NHS-mPEG was prepared from COOH-mPEG as described above for bis-NHS-mPEG. ^{1}H-NMR ($CDCl_3$): δ 4.53 (s, 2H), δ 3.63 (m, 181.2H), δ 3.38 (s, 3H) and δ 2.86 (s, 4H). ^{13}C-NMR ($CDCl_3$): δ 169.24, δ 71.09, δ 67.05, δ 59.56 and δ 26.11.

2.4. Preparation of Gelatin Grafted with PEGylated Peptides (GGG-Gelatin, RGD-Gelatin and PHSRN-Gelatin)

Coupling of peptides and gelatin to the N-hydroxysuccinimide activated esters was accomplished as described below and as shown in Fig. 4. RGD and PHSRN for use in preparation of PEGylated peptides were synthesized following standard solid-phase peptide synthesis techniques utilizing Fmoc chemistry [37, 38]. Product formation was confirmed by ^{1}H-NMR and MALDI mass spectrometry (University of Wisconsin Biotechnology Center, Madison, WI, USA).

Bis-NHS-PEG (1 g, 0.43 mmol) was dissolved in 5 ml of DMF. GGG, RGD or PHSRN (0.52 mmol) was added, followed by N,N-diisopropylethylamine (DIPEA; 0.067 g, 0.52 mmol). After stirring at room temperature for 45 min, the PEGylated peptide solution was poured into a solution of gelatin (0.62 g, 0.008 mol gelatin, 0.21 mol lysine) in 62 ml 1 $\times$ PBS (1%, w/v). The pH was adjusted to 8 through drop-wise addition of 1 M NaOH and the reaction mixture was stirred at room temperature for 60 min. Purification was carried out with a Millipore filter apparatus with a 30 kDa filter, rinsing with pH 4 DI water. A white or off-white powder was obtained following lyophylization.

Percentage of modification of gelatin grafted with PEGylated GGG (GGG-gelatin) was estimated at 80%, which gave a predicted molecular mass of 170 kDa. The average molecular mass from GPC was determined to be 225 kDa using an intrinsic viscosity of 135 ml/g. Percentage of modification of gelatin grafted with PEGylated RGD (RGD-gelatin) was estimated at 56%, which gave a predicted molecular mass of 146 kDa. The average molecular mass from GPC was determined to be 232 kDa using an intrinsic viscosity of 118 ml/g. The percentage of modification of gelatin grafted with PEGylated PHSRN (PHSRN-gelatin) was estimated at 55%, which gave a predicted molecular mass of 152 kDa. The average molecular mass from GPC was determined to be 193 kDa using an intrinsic viscosity of 71 ml/g. Given the polydispersities of both PEG and gelatin, the predicted molecular masses were in good agreement with those experimentally determined by GPC.

2.5. Preparation of Gelatin Grafted with mPEG (mPEG-Gelatin)

NHS-mPEG (1.5 g) was dissolved in 100 ml 1 $\times$ PBS. 1 g gelatin was added to give a 10% (w/v) solution. The reaction mixture was stirred at room tempera-

ture for 60 min, and purification and isolation were carried out as described above for gelatin grafted with PEGylated peptides. Percentage of modification was estimated at 44%, which gave a predicted molecular mass of 122 kDa. The molecular mass from GPC was determined to be 133 kDa using an intrinsic viscosity of 185 ml/g.

2.6. Preparation of Semi-interpenetrating Networks (sIPNs)

sIPNs were prepared as described elsewhere [32]. Briefly, 0.3 g mPEG-gelatin, GGG-gelatin, RGD-gelatin, PHSRN-gelatin, or unmodified gelatin were dissolved in 3 ml Millipore-filtered water (10%, w/v) by heating at 60°C. The gelatin solution was added to 0.24 g PEGdA ($M_n \approx 575$). A stock initiator solution was prepared by dissolving 0.2 g of 2,2-dimethoxyphenylacetophenone (DMPA) in 0.5 g of PEGdA. Initiator solution (60 µl) was added to the gelatin-PEGdA solution to give a gelatin/PEGdA weight ratio of 1:1. For monoculture experiments, the solution was transferred to a Teflon® mold ($d = 8$ mm, $t = 0.75$ mm) and exposed to long-wave UV light ($\lambda_{max} = 365$ nm, 2.5 µW/cm^2 at 2.5 cm) for 5–10 min. The resultant sIPNs (Fig. 2) were dipped into a 70% ethanol solution for sterilization, rinsed three times with 1 × PBS (CellGro) and transferred to 48-well plates. For co-culture experiments, the sIPNs were formed directly within Transwell® polycarbonate membrane inserts (0.4 pore size, $d = 6.5$ mm) (Corning, Lowell, MA, USA), then briefly rinsed with 70% ethanol solution, followed by 1 × PBS (3 times) for sterilization. sIPNs loaded with exogenous KGF were prepared by adding 336 ng human KGF (recombinant, R&D Systems, Minneapolis, MN, USA) to the reaction mixture prior to photopolymerization, as described previously [37]. KGF loading in the latter manner resulted in dosages of approx. 6 ng/ml for 7 days (21 ng per sIPN in 0.5 ml media) and 1 ng/ml for 7 days (9 ng per sIPN in 1.5 ml media) for monoculture and co-culture experiments, respectively. The dosages correlated with a range of 1–10 ng/ml KGF, which was shown to increase proliferation of subconfluent keratinocytes *in vitro*. The loading and release of exogenous KGF was confirmed by immunoassay, as described later.

2.7. Preparation of PEG Hydrogels

PEGdA (0.24 g, $M_n \approx 575$) was added to 3 ml DI water, followed by 60 µl initiator solution, to give a 10% (w/v) aqueous solution of PEGdA. The mixture was transferred to Telfon® molds, exposed to UV light and the resultant hydrogels were sterilized as described previously for sIPNs. PEG hydrogels loaded with exogenous KGF were prepared through the addition of 336 ng KGF to the solution prior to photo-polymerization. As detailed above, KGF loading in the latter manner resulted in dosages of approx. 6 ng/ml for 7 days and 1 ng/ml for 7 days for monoculture and co-culture experiments, respectively.

2.8. Keratinocyte Monoculture

Keratinocytes were seeded onto sIPN, PEG hydrogel and tissue-culture polystyrene (TCPS) surfaces within 48-well plates at a concentration of 3500 cells/cm^2 and

maintained at 37°C and 5% CO_2 in 0.5 ml of KGM supplemented with 5% FBS. TCPS was employed as a positive surface control, while PEG hydrogel was used as an additional surface comparison. To ascertain the impact of exogenous KGF on the positive control, KGF (21 ng) was added directly to the media of TCPS wells. KGF loading corresponded to a dosage of 6 ng/ml for 7 days. mPEG-sIPN and GGG-sIPN were utilized as peptide controls for cell responses to surfaces containing fibronectin-derived peptides (RGD-sIPN and PHSRN-sIPN). The media was changed at the 96-h time point only. At 2, 24, 96 and 168 h, the supernatant was collected for cytokine analysis. The surfaces were rinsed with Dulbecco's PBS (D-PBS) (Mediatech, Herndon, VA, USA) and live adherent cell density was evaluated *via* Live/Dead assay (Invitrogen, Carlsbad, CA, USA). KGF concentration was determined using a Quantikine® colorimetric sandwich ELISA (R&D Systems) and, as mentioned above, was used to confirm loading and release of exogenous KGF by sIPNs. IL-1α, IL-β, IL-6, GM-CSF and TGF-α levels were assessed simultaneously using LINCOPlex multiplex kits (Millipore, Billerica, MA, USA) for Luminex xMap technology. The detectable ranges of the various cytokines were as follows: KGF (<15–2000 pg/ml), IL-1α (1.31–10 000 ng/ml), IL-1β (0.86–10 000 pg/ml), IL-6 (1.29–10 000 pg/ml), GM-CSF (1.75–10 000 pg/ml), TGF-α (3.33–10 000 pg/ml). All concentration values are reported as cumulative release.

2.9. Keratinocyte/Fibroblast Coculture

Fibroblasts were grown to confluence on 24-well plates using the conditions given above. Media was changed to 1.2 ml of KGM plus 5% FBS 24 h and immediately prior to the start of experimentation. sIPNs and PEG hydrogels loaded with our without KGF were prepared on Transwell® PC membranes as described previously. The inserts were placed within the wells, and an addition 0.3 ml of media was added (Fig. 3). Keratinocytes were seeded onto sIPN, PEG hydrogel, and PC at a concentration of 3500 cells/cm^2 and maintained at 37°C and 5% CO_2. PC was employed as a positive surface control, while PEG hydrogel was used as an additional control. Keratinocytes and fibroblasts were also cultured alone in order to better assess the effects of co-culture condition. To ascertain the impact of exogenous KGF on the positive control and the fibroblast monoculture, 21 ng KGF was added directly to the media. KGF loading corresponded to a dosage of 2 ng/ml for 7 days. mPEG-sIPN and GGG-sIPN were utilized as peptide controls for cell responses to surfaces containing fibronectin-derived peptides (RGD-sIPN and PHSRN-sIPN). The media was changed at the 96-h time point only. At 2, 24, 96 and 168 h, the supernatant was collected from the upper (A) and lower (B) chambers of the insert-well assembly for cytokine analysis, and live adherent cell density was evaluated as described above for keratinocyte monoculture. All cytokine concentrations are reported as cumulative release.

2.10. Statistical Analysis

All experimental results are reported as mean ± standard deviation ($n = 3$). Live adherent keratinocyte densities and cytokine concentrations were compared *via* ANOVA followed by independent t-tests with a significance level of $P < 0.05$ using Statgraphics software.

3. Results

3.1. Effect of Surface Type and Exogenous KGF on Cell Adhesion in Keratinocyte Monoculture

Live adherent keratinocyte density on TCPS increased between the 2, 24, 96 and 168-h time points in the presence and absence of exogenous KGF within the monoculture system (Table 1). In contrast, live adherent cell density on sIPNs and PEG hydrogels remained low throughout the course of the experiment. Although initial cell adhesion to RGD-sIPN without exogenous KGF was significantly greater than all other sIPN surfaces without exogenous KGF, the phenomenon did not continue to later time points. There was a trend towards higher adherent cell densities on sIPNs containing fibronectin-derived peptides (RGD-sIPN and PHSRN-sIPN) rel-

Table 1.
Live adherent keratinocyte density on sIPN, PEG hydrogel and TCPS surfaces in monoculture system

Surface	Exogenous KGF	Live adherent keratinocyte density (cells/mm^2) after			
		2 h	24 h	96 h	168 h
MPEG-sIPN	No	0 ± 0	15 ± 6	2 ± 1	1 ± 1
	Yes	0 ± 0	2 ± 0	1 ± 1	1 ± 1
GGG-sIPN	No	0 ± 0	14 ± 7	5 ± 1[a]	1 ± 1
	Yes	0 ± 0	6 ± 28	2 ± 1	0 ± 0
RGD-sIPN	No	7 ± 5[b]	44 ± 27	22 ± 8[a]	4 ± 7
	Yes	1 ± 0	34 ± 2	4 ± 3	2 ± 2
PHSRN-sIPN	No	1 ± 1	26 ± 25	15 ± 11	9 ± 4[a]
	Yes	1 ± 1	8 ± 8	3 ± 2	1 ± 1
Gelatin-sIPN	No	1 ± 0	11 ± 10	3 ± 3	0 ± 0
	Yes	0 ± 0	2 ± 1	1 ± 1	0 ± 0
PEG hydrogel	No	2 ± 2	0 ± 1	0 ± 1	0 ± 0
	Yes	4 ± 6	1 ± 1	0 ± 0	0 ± 0
TCPS	No	3 ± 0	46 ± 35	168 ± 81[c]	188 ± 27[c]
	Yes	3 ± 2	64 ± 41[d]	170 ± 48[d]	145 ± 134[d]

[a] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.
[b] $P < 0.05$ *vs*. all other sIPN substrates prepared without exogenous KGF.
[c] $P < 0.05$ *vs*. sIPN substrates prepared without exogenous KGF at the same time point.
[d] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

ative to the other sIPNs (GGG-sIPN and mPEG-sIPN) at the 96 and 168-h time points without exogenous KGF; however, the values were significantly low when compared to those of TCPS without KGF.

3.2. Effect of Surface Type and Exogenous KGF on Cytokine Concentrations in Keratinocyte Monoculture

Generally, KGF release from keratinocytes grown in monoculture on surfaces that incorporated exogenous KGF was significantly greater than from keratinocytes grown without supplements (Table 2). However, KGF levels in supernatant obtained from wells containing KGF loaded gelatin-sIPNs were significantly lower than concentrations obtained for all other KGF-loaded sIPNs at 96 and 168 h.

KGF-loaded gelatin-sIPNs at 168 h released significantly greater amounts of IL-1α compared to all other KGF loaded sIPNs (Table 3). Keratinocytes adhered to RGD-sIPN released significantly higher levels of IL-1α *versus* the same surface without KGF at 96 and 168 h. At 96 h, IL-1α concentrations were also significantly greater for keratinocytes grown on KGF-loaded PHSRN-sIPN compared to the corresponding unloaded sIPN. Keratinocytes adhered to TCPS had significantly higher levels of IL-1α at 96 and 168 h when exogenous KGF was incorporated into the media relative to KGF-free media. IL-1β was not detectable

Table 2.
KGF concentration (cumulative release) in supernatant from keratinocytes grown on sIPN, PEG hydrogel and TCPS surfaces in the monoculture system

Surface	Exogenous KGF	KGF concentration (pg/ml) after			
		2 h	24 h	96 h	168 h
mPEG-sIPN	No	3 ± 2	2 ± 3	0 ± 0^{a}	1 ± 2^{a}
	Yes	187 ± 128	266 ± 137	174 ± 61	309 ± 68
GGG-sIPN	No	8 ± 11	13 ± 13^{a}	0 ± 0^{a}	0 ± 0^{a}
	Yes	483 ± 451	457 ± 74	124 ± 6	226 ± 12
RGD-sIPN	No	0 ± 0	1 ± 3	0 ± 0^{a}	0 ± 0^{a}
	Yes	43 ± 37	95 ± 45	78 ± 24	93 ± 24
PHSRN-sIPN	No	0 ± 0	1 ± 1	0 ± 0^{a}	10 ± 16^{a}
	Yes	366 ± 287	332 ± 193	167 ± 66	233 ± 72
Gelatin-sIPN	No	9 ± 7	0 ± 0	1 ± 2	1 ± 2
	Yes	0 ± 0	3 ± 3	0 ± 0^{b}	1 ± 1
PEG hydrogel	No	1 ± 2^{a}	7 ± 9	1 ± 1^{a}	5 ± 4^{a}
	Yes	197 ± 59	140 ± 62	147 ± 49	160 ± 49
TCPS	No	2 ± 3^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	Yes	$>2000^{c}$	$>2000^{c}$	$>2000^{c}$	$>2000^{c}$

[a] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.
[b] $P < 0.05$ *vs*. all other sIPN substrates prepared with exogenous KGF at the same time point.
[c] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

Table 3.
IL-1α concentration (cumulative release) of supernatant from keratinocytes grown on sIPN, PEG hydrogel and TCPS surfaces

Surface	Exogenous KGF	IL1-α concentration (pg/ml) after			
		2 h	24 h	96 h	168 h
MPEG-sIPN	No	2 ± 1	2 ± 0	3 ± 2	3 ± 2
	Yes	7 ± 4	3 ± 3	6 ± 1	6 ± 1
GGG-sIPN	No	7 ± 11	1 ± 1	4 ± 1	4 ± 2
	Yes	4 ± 1	2 ± 2	6 ± 2	6 ± 2
RGD-sIPN	No	10 ± 12	0 ± 0	1 ± 0^{a}	2 ± 1^{a}
	Yes	3 ± 3	3 ± 3	6 ± 1	6 ± 1
PHSRN-sIPN	No	7 ± 7	1 ± 0	3 ± 1^{a}	3 ± 1
	Yes	2 ± 1	0 ± 0	6 ± 2	7 ± 2
Gelatin-sIPN	No	4 ± 2	3 ± 0	5 ± 0^{a}	7 ± 0^{a}
	Yes	5 ± 1	5 ± 2	14 ± 5^{b}	26 ± 18^{b}
PEG hydrogel	No	1 ± 2	3 ± 2	7 ± 6	9 ± 6
	Yes	4 ± 1	3 ± 1	8 ± 2	10 ± 3
TCPS	No	9 ± 10	2 ± 1	4 ± 1^{a}	6 ± 1^{a}
	Yes	3 ± 1	0 ± 1	7 ± 1	10 ± 2

[a] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.
[b] $P < 0.05$ *vs*. all other sIPN substrates prepared with exogenous KGF.

(0 ± 0 pg/ml) at any time points for any of the surfaces with or without exogenous KGF.

TGF-α was not detectable (0 ± 0 pg/ml) at any time points for any of the surfaces, regardless of the presence of exogenous KGF. For the most part, GM-CSF was also not detectable (0 ± 0 pg/ml) for any of the surfaces with or without exogenous KGF. However, extremely low levels (2 ± 1 pg/ml) that were significantly greater than those obtained from KGF-loaded sIPNs could be detected from TCPS with exogenous KGF at 96 and 168 h.

Keratinocytes on TCPS surfaces released significantly higher levels of IL-6 when KGF was added to the media relative to KGF free media (Table 4). In contrast, IL-6 concentrations were significantly higher when keratinocytes were cultured on sIPN surfaces without exogenous KGF *versus* those with exogenous KGF.

3.3. Effect of Surface Type, Exogenous KGF and Fibroblast Presence on Keratinocyte Adhesion in Keratinocyte–Fibroblast Co-culture

As was the case for keratinocyte monoculture, live adherent cell density on sIPNs and PEG hydrogels within the keratinocyte–fibroblast co-culture system remained low throughout the course of the experiment compared to that on positive control surfaces, i.e., PC membranes (Table 5). At 96 and 168 h, the live adherent keratinocyte density on PC was significantly greater than that of all other surfaces

Table 4.
IL-6 concentration (cumulative release) in supernatant from keratinocytes grown on sIPN, PEG hydrogel and TCPS surfaces in the monoculture system

Surface	Exogenous KGF	IL-6 concentration (pg/ml) after			
		2 h	24 h	96 h	168 h
MPEG-sIPN	No	18 ± 16	38 ± 6^{a}	87 ± 13^{a}	132 ± 19^{a}
	Yes	9 ± 4	10 ± 6	4 ± 2	4 ± 2
GGG-sIPN	No	26 ± 3	34 ± 14	98 ± 61	132 ± 61^{a}
	Yes	12 ± 10	10 ± 2	6 ± 2	6 ± 2
RGD-sIPN	No	29 ± 11	28 ± 5^{a}	67 ± 19^{a}	308 ± 232
	Yes	12 ± 4	10 ± 5	11 ± 2	11 ± 5
PHSRN-sIPN	No	25 ± 4^{a}	42 ± 3^{a}	117 ± 36^{a}	230 ± 102
	Yes	9 ± 2	8 ± 6	13 ± 5	36 ± 32
Gelatin-sIPN	No	31 ± 22	56 ± 24^{a}	128 ± 2^{a}	231 ± 151
	Yes	11 ± 1	6 ± 7	23 ± 2	42 ± 14
PEG hydrogel	No	21 ± 9	30 ± 4^{a}	23 ± 15	117 ± 75
	Yes	16 ± 11	15 ± 2	36 ± 10	133 ± 171
TCPS	No	24 ± 7	41 ± 3^{a}	117 ± 32^{a}	279 ± 105
	Yes	32 ± 4^{b}	30 ± 3^{b}	250 ± 31^{b}	403 ± 142^{b}

[a] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.
[b] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

regardless of whether exogenous KGF was present or whether the cells were co-cultured with fibroblasts. At 96 h, the adherent cell density to PC in monoculture without KGF was significantly high *versus* the adherent cell density to PC in co-culture. Although the difference was not statistically significant, the latter trend continued to the 168-h time point. At the 2-h time point, live adherent keratinocyte densities on RGD-sIPN and PHSRN-sIPN surfaces that did not incorporate exogenous KGF were significantly greater than those on sIPNs not conjugated to fibronectin-derived peptides (GGG-sIPN and mPEG-sIPN). The levels of adhesion for RGD- and PHSRN-sIPNs without KGF were significantly high compared to levels for the same respective surfaces with KGF loading at the same time point. PHSRN surfaces loaded without KGF maintained a cell density that was significantly higher than all other sIPNs at 24 h.

3.4. Effect of Surface Type, Exogenous KGF and Fibroblast Presence on Cytokine Concentrations in Keratinocyte–Fibroblast Co-culture

Although the differences were not significant, there was a trend for higher KGF levels with KGF-loaded sIPNs (Table 6). KGF was expressed by fibroblasts grown in monoculture on TCPS. A small amount of KGF was also detected from keratinocyte monoculture on PC, indicative of a possible contamination with fibroblasts. For the

Table 5.
Live adherent cell density of keratinocytes co-cultured with fibroblasts on sIPN, PEG hydrogel and PC surfaces

Surface	Exogenous KGF	Live adherent keratinocyte density (cells/mm^2) after			
		2 h	24 h	96 h	168 h
MPEG-sIPN	No	1 ± 1	0 ± 0	0 ± 1	6 ± 6
	Yes	1 ± 1	0 ± 0	1 ± 1	3 ± 1
GGG-sIPN	No	1 ± 1	5 ± 7	0 ± 0	2 ± 2
	Yes	0 ± 0	0 ± 0	1 ± 1	3 ± 3
RGD-sIPN	No	$5 \pm 2^{a,b}$	4 ± 4	0 ± 0	1 ± 1
	Yes	1 ± 1	1 ± 0	1 ± 1	4 ± 5
PHSRN-sIPN	No	$3 \pm 0^{a,b}$	$16 \pm 6^{a,c}$	10 ± 16	10 ± 4
	Yes	0 ± 0	1 ± 2	1 ± 2	4 ± 5
Gelatin-sIPN	No	0 ± 0	3 ± 1	0 ± 1	6 ± 6
	Yes	1 ± 1	2 ± 1	0 ± 0	0 ± 0
PEG hydrogel	No	12 ± 5^{d}	5 ± 3	5 ± 2	25 ± 14
	Yes	7 ± 8	6 ± 10	6 ± 10	1 ± 1
PC (keratinocyte co-culture)	No	12 ± 7^{d}	11 ± 7	25 ± 17^{e}	204 ± 95^{d}
	Yes	13 ± 8^{f}	15 ± 4^{f}	89 ± 105^{f}	156 ± 94^{f}
PC (keratinocyte monoculture)	No	7 ± 0	33 ± 26^{d}	78 ± 21^{d}	332 ± 184^{d}
	Yes	12 ± 6^{f}	14 ± 6^{f}	82 ± 12^{f}	236 ± 153^{f}

[a] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.
[b] $P < 0.05$ *vs*. sIPN control substrates prepared without exogenous KGF.
[c] $P < 0.05$ *vs*. all other sIPN substrates prepared without exogenous KGF.
[d] $P < 0.05$ *vs*. sIPN substrates prepared without exogenous KGF at the same time point.
[e] $P < 0.05$ *vs*. keratinocyte monoculture without exogenous KGF.
[f] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

most part, KGF levels detected in the (B) chambers were not significantly different from KGF levels in the (A) chambers, regardless of the presence of exogenous KGF.

IL-1α concentrations were low for keratinocytes grown on sIPNs with or without exogenous KGF (Fig. 5). At 168 h, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF had significantly greater IL-1α release compared to keratinocytes grown on KGF-loaded sIPN and PEG hydrogel surfaces. The same phenomenon was observed for keratinocytes grown on TCPS in monoculture with exogenous KGF. Only nominal levels of IL-1β were found (0 ± 0 to 1 ± 0 pg/ml) throughout the course of the co-culture study, similar to those observed under monoculture conditions.

With the exception of PHSRN-sIPN without exogenous KGF, only low levels of IL-6 were detected from keratinocytes grown on sIPNs with and without KGF (Table 7). PHSRN-sIPN without exogenous KGF showed a significant amount of IL-6 release at 24 h compared to other sIPNs without KGF. Although the trend continued

Table 6.
KGF concentration in supernatant from keratinocytes co-cultured with fibroblasts on sIPN, PEG hydrogel and PC surfaces

Surface	Chamber	Exogenous KGF	KGF concentration (pg/ml) after			
			2 h	24 h	96 h	168 h
Co-culture						
mPEG-sIPN	A	No	11 ± 2	78 ± 48	73 ± 35	73 ± 35
	B	No	8 ± 7	46 ± 27	22 ± 8	48 ± 27
	A	Yes	267 ± 208	351 ± 247[a]	194 ± 124	247 ± 132
	B	Yes	41 ± 20	95 ± 22	70 ± 25	122 ± 40
GGG-sIPN	A	No	0 ± 0[b]	30 ± 29	21 ± 12	21 ± 12
	B	No	16 ± 15	49 ± 13	9 ± 6	10 ± 6
	A	Yes	28 ± 5	59 ± 71	23 ± 5	108 ± 93
	B	Yes	13 ± 4	25 ± 2	25 ± 22	44 ± 29
RGD-sIPN	A	No	26 ± 22	53 ± 22	30 ± 13	30 ± 13
	B	No	13 ± 16	66 ± 38	32 ± 10	46 ± 15
	A	Yes	471 ± 189[a]	252 ± 180	271 ± 173	306 ± 177
	B	Yes	40 ± 18	130 ± 45	67 ± 12	73 ± 16
PHSRN-sIPN	A	No	14 ± 18[b]	39 ± 22	50 ± 46	52 ± 46
	B	No	18 ± 17	70 ± 18	33 ± 27	34 ± 27
	A	Yes	256 ± 57	377 ± 101[a]	163 ± 72	261 ± 122
	B	Yes	3 ± 5	57 ± 31	16 ± 13	38 ± 16
Gelatin-sIPN	A	No	23 ± 17	62 ± 31	21 ± 5	21 ± 5
	B	No	18 ± 18	63 ± 42	24 ± 16	24 ± 15
	A	Yes	22 ± 11	58 ± 41	13 ± 7	13 ± 7
	B	Yes	4 ± 6	53 ± 17	5 ± 3	5 ± 3
PEG hydrogel	A	No	40 ± 17	40 ± 28[a]	35 ± 12	65 ± 17
	B	No	60 ± 41[c]	144 ± 65	52 ± 22	113 ± 26
	A	Yes	22 ± 43	60 ± 19	28 ± 10	47 ± 22
	B	Yes	10 ± 2	65 ± 33	204 ± 59	126 ± 119
Polycarbonate	A	No	17 ± 8[b]	53 ± 36[b]	37 ± 19[b]	86 ± 38[a,b]
	B	No	30 ± 3[b]	95 ± 22[b]	76 ± 30[b,d]	152 ± 31[b,d]
	A	Yes	>2000[e]	>2000[e]	>2000[e]	>2000[e]
	B	Yes	>2000[e]	>2000[e]	>2000[e]	>2000[e]

at longer time points, the differences were not significant. At 168 h, significantly higher levels of IL-6 were detected from keratinocytes adhered to PC in co-culture, both with and without KGF, relative to keratinocytes adhered to sIPN surfaces. At 96 h, only PC surfaces in co-culture with exogenous KGF possessed higher concentration of IL-6 relative to KGF-loaded sIPNs. Keratinocytes adhered to PC

Table 6.
(Continued.)

Surface	Chamber	Exogenous KGF	KGF concentration (pg/ml) after			
			2 h	24 h	96 h	168 h
Monoculture						
PC (keratinocytes)	A	No	15 ± 16^{c}	23 ± 25^{c}	37 ± 4^{c}	$128 \pm 54^{a,c}$
	B	No	4 ± 3^{b}	4 ± 7^{b}	9 ± 15^{b}	$21 \pm 26^{b,c}$
	A	Yes	$>2000^{e}$	$>2000^{e}$	$>2000^{e}$	$>2000^{c,e}$
	B	Yes	$>2000^{e}$	$>2000^{e}$	$>2000^{e}$	$>2000^{e}$
TCPS (fibroblasts)	B only	No	21 ± 21^{b}	90 ± 25^{b}	73 ± 38^{b}	178 ± 49^{b}
		Yes	>2000	>2000	1198 ± 205	1439 ± 220

Fibroblasts were grown to confluency on 24-well TCPS plates. Supernatant was collected in the absence of a polycarbonate insert.

[a] $P < 0.05$ *vs*. bottom chamber (B) of same substrate.

[b] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.

[c] $P < 0.05$ *vs*. sIPN substrates prepared without exogenous KGF at the same time point.

[d] $P < 0.05$ *vs*. keratinocyte monoculture without exogenous KGF at the same time point.

[e] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

in monoculture without exogenous KGF released significantly greater amounts of IL-6 relative to sIPNs without KGF at the 168 h time point. At the 2-h time point, IL-6 levels were significantly lower within the top chambers (A) *versus* the bottom chambers (B) for all surfaces. The latter trend was not apparent at later time points. At 168 h, the concentration of IL-6 in the supernatant from fibroblasts cultured without exogenous KGF was significantly higher than from fibroblasts that were exposed to KGF.

Consistent with the observations made during the monoculture study, keratinocytes grown on sIPN and PEG hydrogel surfaces, with and without KGF, showed almost no release of GM-CSF, while keratinocytes grown on PC in monoculture and co-culture showed low levels of GM-CSF release at later time points (Fig. 6). At 168 h, keratinocytes grown on PC in co-culture, regardless of the presence of KGF, had significantly greater GM-CSF release compared to keratinocytes grown on sIPN surfaces.

Keratinocytes grown in co-culture on sIPNs and PEG hydrogels, with and without KGF, showed no detectable TGF-α release (Fig. 7). At 96 and 168 h, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF had significantly greater TGF-α release compared to keratinocytes grown on KGF-loaded sIPN surfaces. Also, at 168 h, keratinocytes grown on PC in monoculture without exogenous KGF had significantly greater TGF-α release compared to keratinocytes grown on sIPNs without KGF. TGF-α release from keratinocyte monoculture on PC in the absence of exogenous KGF at 168 h was significantly greater than that of monoculture in the presence of KGF.

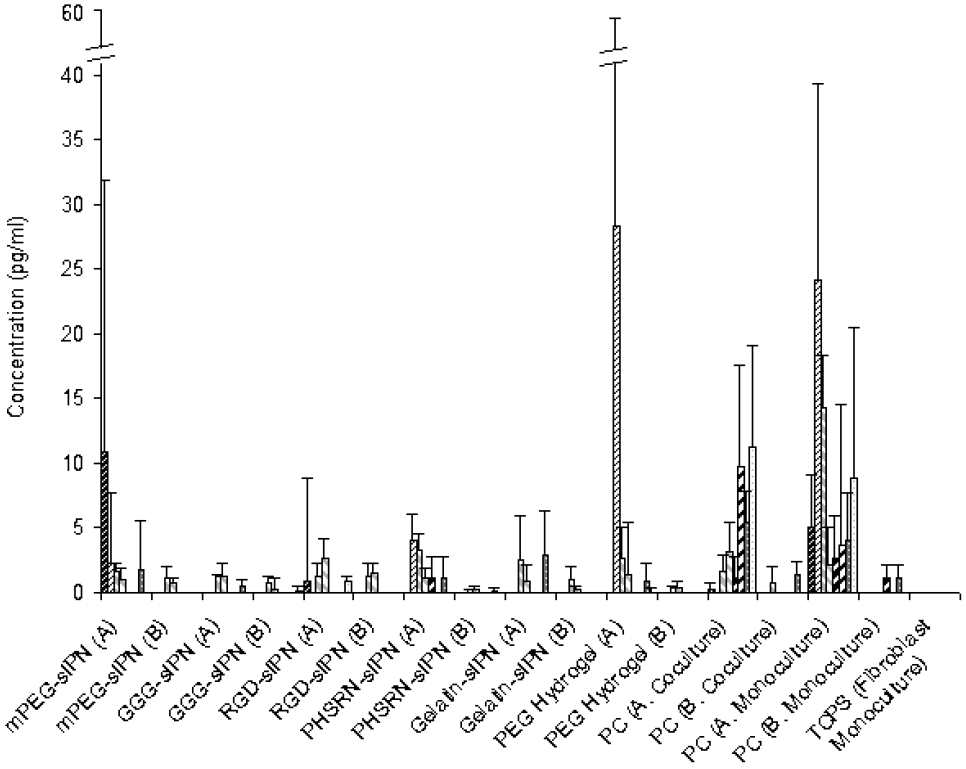

Figure 5. Release of IL-1α from keratinocytes grown in co-culture with fibroblasts on various surfaces. Supernatant was collected and analyzed from the top (A) and bottom (B) chambers of co-culture wells without KGF at 2 h (▨), with KGF at 2 h (▨), without KGF at 24 h (▨), with KGF at 24 h (▨), without KGF at 96 h (▨), with KGF at 96 h (▨), without KGF at 168 h (▨) and with KGF at 168 h (▨). Results are displayed as cumulative release. The error bars represent mean ± SD. Keratinocytes grown on sIPN surfaces showed almost no IL-1α release. At 168 h, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF had significantly greater IL-1α release compared to keratinocytes grown on sIPN surfaces.

4. Discussion

Previous *in vivo* studies have shown sIPNs modified with soluble and covalently-linked bioactive factors accelerate wound healing. In the current study, the re-epithelialization phase was mimicked with a keratinocyte–fibroblast co-culture system. Our belief is that the sIPNs provide points of attachment for keratinocyte migration and alter the cross-talk between the epidermal and dermal cells in a manner that promotes tissue repair. Although incorporation of fibronectin-derived peptides increased keratinocyte adhesion when compared to sIPNs grafted with control peptides, adhesion to sIPN surfaces was significantly low compared to the positive control surfaces of TCPS and PC in monoculture and co-culture systems, respectively (Tables 1 and 5). During a previous study, low adherent keratinocyte densities were observed on sIPNs prepared with unmodified gelatin, regardless of

Table 7.
IL-6 concentration in supernatant from keratinocytes co-cultured with fibroblasts on sIPN, PEG hydrogel and PC surfaces

Surface	Chamber	Exogenous KGF	IL-6 concentration (pg/ml) after			
			2 h	24 h	96 h	168 h
Co-culture						
mPEG-sIPN	A	No	0 ± 0	0 ± 0	0 ± 0	0 ± 2
	B	No	12 ± 13	51 ± 96	1 ± 1	1 ± 4
	A	Yes	0 ± 0^{a}	0 ± 0	161 ± 183	161 ± 183
	B	Yes	8 ± 3	0 ± 0	0 ± 0	0 ± 0
GGG-sIPN	A	No	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	B	No	0 ± 0	1 ± 2	0 ± 0	0 ± 0
	A	Yes	0 ± 0^{a}	0 ± 1	0 ± 0	0 ± 0
	B	Yes	11 ± 3	1 ± 1	0 ± 0	0 ± 0
RGD-sIPN	A	No	0 ± 0	0 ± 1	0 ± 0	0 ± 0
	B	No	0 ± 0	0 ± 0	0 ± 1	0 ± 0
	A	Yes	0 ± 0^{a}	1 ± 1	0 ± 0	0 ± 0
	B	Yes	9 ± 4	1 ± 0	1 ± 1	1 ± 1
PHSRN-sIPN	A	No	0 ± 0	$137 \pm 22^{a,b,c}$	210 ± 456	210 ± 456
	B	No	0 ± 0	$274 \pm 66^{b,c}$	9 ± 12	9 ± 12
	A	Yes	0 ± 0^{a}	0 ± 0	0 ± 0	0 ± 0
	B	Yes	9 ± 3	0 ± 0	0 ± 1	0 ± 1
Gelatin-sIPN	A	No	0 ± 0	0 ± 0	157 ± 331	157 ± 331
	B	No	0 ± 0	0 ± 0	3 ± 1	3 ± 2
	A	Yes	0 ± 0^{a}	3 ± 8	0 ± 0	0 ± 0
	B	Yes	6 ± 1	14 ± 24	0 ± 0	0 ± 0
PEG hydrogel	A	No	$6 \pm 4^{a,d}$	37 ± 16	53 ± 2	191 ± 83
	B	No	92 ± 80	69 ± 26	72 ± 94	262 ± 144^{c}
	A	Yes	$22 \pm 5^{a,e}$	17 ± 23	40 ± 52	58 ± 54
	B	Yes	132 ± 29^{e}	62 ± 33^{e}	31 ± 15	58 ± 36
Polycarbonate	A	No	$12 \pm 4^{a,d}$	102 ± 41	183 ± 127	742 ± 190^{d}
	B	No	143 ± 40^{d}	137 ± 122	453 ± 606	1066 ± 722^{d}
	A	Yes	$28 \pm 14^{a,e}$	82 ± 25	948 ± 746^{e}	1901 ± 930^{e}
	B	Yes	$166 \pm 34^{e,f}$	118 ± 45^{e}	968 ± 530^{e}	1935 ± 779^{e}

whether exogenous KGF was present [34]. The authors purported that PEG within the network minimized protein adsorption and, consequently, cell adhesion [39]. Given that keratinocyte migration occurs by fibronectin binding [27], RGD and PHSRN functionalization of the gelatin within the sIPNs was expected to increase keratinocyte adhesion, particularly in the presence of KGF. Exogenous KGF has

Table 7.
(Continued.)

Surface	Chamber	Exogenous KGF	IL-6 concentration (pg/ml) after			
			2 h	24 h	96 h	168 h
Monoculture						
PC (keratinocytes)	A	No	$7 \pm 1^{a,d}$	20 ± 5^{b}	269 ± 242	835 ± 262^{d}
	B	No	0 ± 1	14 ± 1^{b}	173 ± 30	$793 \pm 164^{b,d}$
	A	Yes	9 ± 4^{a}	9 ± 4	94 ± 64	725 ± 844
	B	Yes	1 ± 1	6 ± 2	112 ± 84	365 ± 165
TCPS (fibroblasts)		No	181 ± 51	640 ± 73	1297 ± 908	3925 ± 1242^{b}
		Yes	147 ± 11	500 ± 99	591 ± 422	1009 ± 427

Fibroblasts were grown to confluency on 24-well TCPS plates. Supernatant was collected in the absence of a polycarbonate insert.

[a] $P < 0.05$ *vs*. bottom chamber of same substrate.

[b] $P < 0.05$ *vs*. all other sIPN substrates prepared without exogenous KGF.

[c] $P < 0.05$ *vs*. same substrate with exogenous KGF at the same time point.

[d] $P < 0.05$ *vs*. sIPN substrates prepared without exogenous KGF at the same time point.

[e] $P < 0.05$ *vs*. sIPN substrates prepared with exogenous KGF at the same time point.

[f] $P < 0.05$ *vs*. keratinocyte monoculture with exogenous KGF (same chamber).

been found to increase cell adhesion to extracellular matrix proteins, including fibronectin, by inducing integrin avidity and a migratory phenotype [4]. The latter changes increase surface exposure of the $\alpha_5\beta_1$ fibronectin receptors, which are often not detectable on terminally differentiated keratinocytes [26]. The changes in integrin topography and phenotype necessary for fibronectin binding may have played a role in the low keratinocyte adhesion levels observed in the current study. However, adhesion did occur to TCPS and PC, indicating that $\alpha_5\beta_1$ receptors were in fact present. Therefore, the density of fibronectin-derived peptides on the sIPN surface may have been too low to offset the effect of PEG within the sIPN that acts to minimize cell adhesion.

At early time points in co-culture, keratinocyte adhesion to KGF-loaded RGD-sIPN and PHSRN-sIPN was significantly less compared to the same surfaces without KGF; however, over the course of monoculture and co-culture experiments, a relationship between keratinocyte adhesion and incorporation of exogenous KGF into the sIPNs could not be established (Tables 1 and 5). The influence of KGF was difficult to ascertain due to the low values for cell adhesion to sIPNs. Keratinocyte adhesion to PC surfaces was independent of the incorporation of KGF, but slightly dependent upon the presence of fibroblasts. At later time points, the adherent cell density to PC in monoculture without KGF was significantly higher than the adherent cell density to PC in co-culture. Despite production of KGF (Table 6), fibroblasts down-regulated cell adhesion to the PC surfaces.

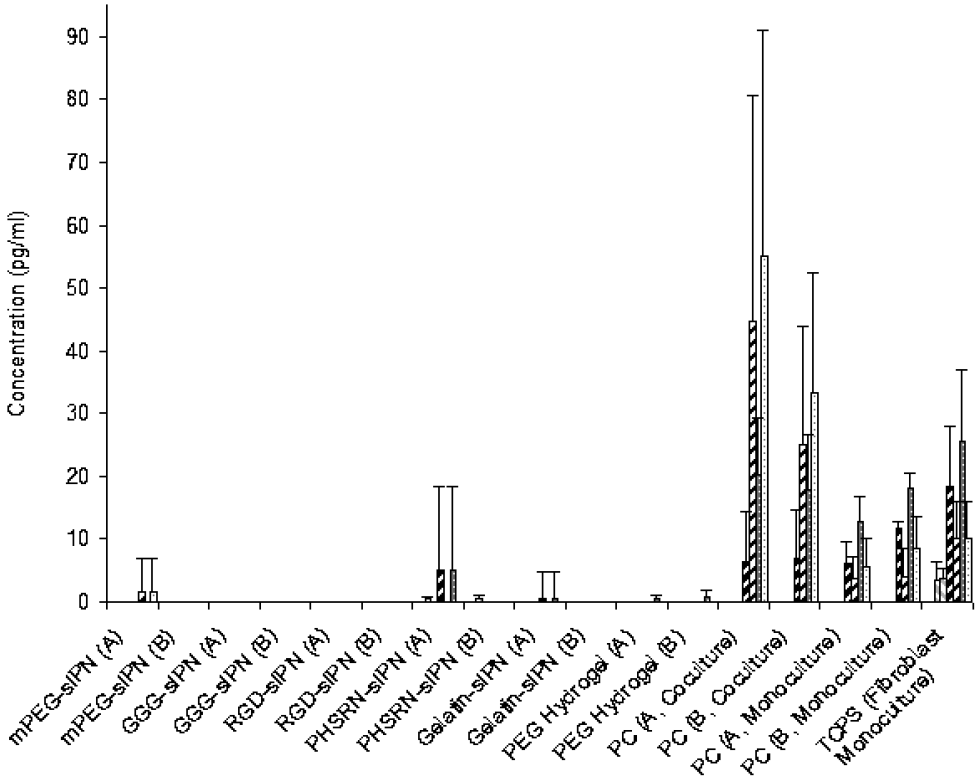

Figure 6. Release of GM-CSF from keratinocytes grown in co-culture with fibroblasts on various surfaces. Supernatant was collected and analyzed from the top (A) and bottom (B) chambers of co-culture wells without KGF at 2 h (▨), with KGF at 2 h (▨), without KGF at 24 h (▨), with KGF at 24 h (▨), without KGF at 96 h (▨), with KGF at 96 h (▨), without KGF at 168 h (▨) and with KGF at 168 h (▨). Results are displayed as cumulative release. The error bars represent mean ± SD. Keratinocytes grown on sIPN surfaces showed almost no IL-1α release. Keratinocytes grown on sIPN surfaces showed almost no release of GM-CSF. At 168 h, keratinocytes grown on PC in co-culture with and without exogenous KGF had significantly greater GM-CSF release compared to keratinocytes grown on sIPN surfaces.

Generally, KGF release from keratinocytes grown in monoculture and co-culture on surfaces that incorporated exogenous KGF was greater than from keratinocytes grown without supplementation (Tables 2 and 6), thereby confirming the loading and release of exogenous KGF from sIPNs and PEG hydrogels. During co-culture, KGF levels detected in the top (A) and bottom (B) chambers were comparable, suggesting that KGF is free to diffuse through the sIPNs, PEG hydrogels and PC membranes. KGF was expressed by fibroblasts grown in monoculture on TCPS; therefore, the KGF detected when cells were co-cultured on sIPNs without exogenous KGF was produced by the underlying fibroblasts. KGF levels in supernatant obtained from monoculture wells containing KGF-loaded gelatin-sIPNs were low relative to other KGF-loaded sIPNs. Additional studies revealed that KGF is released by gelatin-sIPNs when keratinocytes are not present; therefore, the adhered

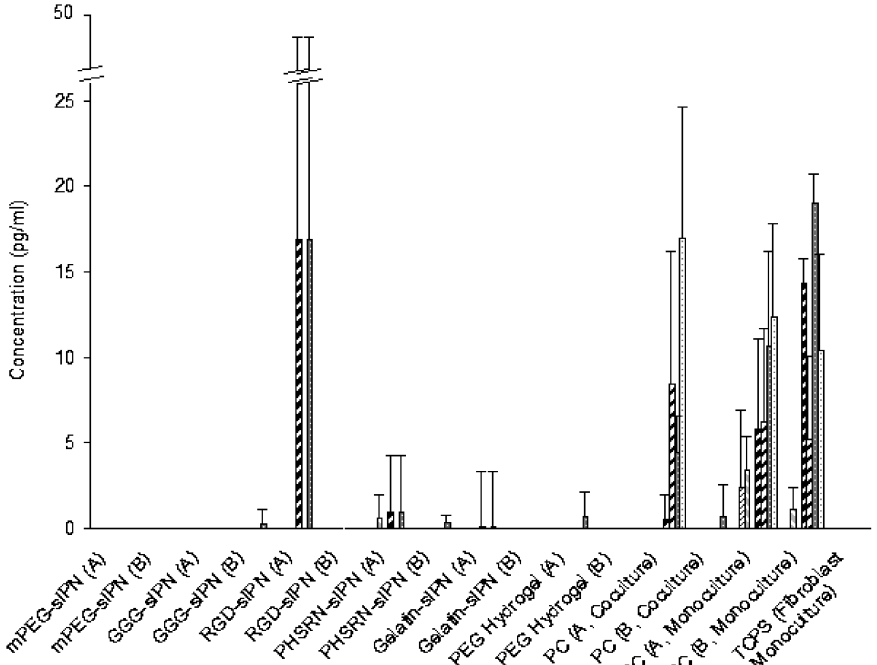

Figure 7. Release of TGF-α from keratinocytes grown in co-culture with fibroblasts on various surfaces. Supernatant was collected and analyzed from the top (A) and bottom (B) chambers of co-culture wells without KGF at 2 h (▨), with KGF at 2 h (▨), without KGF at 24 h (▨), with KGF at 24 h (▨), without KGF at 96 h (▨), with KGF at 96 h (▨), without KGF at 168 h (▨) and with KGF at 168 h (▨). Results are displayed as cumulative release. The error bars represent mean ± SD. Keratinocytes grown on sIPN surfaces showed almost no release of TGF-α. At 96 and 168 h, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF had significantly greater TGF-α release compared to keratinocytes grown on sIPN surfaces. At 168 h, keratinocytes grown on PC in monoculture without exogenous KGF also had significantly greater TGF-α release compared to keratinocytes grown on sIPNs. Monoculture release in the absence of exogenous KGF at 168 h was significantly greater than monoculture in the presence of KGF.

cells were taking up the exogenous KGF. As indicated by significantly greater amounts of IL-1α released by KGF-loaded gelatin-sIPNs at 168 h compared to all other KGF-loaded sIPNs (Table 3), the uptake of KGF may have induced IL-1α expression (Fig. 1). Although keratinocytes do not possess the $\alpha_v\beta_3$ integrin to allow binding to denatured collagen, keratinocyte adhesion to gelatin can occur through fibronectin, which binds to gelatin with a high affinity [40]. Fibronectin bound to gelatin may induce a different cellular response than fibronectin-derived peptides grafted onto gelatin

At later time points within the monoculture system, keratinocytes adhered to KGF-loaded RGD-sIPN, PHSRN-sIPN and TCPS released higher levels of IL-1α *versus* the same surface without KGF (Table 3). Therefore, keratinocytes adhered to RGD-sIPN, PHSRN-sIPN, gelatin-sIPN and TCPS surfaces all appeared to uptake some KGF, which then induced expression of IL-1α. In the co-culture system,

IL-1α concentrations were low for keratinocytes grown on sIPNs, including sIPNs grafted with fibronectin-derived peptides, with or without exogenous KGF (Fig. 5). Potentially, the released IL-1α is being taken up by the fibroblasts (Fig. 1). At 168 h, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF had significantly greater IL-1α release compared to keratinocytes grown on KGF-loaded sIPN and PEG hydrogel surfaces. The same phenomenon was observed for keratinocytes grown on TCPS in monoculture with exogenous KGF. The higher IL-1α levels observed for the positive control surfaces may be a result of the significantly higher KGF concentrations observed when exogenous KGF was added directly to the media *versus* when KGF was incorporated into the sIPNs (Tables 2 and 5). IL-1β levels were expected to follow a trend similar to that seen for IL-1α; however, only nominal levels of IL-1β were found throughout the course of the monoculture and co-culture studies. Keratinocytes lack the proteolytic enzyme necessary to convert pro-IL-1β to active IL-1β; therefore, IL-1β may have been released, but not in an active, detectable form [41, 42]. For TCPS, PC, gelatin-sIPN and sIPNs containing fibronectin-derived peptides, KGF stimulated the release of IL-1α, as would be expected based upon the known autocrine and paracrine regulators of keratinocyte adhesion, migration and proliferation.

For the most part, in keratinocyte monoculture, GM-CSF was not detectable for any of the surfaces with or without exogenous KGF. However, low levels could be detected from TCPS with exogenous KGF at later time points. KGF and IL-1α induce the expression of GM-CSF (Fig. 1); therefore, detectable levels of GM-CSF were anticipated with higher levels of KGF and IL-1α, as were seen from keratinocytes grown on TCPS with exogenous KGF. Consistent with the observations made during the monoculture study, keratinocytes grown on sIPN and PEG hydrogel surfaces, with and without KGF, showed almost no release of GM-CSF, while keratinocytes grown on PC in monoculture and co-culture showed low levels of GM-CSF release at later time points (Fig. 6). At 168 h, keratinocytes grown on PC in co-culture, regardless of the presence of KGF, had significantly greater GM-CSF release compared to keratinocytes grown on sIPN surfaces. KGF, either exogenously added or produced by underlying fibroblasts, induced the production of IL-1α in keratinocytes adhered to PC, which in turn stimulated production of GM-CSF by both fibroblasts and keratinocytes (Fig. 1).

Despite literature claims that KGF stimulates the release of TGF-α by keratinocytes, in monoculture, TGF-α was not detectable at any time points for sIPN, PEG hydrogel and TCPS surfaces, regardless of the presence of exogenous KGF. Similarly, keratinocytes grown in co-culture on sIPNs and PEG hydrogels, with and without KGF, showed no detectable TGF-α release (Fig. 7). However, keratinocytes grown on PC in co-culture and monoculture in the presence of exogenous KGF displayed some TGF-α release at later time points. Although the latter result may in part have been due to the higher KGF concentrations observed when exogenous KGF was added directly to the media *versus* when KGF was incorporated into the

sIPNs, KGF did not have the same influence over cells adhered to TCPS, indicating that TGF-α release is highly surface dependent. In support of this notion, keratinocytes grown on PC in monoculture without exogenous KGF also showed a significant amount of TGF-α release at later time points. Although TGF-α concentrations were expected to increase with the addition of exogenous KGF (Fig. 1), TGF-α release from keratinocyte monoculture on PC in the absence of exogenous KGF was greater than that of monoculture in the presence of KGF.

As expected, keratinocytes grown in monoculture on TCPS surfaces released higher levels of IL-6 when KGF was added to the media relative to KGF free media (Table 4). However, the opposite occurrence was observed for sIPN surfaces. IL-6 concentrations were higher when keratinocytes were cultured on sIPN surfaces without exogenous KGF *versus* those with exogenous KGF. In co-culture, with the exception of PHSRN-sIPN without exogenous KGF, only low levels of IL-6 were detected from keratinocytes grown on sIPNs with and without KGF (Table 7). Potentially, as was the case for IL-1α, the underlying fibroblasts took up the released IL-6. PHSRN-sIPN without exogenous KGF showed a larger amount of IL-6 release compared to other sIPNs without KGF. The response from keratinocytes adhered to PHSRN-sIPN may be correlated to the greater number of live cells adhered to the surface relative to the other sIPNs at early time points. At later time points, higher levels of IL-6 were detected from keratinocytes adhered to PC in co-culture, both with and without KGF, relative to keratinocytes adhered to sIPN surfaces. Keratinocytes adhered to PC in monoculture without exogenous KGF also released significantly greater amounts of IL-6 relative to sIPNs without KGF. At the 2 h time point, IL-6 levels were significantly lower within the top chambers (A) *versus* the bottom chambers (B) for all surfaces, implying that the detectable IL-6 in co-culture is initially released by fibroblasts. At 168 h, the concentration of IL-6 in the supernatant from fibroblasts cultured without exogenous KGF was higher than from fibroblasts that were exposed to KGF. The result is unexpected given that fibroblasts lack the receptor necessary to uptake KGF. A correlation of IL-6 levels with inclusion of exogenous KGF, the presence of fibroblasts, and the surface type could not be established.

In conclusion, the keratinocyte–fibroblast autocrine/paracrine relationship was evaluated in the presence of sIPNs grafted with peptides that mimic those of the extracellular matrix to which keratinocytes adhere during wound closure. The sIPNs were further utilized as vehicles for delivery of exogenous KGF, which promotes keratinocyte proliferation and plays a significant role in the interplay between the dermis and epidermis. As summarized in Fig. 8, exogenous KGF induced the production of IL-1α in both keratinocyte monoculture and co-culture, which in turn stimulated production of GM-CSF. The effects were more noticeable on PC and TCPS surfaces relative to sIPN and PEG hydrogel surfaces. Regulation of the release of TGF-α was surface dependent. TGF-α was not detectable for TCPS, PEG hydrogel and sIPN surfaces; however, TGF-α release was detectable at later time points for keratinocyte monoculture and co-culture on PC. IL-6 re-

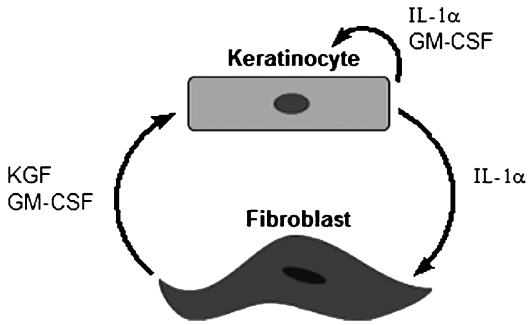

Figure 8. Schematic illustration of the method by which surfaces containing exogenous KGF promote wound healing. KGF taken up by keratinocytes promotes the release of IL-1α, which in turn stimulates the release of GM-CSF by both fibroblasts and keratinocytes.

lease was dependent upon the surface type, the inclusion of exogenous KGF and the presence of fibroblasts. In monoculture, keratinocytes on TCPS released higher levels of IL-6 in the presence of exogenous KGF, while keratinocytes on sIPNs released higher levels of IL-6 when not loaded with KGF. In co-culture, IL-6 was not detectable for keratinocytes adhered to sIPN surfaces, regardless of the presence of KGF. Keratinocytes adhered to PC released significant levels of IL-6 at later time points in both monoculture and co-culture conditions without exogenous KGF. For PC surfaces with KGF, significant levels of IL-6 were only observed for keratinocytes grown in co-culture with fibroblasts. Therefore, the primary role of sIPNs containing soluble bioactive factors in re-epithelialization, as mimicked herein by keratinocyte–fibroblast co-culture, is to provide a conduit for exogenous KGF, which induces the expression of other cytokines associated with keratinocyte response during wound healing (Fig. 8). The latter result in part explains the accelerated tissue repair previously observed *in vivo*.

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