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A Novel Role of Fibrin in Epidermal Healing: Plasminogen-Mediated Migration and Selective Detachment of Differentiated Keratinocytes

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Recent studies have shown that fibrin promotes epidermal regeneration *in vitro* and maintains the stem cell population after transplantation of keratinocytes *in vivo*. As epidermal keratinocytes do not express integrin αvβ3, the receptor for fibrin and fibrinogen, the mechanism through which fibrin affects epidermal cells remains elusive. To investigate the role of fibrin in epidermal wound healing, we developed an *in vitro* model in which fibrin was added to the top of wounded keratinocyte monolayers grown on collagen. With this matrix topology, keratinocytes migrate between the collagen on their basal side and fibrin on their apical side mimicking migration of the epidermis *in vivo*. Using this model, we found that fibrin promoted keratinocyte migration in low and high calcium concentrations by exposing the cells to plasminogen. The migration rate depended strongly on the concentration of fibrinogen and the rate of plasmin-mediated fibrin degradation. Surprisingly, fibrin and fibrinogen caused significant detachment of keratinocytes which was prevented by the addition of calcium. Further examination using flow cytometry revealed that the detached cells were larger, more granular, and had very low levels of β1 integrin, which are all signs of differentiated keratinocytes. Our results suggest a novel dual role of fibrin in epidermal healing. First, fibrin promotes keratinocyte migration indirectly by exposing plasminogen to migrating cells, and second, fibrin selectively disrupts adhesion of differentiated keratinocytes. Our data are novel and may have important implications in understanding wound healing and in the use of fibrin as a biomaterial for protein and gene delivery. ***Key words: fibrin/fibrongin/biomaterials/wound healing/cell migration/calcium/plasmin. J Invest Dermatol 121:1210–1216, 2003***

Natural biomaterials, such as extracellular matrix molecules, act as substrates for cell attachment and migration thus guiding tissue regeneration and promoting wound healing. A common material that immediately forms at the site of skin injury is fibrin, an insoluble matrix used initially by the body to prevent blood loss. It forms when thrombin initiates noncovalent polymerization by enzymatically cleaving fibrinopeptides A and B on fibrinogen. Later, activated transglutaminases covalently cross-link the matrix in a calcium-dependent reaction (Mosesson *et al*, 2001). Approximately 18 to 24 h after injury, keratinocytes migrate from the wound edge, carefully dissecting the fibrin clot and the collagen-rich granulation tissue to eventually re-establish the epithelial barrier (Clark, 1997; Kubo *et al*, 2001). In time, complete re-epithelialization occurs and the fibrin clot (i.e., scab) is degraded and expunged from the external surface of the wound.

Fibrin provides a temporary scaffold that quickly directs cells to the site of injury, beginning the process of new blood vessel formation and creation of a viable dermal compartment (Clark, 2001). As such, fibrin has been used in multiple medical applications, including Achilles tendon repair, remodeling of bone grafts, skin grafting after burn or injury, repair of peripheral nerves, and induction of angiogenesis (Dvorak *et al*, 1987; Silver *et al*, 1995; Jackson, 2001). In addition, fibrin has been used for the delivery of growth factors and genes to promote wound healing. Examples include fibroblast growth factor-1 (Guest *et al*, 1997; Shireman *et al*, 1999; Pandit *et al*, 2000), neurotrophic factors (Cheng *et al*, 1995; Iwaya *et al*, 1999), and epidermal growth factor-encoding DNA (Andree *et al*, 2001). More recently, controlled delivery of growth factors using fibrin gels has been demonstrated with the development of techniques for incorporating peptides that effectively link heparin-binding proteins into the fibrin matrix during polymerization (Schense *et al*, 2000; Sakiyama-Elbert *et al*, 2001).

Although many cell types, including fibroblasts, endothelial, and smooth muscle cells bind to fibrin, the interactions of epidermal keratinocytes with fibrin remain unclear. Earlier studies demonstrated that chronic wound fluid containing high concentrations of fibrinogen was highly toxic to keratinocyte cultures (Shakespeare and Shakespeare, 1991). In addition, keratinocytes can only attach to fibrin gels that contain fibronectin as they lack integrin αvβ3 (Weiss *et al*, 1998; Kubo *et al*, 2001). When genetically modified to express αvβ3, keratinocytes were able to bind fibrin and fibrinogen suggesting that in their natural state epidermal cells may not interact with the fibrin clot (Kubo *et al*, 2001).

Despite the lack of receptors for fibrin, recent studies demonstrated beneficial effects of fibrin in keratinocyte migration and epidermal healing. Keratinocytes suspended in fibrin gels penetrated

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the gel by three-dimensional helical movements, albeit they remained rounded (Ronfard and Barrandon, 2001). Also, fibrin gels accelerated healing in a three-dimensional *in vitro* model of wound healing based on tissue engineered skin equivalents (Geer *et al*, 2002). Finally, *in vivo* transplantation of keratinocytes suspended in fibrin gels improved the "take" rate of keratinocyte grafts and maintained the stem cell population of the epidermis (Horch *et al*, 1998; Pellegrini *et al*, 1999). As keratinocytes do not bind to fibrin, the role that fibrin may play in epidermal healing remains unclear.

In this paper, we devised a model system to study the effects of fibrin on keratinocyte migration by sandwiching wounded cell monolayers between collagen and fibrin. The model was designed to mimic the environment keratinocytes encounter during *in vivo* wound healing, where they migrate by dissecting between the collagen of the dermis and the fibrin clot in the wound space (Clark, 1997). Using this novel system we found that the rate of keratinocyte migration depended on the concentration of fibrinogen in a biphasic manner. Physiologic concentrations of fibrinogen in the fibrin gels accelerated cell migration but higher concentrations severely inhibited wound healing. Further studies revealed that plasminogen activation was necessary for fibrin degradation and accelerated healing. Surprisingly, fibrinogen and fibrin caused significant detachment of differentiated, low β1 integrin expressing keratinocytes in a time- and concentration-dependent fashion. Addition of calcium prevented detachment and caused strengthening of cell–cell interactions as indicated by epithelial sheet migration *versus* individual cell migration observed in low calcium conditions. Our data uncovered previously unsuspected effects of fibrin on keratinocyte adhesion and migration, which may have important implications in understanding wound healing and in the use of fibrin as biomaterial in tissue engineering and drug delivery. The Institutional Review Board reviewed and unanimously approved the study.

MATERIALS AND METHODS

Cell culture Human keratinocytes were isolated from neonatal foreskins with dispase (Gibco, Gaithersburg, Maryland) following the manufacturer's recommendations. The cells were propagated on feeder layers of 3T3-J2 mouse fibroblasts (ATCC, Manassas, Virginia) as described previously (Andreadis *et al*, 2001). Keratinocytes used in experiments were maintained within one to four passages.

For wounding and detachment experiments, keratinocytes were subcultured on rat tail collagen (20 μg per mL; Biomedical Technologies Inc., Stoughton, Massachusetts) in keratinocyte serum-free medium (Gibco) supplemented with epidermal growth factor (5 ng per mL) and bovine pituitary extract (SFM).

Wounding of keratinocyte monolayers and fibrin gel preparation Keratinocytes were seeded in collagen-coated 24-well plates (10^5 cells per well) and grown to confluence. Two days later, the monolayers were scratch wounded with a plastic pipette tip creating a wound approximately 1.4 mm in width. Immediately after wounding, monolayers were washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (Gibco) to remove cell remnants and either fibrin gels (300 μL) or medium (500 μL) were applied completely covering the wound and monolayer. The fibrin gelled in 20 to 40 s and the plates were returned to the incubator. Approximately 20 min after gelation, fresh medium (200 μL) was added to the top of each polymerized gel.

Fibrin gels were prepared by mixing equal volumes (175 μL) of two solutions: one containing 2 to 20 mg per mL of fibrinogen (Sigma, St Louis, Missouri) in PBS and the other containing 5 NIH U per mL of thrombin (Sigma) in serum-free medium. Fibrinogen was pooled from human plasma and contained trace amounts of factor XIII, fibronectin, vitronectin, Von Willebrand factor, and plasminogen (Sigma). Plasminogen-free fibrinogen (Sigma) was also used in some experiments as noted. For some experiments, gels contained 1 to 100 KIU per mL of aprotinin, a plasmin inhibitor (Sigma) or 1 to 2 mM calcium chloride (Sigma). In addition, some wounds were treated with medium that contained the active plasmin enzyme (0.1 U per mL, Sigma).

Analysis of wound healing Images of the wounds at various times postwounding were acquired at × 4 magnification on an inverted microscope (Diaphot-TMD, Nikon Corporation) using a Retiga 1300 digital camera and QCapture2 software, version 1.1 (Quantitative Imaging Corporation, Burnaby, British Columbia, Canada). Digital images were analyzed using public domain ImageJ 1.28 k software (National Institutes of Health Bethesda, MD). The percent healing or healing index represents the number of cells that enter the initial wound area divided by the number of cells in that same area after complete healing. The initial rate of healing was calculated from the initial slope (from 0 to 24 h) of the healing curves and reported as percent healing per hour.

Quantitation of keratinocyte detachment with fibrinogen treatment Keratinocytes were seeded in 96-well plates (5×10^4 cells per well) on collagen and grown to confluence. At that time, serum-free medium containing 1 to 10 mg fibrinogen per mL with or without 2 mM of calcium chloride was used to treat the cells. The number of cells remaining in each well after treatment was measured using the FluoReporter Blue Fluorometric double-stranded DNA Quantitation Kit (Molecular Probes, Eugene, Oregon) as per the manufacturer's protocol. Briefly, the culture medium was aspirated from the wells containing the cells and the plates were frozen at −80°C. At the time of the assay, the plates were thawed, de-ionized H_2O was added (100 μL per well), and the plates were incubated at 37°C for 1 h. Then the plates were frozen at −80°C and thawed at 37°C to cause rapid cell lysis before the addition of Hoechst 33258 in TNE (10 mM Tris, 2M Nacl, 1 mM EDTA) buffer (100 μL per well). Wells with 100 μL of de-ionized H_2O served as blanks. The fluorescence intensity was measured (Ex: 360 nm, Em: 460 nm) with a fluorescence microplate reader (SpectraMax Gemini, Molecular Devices, Menlo Park, California) and the fluorescence intensity of blank wells was subtracted as background. The fluorescence intensity of each sample was normalized to that of the untreated control at the same time point.

Flow cytometry Keratinocytes were seeded in 25 cm^2 culture flasks (1.25×10^6 cells per flask) on collagen and grown to confluence. At that time, monolayers were treated for 3 h with 2.5 mg per mL of fibrinogen in SFM. The supernatant containing the detached cells was removed and the remaining cells were washed once with versene (1:5000; Gibco). The remaining cell population was detached from the substrate with trypsin–ethylenediamine tetraacetic acid (3 mL). The trypsin was inactivated with culture medium containing 10% fetal bovine serum, cells were then centrifuged (5 min; $700 \times g$) and washed with cold PBS. Detached, remaining, and untreated cell populations were incubated (30 min, 4°C) in PBS containing a primary antibody against β1 integrin (10 μg per mL; Clone TS2/16, isolated from hybridoma cells, strain HB-243, ATCC). Cells were washed once with PBS and incubated in 100 μL of the secondary antibody (10 μg per mL; Alexa Fluor 488 highly cross-adsorbed goat anti-mouse IgG) in PBS for 1 h at room temperature. Following two washes with PBS, the samples were assayed using flow cytometry.

Statistical analysis Statistical analysis of the data was performed using a two-tailed Student's t-test ($\alpha = 0.05$).

RESULTS

Biphasic behavior of keratinocyte migration in fibrin-treated wounds It has been proposed that during wound healing, keratinocytes of the epidermis migrate by dissecting between the collagen matrix at the basal side and fibrin clot at the apical side (Clark *et al*, 1982). To study the role of fibrin in keratinocyte migration we developed a two-dimensional model of wound healing that mimics the *in vivo* topology. Keratinocytes were plated on collagen-coated 24-well plates at high cell density (10^5 cells per well) and formed confluent monolayers. At 2 d postconfluence, the monolayers were wounded (**Fig 1*A***) and overlaid with solutions (300 μL per well) containing different concentrations of fibrinogen (1, 2.5, 5, and 10 mg per mL) and thrombin (2.5 U per mL), which gelled quickly (20–40 s) after application to the cell monolayer. In this system, the cells interact with collagen through the basal side and with fibrin through the apical side.

Application of fibrin gels on the wounded monolayers enhanced the rate of healing significantly. Fibrin-treated samples healed by 72 h after wounding (**Fig 1*B***), whereas untreated

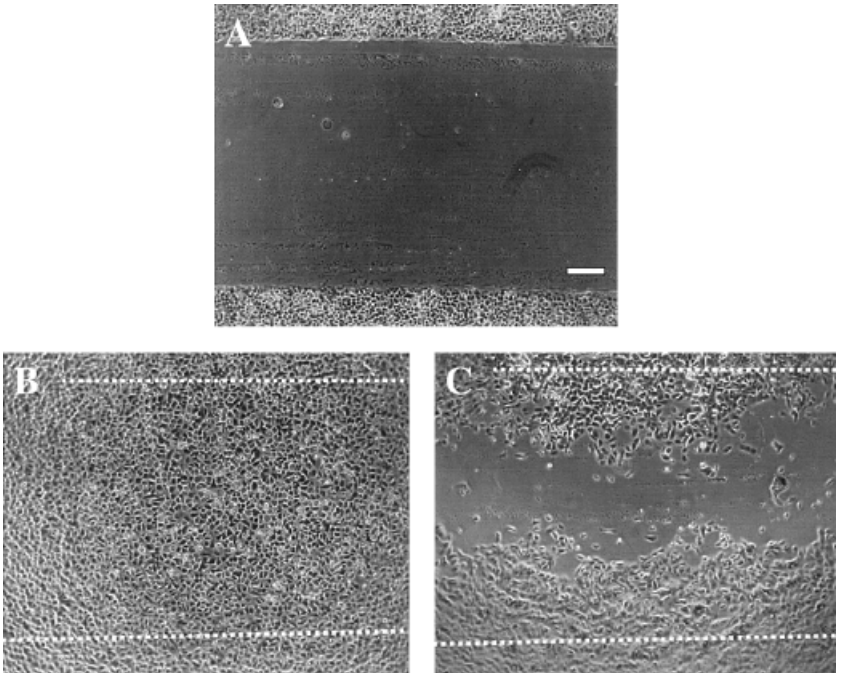

Figure 1. Fibrin promotes epidermal wound healing. Keratinocytes were grown on collagen-coated plates to confluence and wounded 2 d later. The wounds were approximately 1.4 mm wide (*A*). At 72 h, fibrin-treated wounds (*B*) healed completely, whereas untreated (*C*) showed only ≈50% closure. *Scale bar* = 200 μm. Original magnification × 4.

monolayers healed by only ≈50% in the same time (**Fig 1*C***). In addition, the rate of healing depended strongly on the concentration of fibrinogen in the fibrin gels. Monolayers treated with physiologic concentrations of fibrinogen (1 and 2.5 mg per mL) healed ≈2.3 times faster than untreated controls or 3.32 ± 0.36% of wound coverage per hour. Surprisingly, increasing the concentration of fibrinogen decreased the rate of healing significantly. Specifically, 5 mg fibrinogen per mL decreased the healing rate of fibrin-treated samples to the levels of untreated monolayers and 10 mg fibrinogen per mL inhibited healing completely (**Fig 2**).

In addition to the rate of wound healing the overlaying fibrin gels affected the mode of keratinocyte migration. In fibrin-treated samples, keratinocytes dissociated from the wound edges and migrated as individual cells (**Fig 3*A***). In contrast, cells in untreated monolayers dissociated from their neighbors to a lesser extent and migrated by colony expansion from the wound edge (**Fig 3*B***, asterisks). Interestingly, the original wound edges were visible in untreated controls (**Fig 3*B***, *arrows*) but disappeared in fibrin-treated monolayers, suggesting that fibrin may be aiding the proteolytic breakdown of accumulated cell debris and extracellular matrix at the wound margin.

Fibrinolysis is required for epidermal healing As keratinocytes migrated to heal the wound they also degraded the fibrin gels. Complete fibrinolysis occurred in 12 to 24 h when gels contained low concentrations of fibrinogen and 48 h for higher fibrinogen concentrations. To examine if degradation of the fibrin gels was necessary for keratinocyte migration we used

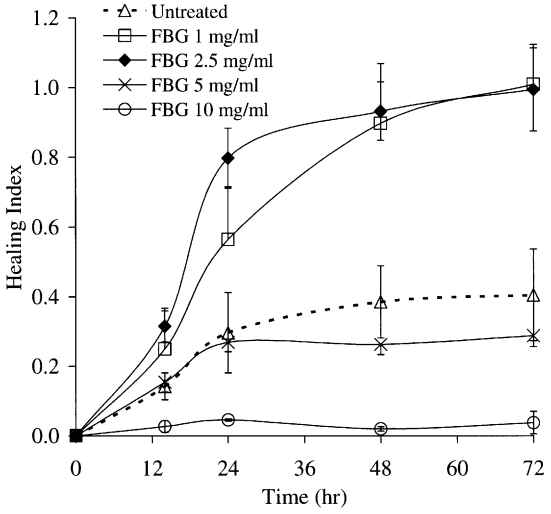

Figure 2. Keratinocyte migration depends on the concentration of fibrinogen in the fibrin gels in a biphasic way. Keratinocytes were grown on collagen-coated plates to confluence and wounded 2 d later. The rate of healing was determined by counting the number of cells into the wound area at various times postwounding for 0, 1, 2.5, 5, and 10 mg fibrinogen per mL. Differences between 0, 2.5, and 10 mg fibrinogen per mL were statistically significant ($p < 0.05$). All values are the mean ± SD of quadruplicate samples in a representative experiment. The results are representative of at least three independent experiments ($n = 3$). FBG, fibrinogen.

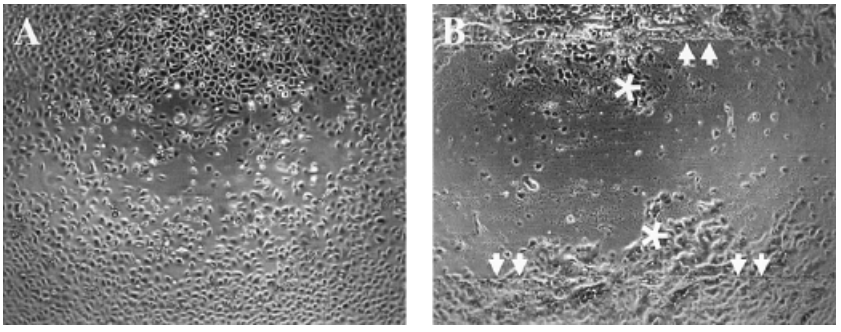

Figure 3. Fibrin-treated monolayers exhibit different locomotive behavior. At 48 h postwounding, cells in fibrin-treated monolayers (*A*) dissociate from the wound edge and migrate as single cells to heal the wound. In contrast untreated monolayers (*B*) migrate by colony expansion from the wound edges. *Arrows* denote original wound edges and asterisks denote extending colonies (original magnification × 4).

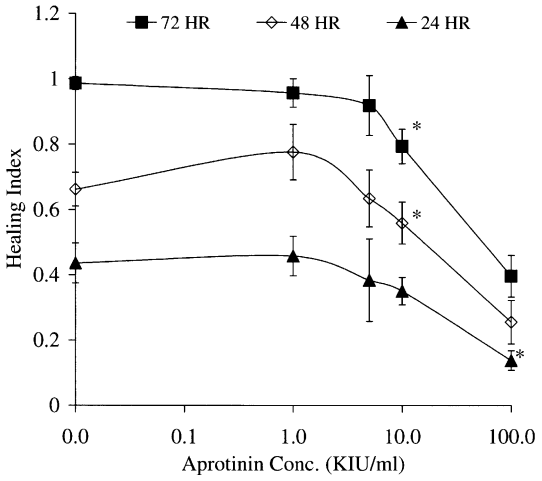

Figure 4. Fibrinolysis is required for keratinocyte migration. Keratinocytes were grown on collagen-coated plates to confluence and wounded 2 d later. The rate of healing was determined by counting the number of cells in the area of the wound at various times postwounding in the presence of aprotinin at 0, 1, 5, 10, and 100 KIU per mL. Values are the mean ± SD for quadruplicate samples in a representative experiment (n = 3). The asterisk (*) denotes a significant change ($p < 0.05$) in the healing response of aprotinin-treated samples as compared with controls (no aprotinin) for the same time point.

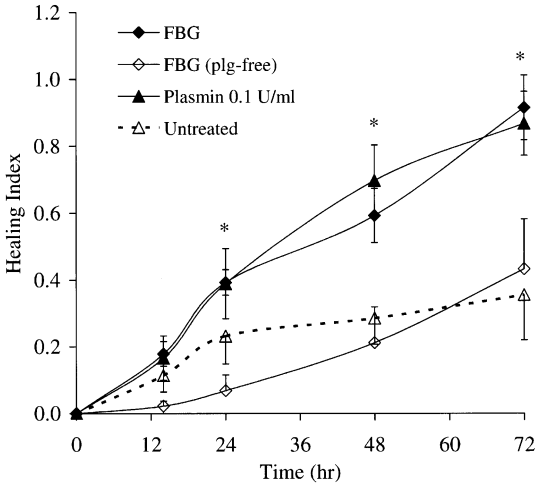

Figure 5. Plasminogen conversion to plasmin is required for wound healing. Keratinocytes were grown on collagen-coated plates to confluence and wounded 2 d later. Monolayers were then either treated with fibrin containing plasminogen-free fibrinogen (2.5 mg per mL) or plasmin (0.1 U per mL) and kinetics were recorded by counting the number of cells in the wound space. Untreated samples and fibrin containing fibrinogen (2.5 mg per mL) were used as negative and positive controls, respectively. Values are the mean ± SD of quadruplicate samples in a representative experiment (n = 3). The asterisk (*) denotes a significant ($p < 0.05$) difference between fibrinogen- and plasmin-treated wounds *versus* untreated and plasminogen-free fibrinogen samples for the same time point. FBG, fibrinogen.

aprotinin, a plasmin inhibitor that prevents fibrinolysis. Aprotinin decreased wound healing of fibrin-treated samples in a concentration-dependent way (**Fig 4**) but did not affect migration when added in the medium of untreated controls (data not shown). In addition, aprotinin prevented degradation of the matrix and cell debris at the wound edge (data not shown; similar to **Fig 3*B***) suggesting that fibrin gels may promote healing by accelerating the proteolytic degradation of extracellular matrix.

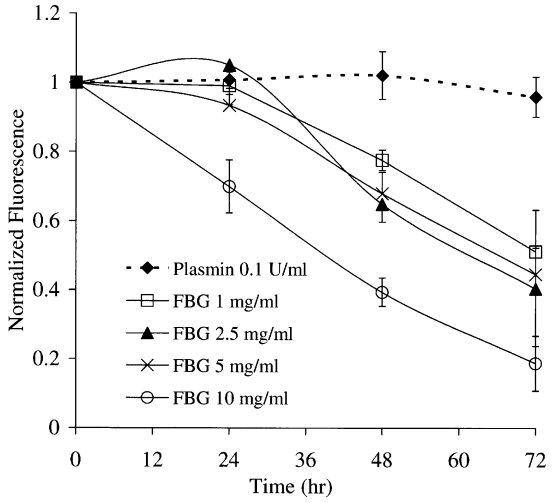

Figure 6. Fibrin(ogen) (FBG) detaches keratinocytes in a concentration-dependent and time-dependent way. Keratinocytes were grown on collagen-coated 96-well plates to confluence and treated with 1, 2.5, 5, or 10 mg per mL of fibrinogen or 0.1 U per mL of plasmin for 24, 48, and 72 h. At the indicated times, PBS was used to wash away detached cells. The remaining cells were lyzed and total DNA was measured with Hoechst 33258. Total fluorescence was measured using a fluorescence microplate plate reader. At each time point the fluorescence intensity was normalized to that of untreated cells. Values are the mean ± SD of quadruplicate samples in a representative experiment (n = 3). FBG, fibrogen.

Plasmin accelerates keratinocyte migration To determine the role of plasminogen activation in accelerating keratinocyte migration we prepared fibrin gels with plasminogen-free fibrinogen at an optimum concentration of 2.5 mg per mL. In contrast to regular fibrin preparations, plasminogen-free fibrin gels did not promote healing (**Fig 5**) indicating a strong dependence of migration on the activation of plasminogen to plasmin. Indeed, addition of plasmin (0.1 U per mL) to the medium of untreated monolayers increased the rate of healing to the same extent as fibrin. These results suggest that fibrin gels promote keratinocyte migration through the conversion of plasminogen into plasmin.

Fibrin(ogen) causes cell detachment Throughout our experiments we observed that treatment of confluent cell monolayers with fibrinogen or plasminogen-free fibrinogen caused noticeable rounding and detachment of cells, a phenomenon not seen with untreated or plasmin-treated samples. **Figure 6** shows that treatment with fibrinogen detached keratinocytes in a time- and concentration-dependent fashion. Treatment with 2.5 or 10 mg per mL for 3 d resulted in detachment of ≈60% and ≈80% of the cells, respectively. Conversely, plasmin was unable to detach cells from the surface suggesting that fibrinogen may affect keratinocyte attachment through a plasmin-independent mechanism.

Fibrin(ogen) detached keratinocytes exhibit low level of β1 integrin on the cell surface Interestingly, whereas some of the cells in untreated monolayers were large and granular (**Fig 7*A***, asterisks), the cells that remained adherent after fibrinogen treatment were small and cuboidal suggesting that the detached cells (**Fig 7*B***, *arrow*) might be differentiated keratinocytes. To determine the differentiation stage of detached and adherent keratinocytes after short treatment (3 h) with fibrinogen we performed flow cytometry on detached, adherent, and untreated cell populations. Detached cells had similar forward scatter (an

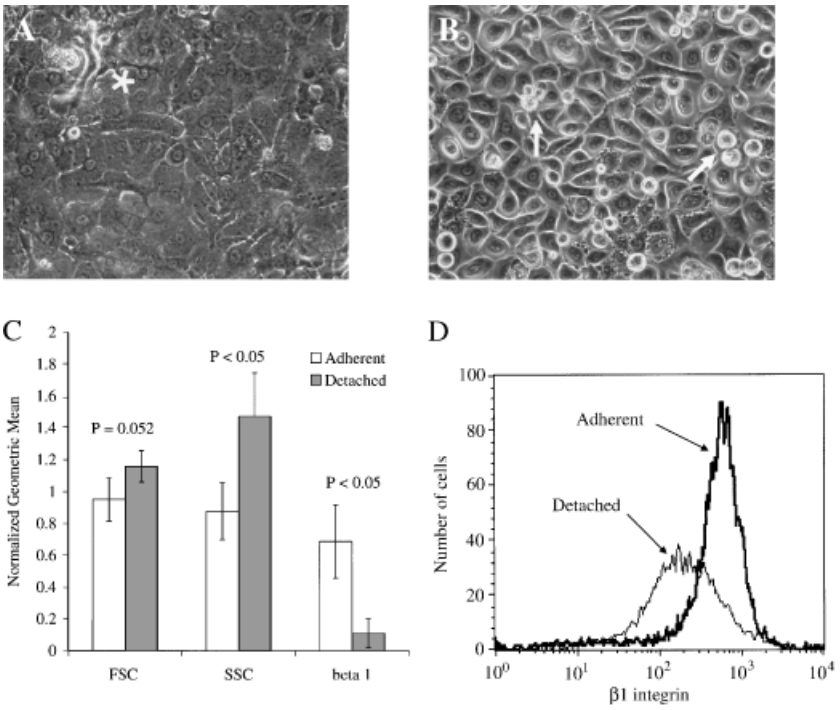

Figure 7. Fibrin(ogen) causes detachment of differentiated keratinocytes. Keratinocytes were grown on collagen-coated plates to confluence and treated with 2.5 mg per mL of fibrinogen (*A*) or remained untreated (*B*) (original magnification × 4). Detached and adherent cell populations were analyzed using flow cytometry. The geometric mean of the forward scatter (FSC; indicator of cell size), side scatter (SSC; indicator of granularity) and fluorescence intensity of each sample was normalized to that of the untreated control for each experiment (*C*). Histogram represents β1 surface expression (*D*). Values represent the mean ± SD of three independent experiments.

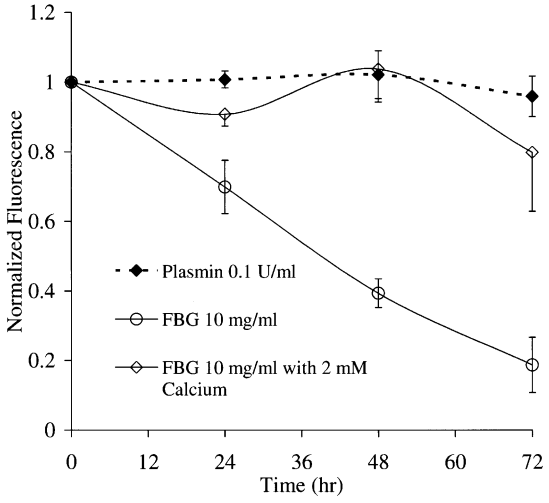

Figure 8. Calcium prevents fibrinogen-mediated detachment of keratinocytes. Keratinocytes were grown on collagen-coated plates to confluence and then treated with 10 mg per mL of fibrinogen without or with addition of Ca^{2+} (2 mM) for 24, 48, and 72 h. Some samples received plasmin (0.1 U per mL), whereas untreated samples served as controls. At the indicated times the detached cells were washed with PBS and the remaining cells were lyzed and total DNA was measured with Hoechst 33258. Total fluorescence was measured using a fluorescence microplate plate reader. At each time point the fluorescence intensity was normalized to that of untreated cells. Values are the mean ± SD of quadruplicate samples in a representative experiment (n = 3). FBG, fibrinogen.

indicator for cell size), but markedly increased side-scatter (an indicator of granularity) as compared with adherent cells (**Fig 7*C***). Most important, the detached cells had significantly lower levels of β1 integrin (**Fig 7*C,D***). The adherent cells also showed a small but statistically significant decrease in the surface expression of β1 integrin.

Calcium prevents fibrinogen-mediated detachment of keratinocytes As Ca^{2+} is important in cell–cell and cell–matrix interactions, we evaluated the effects of Ca^{2+} on fibrinogen-induced detachment of keratinocytes. Addition of Ca^{2+} in serum-free, low-Ca^{2+} (0.09 mM) medium to a final concentration of 2 mM prevented cell detachment even after prolonged treatment (3 d) with 1, 2.5, 5, or 10 mg fibrinogen per mL (**Fig 8**). Interestingly, high Ca^{2+} concentration resulted in a concerted, "sheet-like" migration in contrast to individual cell migration observed under low calcium conditions (**Fig 9**). These results show that fibrinogen-induced detachment depends on the concentration of Ca^{2+}, which alters the mode of cell migration without significantly affecting the rate of wound healing (data not shown).

DISCUSSION

During re-epithelialization, keratinocytes migrate under the fibrin clot while maintaining adhesion to the collagen substrate (Krawczyk, 1971; Clark *et al*, 1982). To study the effects of fibrin on keratinocyte migration we developed a wound-healing model in which keratinocytes were sandwiched between fibrin and collagen thus mimicking the *in vivo* topology. Using this model, we found that fibrin and fibrinogen enhanced the rate of keratinocyte migration on collagen to the same extent as exogenous plasmin. As keratinocytes do not express the fibrin receptor αvβ3 (Kubo *et al*, 2001), we examined the possibility that fibrin gels may affect keratinocyte migration indirectly through activation of plasminogen to plasmin. We found that plasminogen-free fibrin does not enhance the rate of migration, suggesting that the fibrin clot may be responsible for providing and exposing plasminogen to keratinocytes thus enhancing keratinocyte migration through the formation of plasmin.

Plasminogen is converted by cells into plasmin via plasminogen activators that are secreted and confined locally on the cell surface by plasminogen activator receptors (Collen, 2001). In turn plasmin activates matrix metalloproteinases, which are known to be crucial for epithelial cell migration *in vitro*. (Ravanti and Kahari, 2000; Mirastschijski *et al*, 2002). In addition, plasmin has been found to be involved with the conversion of latent growth factors, such as transforming growth factor β and hepatocyte growth factor, which are important in wound healing (Naldini *et al*, 1992; Odekon *et al*, 1994). The importance of plasmin

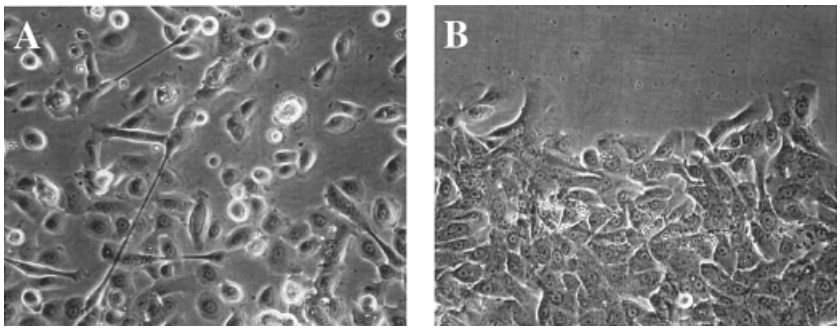

Figure 9. Keratinocytes migrate as an epithelial sheet in the presence of high Ca^{2+}. Keratinocytes were grown on collagen-coated plates to confluence and wounded 2 d later. Fibrin gels were added to wounded monolayers without (*A*) or with addition of 2 mM Ca^{2+} (*B*). At 24 h postwounding, the monolayers were photographed using a digital camera mounted on an inverted Nikon Diaphot microscope. At low Ca^{2+} keratinocytes migrate as individual cells but at high Ca^{2+} they migrate as an epithelial sheet (original magnification × 20).

formation in wound healing has been demonstrated *in vivo* using plasminogen-deficient mice that displayed severe impairment of the healing response, whereas fibrinogen-deficient mice did not exhibit these effects (Bugge *et al*, 1996). In agreement with this *in vivo* data our results suggest that fibrin may act as a carrier for plasminogen, which ultimately promotes healing.

Most important, our results uncovered a novel and unexpected role of fibrin in wound healing. We found that fibrin and fibrinogen caused detachment of keratinocytes from the collagen substrate. Closer examination showed that the detached cells were more granular and expressed very low levels of β1 integrin, which are both characteristics of differentiated keratinocytes (Jones and Watt, 1993; Levy *et al*, 2000). Although the physiologic significance of fibrinogen-mediated cell detachment remains unknown, removal of differentiated keratinocytes from the wound may result in tissue regeneration by stem and transit amplifying cells, which have higher growth potential. This hypothesis is currently under investigation in our laboratory.

The epidermis is characterized by gradients of calcium and integrins across the tissue. As epidermal keratinocytes differentiate and migrate upwards, their Ca^{2+} content increases and the levels of integrins decrease significantly (Vicanova *et al*, 1998; Watt, 1998). Disruption of the epidermis by wounding immediately lowers the Ca^{2+} and Mg^{2+} in the wound (Njau *et al*, 1991; Grzesiak and Pierschbacher, 1995b), providing an environment that facilitates the interaction of the fibrin clot with the low integrin expressing, differentiated keratinocytes. Indeed our experiments show that fibrin-induced detachment of differentiated cells occurred at low Ca^{2+} concentration (0.09 mM), but it was prevented at higher concentrations of Ca^{2+} (2 mM), suggesting that the environment of the wound may promote detachment of differentiated keratinocytes by fibrinogen. The mechanism through which fibrinogen or fibrin mediates detachment of differentiated keratinocytes, however, is not known.

As Ca^{2+} is known to mediate integrin binding to extracellular matrix (Grzesiak and Pierschbacher, 1995a) and each molecule of fibrinogen has three Ca^{2+} binding sites (Lounes *et al*, 2001), it is possible that fibrinogen may weaken the interactions of the cells with the surface by binding Ca^{2+} and reducing its effective concentration. Simple calculations, however, show that the concentration of fibrinogen that caused cell detachment (2.5 mg per mL or 7.35 μM) could only bind ≈ 22 μM of Ca^{2+} (7.35 μM × 3 Ca^{2+} binding sites). The remaining Ca^{2+} (≈70 μM) is enough to maintain cell adherence as dilutions (1:1) of the serum-free medium with Ca^{2+}-free PBS had no effect on cell attachment or viability (data not shown). Consequently, fibrinogen does not mediate keratinocyte detachment by lowering the effective concentration of Ca^{2+} in the medium.

On the other hand the extracellular matrix may be degraded by the action of plasmin. It has been reported that plasmin abrogates αvβ5-mediated adhesion of keratinocytes by proteolytic cleavage of the extracellular protein vitronectin (Reinartz *et al*, 1995). Our data do not support this hypothesis as use of plasminogen-free and plasminogen-containing fibrinogen induced cell detachment to the same extent and the exogenous addition of plasmin did not cause keratinocyte detachment.

As flow cytometry revealed decreased levels of integrin β1 on the surface of fibrinogen-treated cells it is possible that fibrin mediates keratinocyte detachment by downregulating integrin expression. The mRNA levels of β1, however, remained unchanged even after treatment with fibrinogen for 4 h (data not shown), suggesting that fibrinogen does not modulate the steady-state levels of β1 integrin mRNA.

Alternatively, fibrin and fibrinogen may interfere with integrin-mediated cell attachment by disrupting the connections of the cells with the extracellular matrix. Indeed, a recent study showed that fibrinogen abrogated keratinocyte interactions with fibronectin by partially blocking the α5β1 binding sites (Kubo *et al*, 2001). As differentiated keratinocytes have a lower number of integrins and lower integrin avidity (Hertle *et al*, 1991; Watt, 2002), they may be more prone to detachment upon treatment with fibrinogen.

In this study, we present data that suggest a novel role of fibrin in epidermal regeneration. Fibrin and fibrinogen modulate both adhesion and migration of wound keratinocytes even though these cells do not bind to either substrate due to the lack of integrin αvβ3 (Kubo *et al*, 2001). We report that fibrin enhances keratinocyte migration through the activation of plasminogen to plasmin and illustrate a previously unreported role for fibrin in the wound environment. Specifically, we found that fibrinogen and fibrin preferentially detach differentiated keratinocytes thus preventing them from migrating into the wound. This novel role of fibrin may serve to promote healing by cells with high growth potential ultimately ensuring the long-term survival of the regenerated tissue. Current investigations in our laboratory are focused on the mechanism of fibrinogen-mediated detachment of keratinocytes in culture and the role of fibrin in more complex three-dimensional tissue engineered epidermis (Geer *et al*, 2002). Our data may have important implications in wound healing and for the use of fibrin as a biomaterial for tissue engineering and protein and gene delivery.

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