Temporal Stimulation of Corneal Fibroblast Wound Healing Activity by Differentiating Epithelium In Vitro

Julie T. Daniels¹ and Peng T. Khaw¹,²

PURPOSE. To determine whether differentiating corneal epithelium can temporally stimulate fibroblast activity.

METHODS. Corneal epithelial cells were cultured to confluence and then stimulated to mature into multilayered epithelia with addition of serum-containing medium. Differentiation was assessed morphologically and immunocytochemically using a monoclonal antibody to cytokeratin 3. At intervals after onset of differentiation serum-free conditioned medium was collected up to 28 days. Preliminary experiments deduced the optimum concentration of conditioned medium to be used for assessing fibroblast activity. Conditioned medium (25% vol/vol) was added to donor-matched corneal fibroblasts in migration chambers, WST-1 reagent proliferation assays, and fibroblast-populated collagen gel contraction assays. Platelet-derived growth factor (PDGF)-AB and interleukin (IL)-1β in conditioned media were quantified by enzyme-linked immunosorbent assay (ELISA). Fibroblast migration and collagen contraction assays were performed with concentrations of PDGF-AB.

RESULTS. Conditioned medium collected from differentiating epithelium stimulated fibroblast migration and collagen gel contraction, with activity peaks occurring with medium collected on day 14 (P < 0.05). No significant difference was detected between fibroblast growth rates in each of the conditioned media. Levels of PDGF-AB increased during epithelial culture up to 22 days (up to approximately 360 pg/ml) with a subsequent decrease by 28 days. IL-1β inversely correlated with fibroblast activity induced by conditioned medium, with a trough in concentration (2 pg/ml) occurring at 14 days. Both fibroblast migration and collagen contraction were stimulated in a dose-dependent manner by PDGF-AB.

CONCLUSIONS. Corneal epithelium is capable of temporally stimulating fibroblast wound-healing characteristics during its differentiation. One of the growth factors potentially involved in this epithelial–stromal interaction is PDGF. This work demonstrated that developing epithelium (possibly similar to repairing epithelium in vivo) regulated fibroblast behavior and may indicate a mechanism of fibroblast recruitment to a wound after epithelial closure. (Invest Ophthalmol Vis Sci. 2000;41:3754–3762)

Corneal scarring (haze) after penetrating injury and/or surgery is an important cause of visual impairment. Postkeratectomy keratocytes are reported to die in the periphery of the ablation zone by apoptosis. The release of interleukin (IL)-1 appears to stimulate Fas ligand production by corneal fibroblasts that do not ordinarily produce this ligand, thereby stimulating autocrine suicide. It has been suggested that this apoptotic event initiates the wound-healing response.¹,²

The extent of the influence of injured epithelial cells over keratocytes in a wound has been demonstrated after different surgical techniques of photorefractive keratectomy (PRK). The number of apoptotic keratocytes localizes around areas of epithelial damage increases with the magnitude of epithelial injury (e.g., many are associated with an epithelial scrape wound, whereas fewer are found after transepithelial PRK).³

The recruitment of large numbers of active fibroblasts to the wound site may result in excess deposition and contraction of tissue resulting in scarring. The mechanism of activated keratocyte (fibroblast) migration to a corneal wound site after the initial apoptotic phase has not yet been defined.

It is thought that in the cornea the migration of fibroblasts to the injured area may begin when epithelial cells first resurface the wound, followed by maturation of fully differentiated, multilayered epithelium.⁴ An experimental model of PRK in the rabbit in which epithelium was gently scraped from the stroma before photoablation elegantly demonstrated that the early stages of re-epithelialization occur by day 7, whereas the anterior stroma remains predominantly acellular until later time points.⁵ Re-epithelialization begins with epithelial cell migration (slide) from the wound edge. After wound closure, the processes of proliferation and differentiation restores the
epithelium to its normal structure. A number of studies have demonstrated the presence and significance of paracrine mediators of epithelial-stromal interactions in homeostasis and corneal tissue repair. In particular, it has been shown that IL-1β, IL-1α, and platelet-derived growth factor (PDGF) are expressed by epithelial cells, whereas the receptors for these growth factors are predominantly expressed by fibroblasts. A variety of growth factors including PDGF have been found to be chemotactic to human corneal keratocytes. A recent study has demonstrated the presence of PDGF isoforms and receptors associated with epithelial, fibroblast, and endothelial cells in vivo. Furthermore, the authors elected PDGF as a potential candidate for recruitment of fibroblasts to a corneal wound site. The current study was undertaken to determine whether differentiating epithelium could temporally stimulate fibroblast wound healing activity through the release of soluble mediators, to indicate a possible stimulus for fibroblast recruitment to a corneal wound after initial epithelial cover.

METHODS

Cell Culture

Human corneal epithelial and fibroblast cells were isolated from limbal rim donors for research from the Eye Bank at Moorfields Eye Hospital (London, UK). The research followed the tenets of the Declaration of Helsinki. Cells were also isolated from the redundant corneas of Dutch Belted rabbits used in unrelated studies. Corneal tissue was cut into pieces and incubated with 2 mg/ml dispase (neutral protease II; Boehringer–Mannheim, Lewes, UK) fibroblast culture medium (FCM) for 3 hours at 37°C in 5% (vol/vol) CO2, in air. FCM consisted of Dulbecco’s modified Eagle’s medium (DMEM; Sigma, Dorset, UK) supplemented with 10% (vol/vol) newborn calf’s serum, 2 mM l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml antimycotic (all supplied by Gibco Life Technologies, Paisley, UK). Epithelial cells were then scraped into 3 ml phosphate-buffered saline (PBS; Sigma, Dorset, England) and centrifuged at 80 g for 5 minutes. The cell suspension was resuspended in 5 ml keratinocyte serum-free medium (K-SFM) supplemented with 50 µg/ml bovine pituitary extract; 5 ng/ml each of epidermal growth factor, penicillin, and streptomycin (all supplied by Gibco); and 0.03 mM calcium chloride (Sigma). The cells were seeded onto a feeder layer of ST3 fibroblasts previously grown at 4 µg/ml mitomycin C (Kyowa Hakko, London, UK) for 2 hours and cultured as described. Epithelial and fibroblast cells from five different human and three rabbit donors were cultured separately and were used for experiments at passage 3 or between passages 3 and 6, respectively.

Production of Corneal Epithelium

Epithelial cells of human and rabbit origin were cultured to confluence separately with K-SFM in 175-cm2 tissue culture flasks. To initiate differentiation and promote the development of mature multilayered epithelium, culture medium was changed to the serum-supplemented recipe developed by Rheinwald and Green, which contains physiological levels of calcium (approximately 2 mM), referred to in this study as epithelial differentiation medium (EDM). The still-healthy cultures were subsequently discarded after 35 days in culture.

Confirmation of Differentiation

Human epithelial cells from one donor were cultured in the same manner in chamber slides (Nalge Nunc, Rochester, NY). On days 2, 7, and 14, the culture medium was removed, the cells were washed three times with PBS, fixed for 15 minutes at room temperature in 4% (wt/vol) paraformaldehyde, and treated with 20% (wt/vol) sucrose before storage at −20°C. For immunocytochemistry the cells were thawed and dried for at least 30 minutes before addition of 0.5% (wt/vol) blocking reagent (Boehringer–Mannheim) in Tris-buffered saline (TBS) for 20 minutes. The mouse monoclonal antibody for cytokeratin 3 (AE5, 5 µg/ml diluted in blocking reagent; ICN Pharmaceuticals, Basingstoke, UK) or blocking reagent only (negative control) was added for 3 hours. The remaining protocol followed that previously described. The biotin-labeled rabbit anti-mouse bridging antibody (1:300 dilution; Dako, Glostrup, Denmark) was added, and the cells were left in a moist chamber for 45 minutes followed by a further 45 minutes in streptavidin-avidin-alkaline phosphatase complex (Dako). Red AP substrate (Vector, Burlingame, CA) was added until a sufficient color reaction had occurred (less than 30 minutes). The cells were washed briefly to remove the red substrate, counterstained with Meyers’ hematoxylin, dehydrated through xylene and mounted for observation. All incubations were performed at room temperature, and each step was interspersed with 2 × TBS rinses followed by a 2-minute TBS immersion.

Collection of Conditioned Medium

Individual epithelial cultures (five human and three rabbit) were washed three times with serum-free collection medium consisting of a 1:1 ratio of DMEM–Ham’s F12 (Sigma) containing 1% (wt/vol) bovine serum albumin (BSA; Sigma) and incubated for 16 hours. The resultant conditioned medium was centrifuged, aliquoted into siliconized tubes (Eppendorf, Freeport, CA) and stored at −70°C until use. Conditioned medium was collected from differentiating (maturing) cells on days 2, 7, 14, 22, and 28. For purposes of comparison, baseline BSA control medium (nonconditioned collection medium containing 1% wt/vol BSA) was designated to represent time 0. Cultures were photographed at each time point and then returned to EDM.

Optimization Experiments

Conditioned media collected on day 14 from rabbit epithelium was prepared with collection medium to the following dilutions: 0%, 5%, 10%, 25%, 50%, and 100% (vol/vol). Preliminary corneal fibroblast proliferation, migration, and contraction assays (described later) were performed to identify a reasonable dilution of conditioned medium with which to conduct all the fibroblast activity assays. Epithelial-stromal interaction studies were performed with matched conditioned medium and fibroblasts from the same individual. After the optimization assays, all subsequent experiments were conducted using 25% (vol/vol) conditioned medium, unless otherwise stated.

Proliferation

Fibroblast proliferation was monitored using a kit incorporating the reagent WST-1 (Boehringer–Mannheim) according to the manufacturer’s instructions. Briefly, fibroblasts were seeded into 96-well tissue culture plates, at a density of 2.5 × 103 per well in FCM and cultured for 4 hours. FCM was
removed, and the cells were washed three times with PBS before serum-free DMEM supplemented with 1% (wt/vol) BSA was added for a 16-hour serum starvation period. Conditioned media and BSA baseline control medium (100 μl) were added to the cells (six replicates). Four plates per assay were seeded for harvesting at 2 hours and 1, 3, and 7 days. At each time interval, 10 μl of WST reagent was added to each well, and the plate was incubated at 37°C for 2 hours. The absorbance, related to the number of viable cells converting the reagent to colored formazan crystals, was read at 450 nm. On day 3 the cells for harvesting on day 7 were refed.

Migration
Membrane chambers with a pore size of 0.8 μm (Transwell; Costar Corning, Cambridge, MA) were used to assess migration in the presence of conditioned medium. Fibroblasts were seeded into the upper chambers of the membranes at a density of 8000 per well in 100 μl serum-free DMEM and allowed to attach for 4 hours. A volume of 600 μl conditioned media or BSA baseline control medium was added to the lower chambers (six replicates). The cells were incubated for 16 hours to permit migration. The chambers were washed in PBS for 5 minutes to remove excess protein, fixed in 90% (vol/vol) methanol (BDH, Dorset, UK) for 10 minutes, stained with Harris’s hematoxylin (Shandon–Life Sciences, Runcorn, UK) for 30 minutes, and rinsed in tap water. Settled cells in the upper wells were removed using cotton swabs. The total number of migrated cells per membrane was counted using an inverted microscope. With this protocol, human fibroblasts from two of the donors (three replicates per experiment) were assessed for their migratory activity toward concentrations of PDGF-AB or a BSA baseline control.

Collagen Contraction
Fibroblast-populated collagen gels were prepared in a manner similar to that originally described by Bell et al. but with the omission of serum. Gels were prepared by mixing 2.8 ml concentrated DMEM with 4.8 ml type I collagen (5 mg/ml; Sigma). The pH was raised to 7.4 by drop-wise addition of 0.1 M NaOH before addition of 1.2 ml of concentrated DMEM containing fibroblasts to give a final cell density of 1 × 10^5/ml gel mixture. A gel volume of 150 μl per well was polymerized at 37°C for 3 to 4 hours. Conditioned media or collection medium as a baseline BSA background control (500 μl) was added to the gels (six replicates). Each gel was completely detached with a pipette tip so that it was free floating. Gels were refed on day 3. At each time point the gels were photographed with a digital camera (Casio Computer, Tokyo, Japan) and the gel areas measured in pixels using image analysis software (Image Tool; UTHSCSA, San Antonio, TX). Fibroblasts from two human donors (three replicates per experiment) were also assessed for their ability to contract collagen (as described above) in the presence of concentrations of PDGF-AB or a BSA baseline control.

Statistical Analysis
Paired T-tests were applied to compare the effect of conditioned media or PDGF-AB on fibroblasts compared with the baseline BSA control. Values of 0.05 were considered to be statistically significant. Conditioned media from the epithelial cultures of five separate human individuals were analyzed. Within each separate experiment six replicates were included. The results section shows fibroblast activity in the presence of conditioned media collected from one human donor and is representative of the trends demonstrated by all five cultures and media tested. For the PDGF-AB experiments cells from two human donors were assessed with three replicates within each experiment.

Growth Factor Quantification
Levels of IL-1β and PDGF-AB in undiluted conditioned media collected from human cells were measured using ELISA kits.
RESULTS

Epithelial Maturity

The typical morphology of the maturing (differentiating) human epithelium from which conditioned media were collected is demonstrated in Figure 1. Photographs in Figures 1A through 1E show the development of the cultures from cells that had just attained confluence to mature epithelium. Photographs of the differentiating cultures were taken at the end of the 16-hour serum-starvation period, and morphology did not change throughout the duration of the collection periods. Longer periods of collection (more than 24 hours) resulted in breakdown of epithelial cell–cell contacts and deterioration of morphology (data not shown). By day 2 (Fig. 1A) the cultures started to take on the appearance of the early stages of differentiation, indicated by diminishing intercellular spaces (onset of gradual reduction in bright halos between cell boundaries), and the onset of cell enlargement in small areas. More patches of large differentiated cells started to appear by day 7 (Fig. 1B, arrow) and subsequently increased throughout the cultures with time (Fig. 1C, arrow). By day 22 areas of differentiated cells started to whorl (Fig. 1D) in a manner similar to that described during in vivo re-epithelialization. Conditioned medium was not collected beyond 28 days when the majority of the visible cells had taken on a large, fully differentiated morphology (Fig. 1E). However, the cultures remained intact with good morphology until they were discarded after 35 days.
cytokeratin 3 increased with time, indicating differentiating epithelium. Photographs in Figures 2A, 2C, and 2E show negative controls for cultures on days 2, 7, and 14, respectively. On day 2 (Fig. 2B) several areas contained enlarging cells with cytokeratin 3 expression (arrowhead). By day 7 (Fig. 2C) cells were sloughing from the culture surface (arrowhead), and approximately 40% of the cells were expressing cytokeratin 3 (Fig. 2D). By day 14 sloughing continued (Fig. 2E), and the majority of the cells expressed cytokeratin 3 (Fig. 2F).

Optimization Experiments
The results of the optimization experiments using rabbit cultures are shown in Figure 3.

Proliferation. Conditioned media collected from rabbit epithelial cultures at 14 days stimulated rabbit fibroblast proliferation above that of the BSA baseline control medium in a concentration-dependent manner up to 50% (vol/vol). By day 7 each concentration started to inhibit growth, particularly the highest concentration (100% Fig. 3A).

Migration. Fibroblast migration was stimulated in a concentration-dependent manner (Fig 3B).

Collagen Contraction. Fibroblast-mediated collagen contraction increased with conditioned medium concentration up to 25% (vol/vol) over the 7-day assay period (Fig 3C). Undiluted (100% vol/vol) conditioned medium was not tested in this assay for sample economy. A dilution of 25% (vol/vol) was chosen for subsequent human cell-conditioned media experiments, because this concentration provided reasonable results for each of the parameters tested.

Fibroblast Proliferation
Throughout the time course, no significant difference between the conditioned media was detectable in the support of human fibroblast proliferation (Fig. 4). However, by day 3 of the assay all conditioned media could sustain proliferation above that of the baseline BSA control medium (represented as collection day 0; $P < 0.05$).

Fibroblast Migration
The profile of human fibroblast migration in the presence of conditioned media collected from human differentiating epithelium is demonstrated in Figure 5. Migratory activity increased to a peak with medium collected on day 14 and decreased again with conditioned media collected at later time points. All media supported a statistically significant migratory response compared with the BSA baseline control medium ($P < 0.05$). This trend occurred with each cell culture tested of both human and rabbit origin.

Fibroblast-Mediated Collagen Contraction
Figure 6 demonstrates the ability of human conditioned media to support human fibroblast-mediated collagen contraction. On assay days 3 and 7, little contraction was associated with the baseline BSA control medium. On contraction assay day 3, conditioned medium collected from epithelial cultures at day 7 and onward supported fibroblast-mediated collagen contraction above that of the BSA baseline control ($P < 0.05$). By day 7 of the assay, the ability of the day 2 conditioned medium to provoke contraction also became statistically significant compared with the control ($P < 0.05$). Correlating with fibroblast migration in the membrane chamber assay system (Transwell; Costar), peak contractile activity occurred on both assay days 3 and 7 in the presence of conditioned media collected on day 14.

Growth Factor Quantification
The levels of IL-1β and PDGF-AB present in the conditioned media collected over time from one of the maturing human epithelia are shown in Figures 7A and 7B, respectively. Some variation between the actual amounts of IL-1β and PDGF-AB produced by the five different human cultures was found.
however, the trends were very reproducible. In this example, between days 2 and 14, IL-1β levels decreased to approximately 2 pg/ml and then increased again in media collected at later time points. Conversely, the amounts of PDGF-AB increased with time, peaked at day 22 (360 pg/ml) and started to decline by day 28.

**Fibroblast Response to PDGF-AB**

Human corneal fibroblasts were stimulated to migrate (Fig. 8A) and contract collagen (Fig. 8B) in the presence of the AB isoform of PDGF in a concentration-dependent manner. The number of cells migrating toward all concentrations of growth factor tested was significantly higher than toward the BSA baseline control ($P < 0.05$). For the contraction assay concentrations of 5 ng/ml and above were required to support a statistically significant response in this assay ($P < 0.05$).

**DISCUSSION**

This investigation was designed to determine whether maturing (differentiating) corneal epithelium could temporally stimulate fibroblast wound healing activity. After injury to the epithelium fibroblasts die at the periphery of the wound site by apoptosis.$^{1,2,18}$ Only after wound closure by the repairing epithelium do the fibroblasts return.$^4$ Re-epithelialization is achieved by several cell layers participating in migration; however a large portion of the defect is initially covered by a single cell layer. Subsequent proliferation and differentiation of epithelial cells ultimately restores the epithelium to its usual structure.$^6$ The model of differentiating epithelium used in this study was chosen to attempt to mimic in vitro the transition of an intact monolayer of epithelial cells, such as that which had just closed a wound, through differentiation to a mature homeostatic epithelium. The culture model chosen was based on the pioneering work of Rheinwald and Green in which skin keratinocytes were cultured into multilayered epithelium for grafting.$^{19}$ The morphology of the cultures was typical of that previously observed for differentiating corneal epithelium in culture.$^{20}$ As the cultures matured, the cells characteristically enlarged and intercellular spaces diminished as the cells became confluent$^{21}$ as seen by the reduction in the number and intensity of bright halos around individual cells with time. In our previous studies a multilayered epithelium that could be manipulated and grafted after 7 days of culture demonstrated
that the culture method used indeed stimulated the development of a multilayered, differentiating corneal epithelium in culture (Daniels and Khaw, unpublished data, 1999). In addition, by 14 days in EDM in this study, many cells were expressing cytokeratin 322 and could be seen sloughing off the epithelium into the medium, indicating that the epithelium was reaching or had reached maturity and mimicking normal epithelial homeostasis in vivo. The timing and stages of differentiation in culture correlated with that previously observed in vivo. Therefore, the model was considered to be useful for the study of potential temporal influence of differentiating epithelium on corneal fibroblasts.

Optimization of the concentration of conditioned medium to be used throughout the study was achieved with cells isolated from the corneas of rabbits from unrelated studies. The reason for this was twofold. First, it conserved conditioned medium collected from human cells, and, second, while maximizing use of animal tissues from unrelated studies, it provided corroborating data for the trends seen using human cells isolated from the corneas of rabbits from unrelated studies. The epithelium developing up to 14 days in culture may correspond to the interval immediately after wound closure in vivo, and the 28-day-old cultured epithelium may reflect homeostasis with concomitant reduction in stimulatory factors. If so, a correlation between epithelial regeneration, delivery of cues for fibroblast recruitment to a wound, and subsequent suppression of fibroblasts at the end of repair (either passively by the reduction of stimulatory factors or actively by the release of inhibitors) could be drawn. These data appear to corroborate the time sequence of events occurring in a rabbit wound healing model after PRK.5

In the reported study re-epithelialization was completed by day 7, although the depth of the epithelial tissue was 30% thinner than normal. At this time the anterior stroma remained predominantly acellular. However, by day 14 a higher density of spindle-shaped cells in the anterior stroma was observed, and the epithelium reached its maximal thickness by 14 days. By 4 weeks, no spindle-shaped cells were detected, which suggests that the cells underwent a transition from a migratory to a stationary phenotype once they had repopulated the acellular zone.5 Our data complement these in vivo findings and strongly suggest a functional role for differentiating epithelium in stromal wound healing.

The greatest stimulation of fibroblast-mediated collagen contraction was also associated with conditioned medium collected from multilayered epithelium (day 14). There are several hypotheses about the mechanism of matrix contraction. Some reports have demonstrated that contraction results from locomotory forces generated by fibroblast migration through matrix 23–25 Other studies have suggested that stationary cells extending and retracting pseudopodia cause contractions,26 in that it is postulated that individual migrating fibroblasts do not generate enough force to bring about matrix reorganization.27 Whichever mechanism or combination of events actually occurs, this study has shown that conditioned medium collected from epithelium during differentiation temporally stimulated fibroblast-mediated collagen contraction.

IL-1β and PDGF have been shown to be produced by corneal epithelium, whereas the receptors for these growth factors are predominantly expressed by fibroblasts in the stroma, which suggests a potential mechanism for paracrine interaction.7 For this reason the temporal release of these factors into medium during differentiation of epithelium was quantified.
It was interesting that the concentration of IL-1β produced by the epithelium inversely correlated with the stimulus activity of the conditioned medium toward the fibroblasts. IL-1β has been thought to be an indicator of epithelial cell damage or cellular stress.23 It is possible that some cells were stressed shortly after plating, leading to the initial peak. Then, as the cells proliferated, less cell death may have occurred until the cultured became multilayered, and the superficial epithelial cells sloughed and released IL-1β. However, it is difficult to concede that the epithelium cultured in these experiments was producing IL-1β only as a result of injury or cell stress because the morphology of the cultures was excellent, even during serum starvation, throughout the time course studied and beyond. If conditions of culture of epithelium are not optimal for maintaining integrity, the morphology changes, very quickly alerting the observer to culture stress. The conditioned medium was centrifuged before IL-1β was measured, and the possibility of sloughed cells’ contributing to trend was therefore negated. It is possible that IL-1β may be used by the epithelial cells as a means of self-regulation as suggested in a previous study.7

PDGF naturally occurs in three isoforms; AA, BB, and AB. Several studies have reported the influence of the AA and BB isoforms on corneal fibroblast activity.10,11,29 Less information regarding the production of PDGF-AB by corneal epithelial cells and its potential utilization by fibroblasts is available. This study has shown that corneal epithelial cells can produce this isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations. In the laboratory it was not possible to stimulate fibroblast activity with the same isoform in picogram concentrations and that fibroblasts can respond to it in nanogram concentrations.

A study published since the completion of this work12 demonstrated the presence of PDGF isoforms and receptors associated with epithelial, fibroblast, and endothelial cells in vivo. Furthermore, the authors elected PDGF as a potential candidate for recruitment of fibroblasts to a corneal wound site. The important point in our study is that corneal epithelium produces PDGF during differentiation in varying amounts, and this change in concentration gradient influences fibroblast chemotaxis and matrix contraction in culture and may direct cellular activity in vivo. Therefore, our model appears to corroborate the in vivo data.12

To our knowledge this is the first temporal study of the influence of differentiating epithelium on fibroblast activity. These data support increasing evidence of the importance of an intact, healthy epithelium for corneal wound repair and tissue maintenance. This model of regenerating epithelium could be useful in the search for epithelium-derived paracrine mediators that regulate fibroblast activity and help to prevent scarring.

Acknowledgments

The authors thank G. Astrid Limb for expert advice about immunocytochemistry and Ismay Blood for her willing assistance.

References


