Inhibition of Human Corneal Epithelial Production of Fibrotic Mediator TGF-β2 by Basement Membrane–Like Extracellular Matrix

Adriana J. LaGier,1 Sonia H. Yoo,1 Eduardo C. Alfonso,1 Sally Meiners,2 and M. Elizabeth Fini1

PURPOSE. Transforming growth factor (TGF-β2) is a major epithelial mediator of fibroblast marker expression during corneal repair in mice. Production of TGF-β2 protein by cultured rabbit corneal epithelial cells is reduced by plating on a basement membrane–like extracellular matrix extract (Matrigel; BD Biosciences, Bedford, MA). The goal of the present study was to understand further the nature of Matrigel regulation.

METHODS. TGF-β2 protein, mRNA, and gene transcriptional promotion were characterized in cultured human corneal epithelial cells.

RESULTS. TGF-β2 production was inhibited by Matrigel at the level of mRNA accumulation and activity of the gene transcriptional promoter. This effect of Matrigel was not explained by (1) growth factor contaminants, as growth-factor reduced Matrigel also inhibited TGF-β2; (2) independent matrix components, as the pure forms of the major ECM components laminin and collagen IV did not reproduce the effect; or (3) inhibition of a constitutive TGF-β2 autocrine feedback loop, as addition of exogenous TGF-β2 increased p-Smad3 and restored TGF-β2 mRNA levels. In addition, Matrigel's ability to reduce TGF-β2 was not explained by its geometry, as TGF-β2 production was not inhibited by plating cells on a synthetic nanofiber matrix with a three-dimensional topography similar to Matrigel. Matrigel caused a reduction of ezrin, a member of the ezrin-radixin-moesin (ERM) family, which plays a role in establishing polarity of epithelial cells in tissues through the Rho signaling pathway.

CONCLUSIONS. These findings indicate that Matrigel inhibits TGF-β2 gene expression and point to a mechanism dependent on Matrigel composition and structure. The capacity of Matrigel to reduce ezrin is consistent with this idea and directs the focus of future studies toward the ERM/Rho pathway. (Invest Ophthalmol Vis Sci. 2007;48:1061–1071) DOI:10.1167/iovs.06-0772

Penetrating injuries in adult mammals are typically repaired via a fibrotic response, initiated by a diverse array of cytokines released into the wound environment that stimulate cells to divide, reorganize actin into stress fibers, express new repair-associated proteins, and deposit extracellular matrix.1–4 The fibrotic tissue repair response results in a rapid filling of space, which can be organ saving and life saving, but does not regenerate function. However, under some circumstances, the repair response to injury can be quite regenerative (e.g., in the first embryonic trimester).5 Studies comparing regenerative and fibrotic repair have implicated transforming growth factor (TGF)-β as a key cytokine involved in giving repair tissue its fibrotic character.6–8 Pharmacologic agents that target TGF-β activity have been effective in giving repair a more regenerative character with less scarring.5,7

Cellular changes of repair are controlled in skin by proteins that are released from platelets, including platelet-derived growth factor (PDGF) and TGF-β.9 Like skin, the cornea is composed of a collagenous stroma surfaced by epithelial cells, but unlike skin, it contains no blood vessels, and thus there are no platelets to serve as the source of repair cytokines. Yet corneal injuries due to trauma or surgical procedures that penetrate the epithelial basement membrane and Bowman's layer typically stimulate a fibrotic repair response that results in deposition of a "hazy" repair tissue that interferes with corneal clarity.9 The clinical impression has been that the epithelium may be a major source of cytokines controlling fibrosis in the cornea.10 Recently, our research group determined that the epithelial cells of the cornea substitute for platelets in controlling the fibrotic phenotype by producing TGF-β—in the mouse, specifically, the isoform TGF-β2. We have further shown that the release of TGF-β2 from the mouse corneal epithelium and activation of fibrotic gene expression in corneal stromal repair cells correlates in vivo with the absence of basement membrane.11,12 Therefore, epithelial TGF-β2 appears to be a key modulator of fibrotic repair in the cornea when epithelial-stromal interactions are initiated after penetrating injuries disrupt the basement membrane.

Epithelial cells, including those from cornea, produce many regulatory cytokines (reviewed in Ref. 13). The net effect on the underlying mesenchymal tissue is due to the sum of activities released. In a study to understand how corneal epithelial cells control collagenase synthesis by corneal stromal cells, we identified the major stimulator as IL-1α and the major inhibitor as TGF-β2.14,15 The mechanism regulating the release of each cytokine was quite different. IL-1α release was inversely related to cell density, released at much higher rates in cells with limited contact. In contrast, release of TGF-β2 was not affected by cell density.15 However, the findings just described that have implicated basement membrane in control of fibrosis suggest that this may be the determinant of TGF-β2 release. We investigated the role of basement membrane in a rabbit corneal epithelial cell culture model12 and confirmed our hypothesis, showing that epithelial cells produce much less TGF-β2 pro-

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tein when plated on Matrigel, a complex basement membrane–like extracellular matrix extract.

Pharmacologic targeting of TGF-β2 production by corneal epithelial cells could be a novel means for improving the regenerative quality of repair.16,17 The goal of the present study was to explore this initial finding concerning Matrigel regulation of TGF-β2, so as to acquire information necessary for development of a pharmacologic strategy. We developed and applied a human corneal cell culture model for these new investigations, to increase the clinical relevance.

Materials and Methods

Corneal Tissue and Cell Lines

Central corneal tissue was obtained from the National Disease Research Interchange (Philadelphia, PA). Human donor corneoscleral rims preserved in Chen’s medium were provided by the Florida Lions Eye Bank located at the Bascom Palmer Eye Institute in accordance with the guidelines in the Declaration of Helsinki for research involving human tissue. Mink lung epithelial (Mv1Lu) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as recommended.

Human Corneal Epithelial Cell Culture

Human corneal epithelial cells (HCECs) were derived from human donor corneoscleral rims by a previously described method.18 Briefly, sclera and iris remnants were trimmed from each corneoscleral rim, and each rim was dissected into eight equal segments. Each segment was then placed in 1 well of a 24-well culture plate (Nunclon; Nalge Nunc International, Naperville, IL). The explant was treated with 2.5% wt/vol Dispase for 15 minutes at 37°C, washed, and cultured in keratinocyte-serum-free medium (SF; Invitrogen, Carlsbad, CA), a low calcium, serum-free medium. The manufacturer provides epidermal growth factor (EGF) and fibroblast growth factor (FGF)-2 to be added as supplements to stimulate cell growth, but we did not use these particular supplements, instead substituting 10% fetal bovine serum (FBS). The medium was also supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.5 μg/mL amphotericin B at 37°C under 95% humidity and 5% CO2. After 3 weeks, epithelial cells from three segments of specific basement membrane preparation were noted between cells cultured on Aclar and those cultured on glass (S. Meiners, unpublished data, 2005).

Immunostaining

Tissue was formalin fixed, paraffin processed, sectioned, cleared, and rehydrated. The cultured cells were methanol fixed. The cells were immunostained with (1) mouse anti-human cytokeratin 3 (clone AE5; Chemicon, Temecula, CA), confirming that they were epithelial cells (see Figs. 1, 3). For experiments examining ECM’s effects on TGF-β2 expression, the wells were left untreated or pretreated with 10 μg/cm² of specific ECM substrates: BD Matrigel matrix (BD Biosciences, Bedford, MA), or growth-factor-reduced (GFR) BD Matrigel matrix (GFR-Matrigel), laminin, fibronectin, or collagen type IV, all obtained from BD Biosciences (Bedford, MA). Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycan (primarily perlecan), entactin, and nidogen. It also contains some proteinases, TGF-β, and other growth factors naturally present in the tumor. At room temperature, Matrigel polymerizes to produce a biologically active matrix material resembling the mammalian collagen basement membrane. GFR-Matrigel is useful as an alternative to Matrigel when a more highly defined basement membrane preparation is desired. The product is a 20% ammonium sulfate-extracted version of Matrigel.20 Most of the major ECM components of Matrigel are conserved by the process except for heparan sulfate proteoglycan, which is reduced by 40% to 50%. Extraction selectively targets low-molecular-weight proteins, thus effectively removing growth factors except for TGF-β3 which may be bound to collagen IV and/or sequestered in a latent form that partitions with the major components in the purification procedure. In some of our experiments, GFR-Matrigel, which has ~1.7 ng/mL residual TGF-β2, was pretreated with antibody to neutralize the TGF-β2 (R&D Systems, Minneapolis, MN), 0.3 μg/mL per 1 ng/mL TGF-β2 in GFR-Matrigel.

To ensure that cell attachment occurred with equal efficiency on the different ECM coatings, we stained the cells with a fluorescence staining reagent (Cyanquant; Invitrogen) and counted them. The results indicated that cell adherence occurred equally on plastic and on the different ECM coatings used in our experiments (data not shown). For experiments requiring plating of cells on coverslips, 12-well dishes were used. These experiments used Aclar (polychlorotrifluoroethylene [PCTFE], Honeywell Specialty Materials, Morristown, NJ) coverslips electrosprayed with polyclonal antibodies, with or without amines (Ultra-Web; Donaldson Co., Inc., Minneapolis, MN), and coated with SuroMedics (Eden Prairie, MN).21 Aclar coverslips were used instead of glass because of the need to ship the coverslips from New Jersey to Miami with their preservation in an ice pack. No statistical differences in terms of cell morphology or function have been noted between cells cultured on Aclar and those cultured on glass (S. Meiners, unpublished data, 2005).

Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from the cells with a total aurum kit, as per the manufacturer’s instructions (Bio-Rad, Hercules, CA). Total RNA was quantified spectrophotometrically at 260 nm, and 10 ng/μL RNA was reverse transcribed (iScript cDNA Synthesis Kit; Bio-Rad). One-tenth of the cDNA was subjected to qRT-PCR (iQ SYBR Supermix; Bio-Rad) and primers developed for 18S rRNA or GAPDH and TGF-β2 (GFRMatrigel, lami- nin, fibronectin, or collagen type IV, all obtained from BD Biosciences [Bedford, MA]). Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycan (primarily perlecan), entactin, and nidogen. It also contains some proteinases, TGF-β, and other growth factors naturally present in the tumor. At room tem- perature, Matrigel polymerizes to produce a biologically active matrix material resembling the mammalian collagen basement membrane. GFR-Matrigel is useful as an alternative to Matrigel when a more highly defined basement membrane preparation is desired. The product is a 20% ammonium sulfate-extracted version of Matrigel.20 Most of the major ECM components of Matrigel are conserved by the process except for heparan sulfate proteoglycan, which is reduced by 40% to 50%. Extraction selectively targets low-molecular-weight proteins, thus effectively removing growth factors except for TGF-β3 which may be bound to collagen IV and/or sequestered in a latent form that partitions with the major components in the purification procedure. In some of our experiments, GFR-Matrigel, which has ~1.7 ng/mL residual TGF-β2, was pretreated with antibody to neutralize the TGF-β2 (R&D Systems, Minneapolis, MN), 0.3 μg/mL per 1 ng/mL TGF-β2 in GFR-Matrigel.

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Pharmacologic targeting of TGF-β2 production by corneal epithelial cells could be a novel means for improving the regenerative quality of repair.16,17 The goal of the present study was to explore this initial finding concerning Matrigel regulation of TGF-β2, so as to acquire information necessary for development of a pharmacologic strategy. We developed and applied a human corneal cell culture model for these new investigations, to increase the clinical relevance.
TGF-β2 promoter gene was generated by PCR from a 1q41 BAC, RP11-224O19 (Children's Hospital Oakland Research Institute, Oakland, CA). The primers (TGF-β2 promoter-F, 5′-GAAGAGTTCTCAGTG-GCTTG-3′; TGF-β2 promoter-R, 5′-TTGTGGTTTGTATGGCATA-3′) (Invitrogen) were used to create a 3,230-bp product between 50,731 and 53,961 on RP11-224O19 that corresponds to the sequence for the TGF-β2 promoter, −40 to −3270 upstream of the start of exon 1 (M87843, gi:339565) and includes the transcriptional start site at position 153,961 on RP11-224O19 that corresponds to the sequence for the TGF-β2 promoter. The membranes were blocked with 5% dry milk or normal goat serum (132, 90, 55, 43, 34, and 23 kDa) was used as an internal size standard. The reporter construct was confirmed by primer walking (GeneWiz, New Brunswick, NJ).

Using a ubiquitin-C-bla(M) (UBC-bla[M]) vector (Invitrogen), we determined that the bla(M) reporter construct approach would work in HCECs and that transfaction occurred in ~15% to 20% of the HCECs. The DNA coding for the human TGF-β2 promoter was linked to the β-lactamase (bla(M)) reporter gene to create the TGFβ2-bla(M) construct. The capacity of our TGF-β2 promoter construct to drive bla(M) expression was confirmed in Mv1Lu cells, which have been shown to promote TGF-β on exposure to TGF-β (see Fig. 4B).23

The TGF-β2 reporter construct (TGFβ2-bla(M)) or empty vector (empty-bla(M)) was cotransfected into HCECs in serum-free conditions (Transfectin; Bio-Rad), as per the manufacturer’s instructions. Two days after transfection, cells were loaded with a green fluorescent substrate, CCE2. Green fluorescence and blue fluorescence, a measure of TGFβ2-bla(M) gene transcriptional activity, was visualized by an inverted scope equipped with long-pass filters 460/520 nm (Filter 41031; Chroma, Technology Corp., Rockingham, VT) and quantified with the background fluorescence obtained at each wavelength was subtracted.

In additional experiments, we ensured that transfaction occurred with equal efficiency on the different ECM coatings. HCECs were cotransfected with our reporter constructs—empty-bla(M) and TGFβ2-bla(M)—and a dsRed vector at a 5:1 ratio. We counted the percentage of successfully transfected HCECs (red cells) that were positive for TGF-β2 gene transcriptional activity (blue cells).

**Immunoblot Analysis**

Cell lysates were prepared in lysis buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 150 mM NaCl, 10 μg/ml aprotinin [Roche], leupeptin [Roche], and 5 mM sodium orthovanadate [Sigma-Aldrich, St. Louis, MO)]. Lysates and conditioned media were cleared by centrifugation, and protein concentrations were determined with a protein assay (Bio-Rad), according to the manufacturer’s instructions. Samples with 5 to 10 μg total protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% to 15% Tris-glycine gels (Bio-Rad) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Billerica, MA). A molecular weight standard (Cruz Marker; Santa Cruz Biotechnology) that consists of six bands (152, 90, 55, 43, 34, and 23 kDa) was used as an internal size standard. The membranes were blocked with 5% dry milk or normal goat serum (NGS) in Tris-buffered saline (TBS)/Tween20 and probed with (1) 1:500 of anti-TGF-β antibody (Santa Cruz Biotechnology) in 1% dry milk, (2) 1:500 of anti-phospho-Smad3 (p5425/425) antibody (Bio-source, Camarillo, CA) in 1% NGS, or (3) 1:1000 of ezrin-radixin-moesin (ERM) antibody (Cell Signaling Technology, Beverly, MA) in 1% NGS, followed by incubation with horseradish peroxidase (HRP)-linked anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). HRP was visualized by enhanced chemiluminescence (Sigma-Aldrich). The blots were stripped (Pierce Biotechnology, Rockford, IL) and reprobed with, respectively, (1) 1:2000 of anti-β-actin (Sigma-Aldrich) in 1% dry milk or NGS, or (2) 1:500 of anti-Smad 2/3 (BD Biosciences), followed by incubation with HRP-linked anti-mouse or rabbit antibody (Jackson ImmunoResearch) and visualized as above. The TGF-β2, phospho-Smad3 and ERM protein levels were normalized to internal β-actin or Smad2/3. Protein levels were quantified as total adjusted volume of bands corrected for background (Quantity One software, ver. 4.4.1; Bio-Rad).

**Results**

**Prominence of the TGF-β Isoform in the Human Corneal Epithelium and Cultured HCECs**

Past reports have localized TGF-β isoforms in human corneas and cultured HCECs24–30; however, these studies did not directly compare the relative levels of each isoform in the different corneal tissues. To ensure that our previous findings in mouse/rabbit,12 on the prominence of the TGF-β2 isoform in the epithelium could be extended to the human species, we repeated our experiments in the new model (Fig. 1). Immuno-reactive protein for both isoforms was found in epithelium and stroma. However, as we previously observed in the mouse,12 TGF-β2 was strikingly localized to the epithelium, whereas TGF-β1 was primarily concentrated in the stroma (Fig. 1A). In addition, HCECs grown out of corneoscleral rims and plated on plastic without serum were strongly TGF-β2 positive, showing only a minimal TGF-β1 immunoreaction, similar to that seen in the whole epithelium (Fig. 1B). The HCECs were also cytokertatin 3/12 positive, indicating that they are mature central corneal epithelial cells31 (Fig. 1C). These experiments indicated that the human pattern of TGF-β1/TGF-β2 localization in human corneas and cultured HCECs is the same as we found in mouse and rabbit,12 with TGF-β2 being the prominent epithelial isoform.

**Effect of GFR-Matrigel on TGF-β2 Production at the Level of Steady State mRNA and Transcriptional Promoter Activity**

We previously determined that plating of corneal epithelial cells on Matrigel leads to a significant reduction in the amount of TGF-β2 protein produced.12 Matrigel is a complex mixture of ECM proteins, the major component being laminin, followed by collagen IV (approximately 60% and 50% by weight respectively, according to the manufacturer). Matrigel also contains heparan sulfate proteoglycans, as well as TGF-βs and other growth factors that occur naturally in the tumor from which it is extracted. Using a fractionation approach, we sought to determine whether a specific component of Matrigel could mediate Matrigel’s effects on TGF-β2 production by corneal epithelial cells. At the same time, we took one step back in the sequence of events in TGF-β2 gene expression to determine whether Matrigel also reduces the steady state levels of TGF-β2 mRNA. We started by comparing cells plated on plastic or Matrigel to those plated on GFR-Matrigel, an amnomium sulfate-extracted product with reduced levels of growth factors and about half the amount of heparin sulfate proteoglycan.
production if the growth factor is in an active form. To rule out involvement of TGF-β endogenous to Matrigel in mediating Matrigel's inhibitory effect, we treated GFR-Matrigel-coated plates with an anti-TGF-β2 neutralizing antibody before plating HCECs. Despite this neutralization, the level of TGF-β2 mRNA was the same when HCECs were plated on GFR-Matrigel (rM) or neutralized reduced-Matrigel (rM*; Fig. 2B).

The intracellular level of a specific protein is determined by its rate of synthesis and degradation. If TGF-β2 production is controlled by Matrigel at the level of intracellular TGF-β2 mRNA, then reduction in the level of intracellular TGF-β2 protein should follow the reduction in intracellular mRNA. To examine this point, we observed the time course of the Matrigel-mediated reduction of intracellular TGF-β2 protein levels by using the immunoblot assay. Representative results are shown in Figure 3. As previously observed in the rabbit model,
Matrigel reduced the intracellular level of TGF-β2 protein in HCECs—in this case in comparison to laminin. However, in contrast to our finding that the reduction in the intracellular mRNA level occurred within 24 hours, the reduction in protein level was not observed until day 5 after plating and was maintained at day 7 (Fig. 3A). The reduction in the level of intracellular TGF-β2 at day 7 could also be observed by immunostaining (Fig. 3B). Immunoblots of conditioned media probed with TGF-β2 antibody (top). Graph of immunoblot data (bottom). TGF-β2 protein values were determined from the TGF-β2 band densities per 5 µg total protein and are compiled from two independent experiments (average ± high/low). Recombinant human TGF-β2 (10 ng; T) was run as a positive control. TGF-β2 bands from lysates are 25 kDa and from conditioned media are 95 kDa, and β-actin bands are 42 kDa based on molecular size markers (not shown). *P < 0.05, GFR-Matrigel versus laminin.

We ran additional experiments to demonstrate that transcription efficiency did not impact our findings. HCECs were cotransfected with reporter gene constructs and a dsRed vector. The percentage of successfully transfected HCECs, red cells, which had positive TGF-β2 gene transcriptional promoter activity, blue cells was quantified (Fig. 4D). Matrigel reduced the number of successfully transfected cells that had active transcriptional promotion of the TGF-β2 gene.

These results indicate that (1) Matrigel reduces both the steady state levels of TGF-β2 mRNA and the activity of the transcriptional promoter of the TGF-β2 gene.
TGF-β2 transcriptional promoter in corneal epithelial cells, as previously observed for TGF-β2 protein production; (2) the growth factor contaminants and heparin sulfate proteoglycans present in Matrigel can be reduced without altering this effect; (3) neither laminin nor collagen IV—the two major components of Matrigel—is individually sufficient to mediate this effect.

Failure to Implicate TGF-β2 Autocrine Loop Inhibition as the Matrigel Mechanism

TGF-βs can regulate activity of the TGF-β genes in cells that produce them via an autocrine feedback loop. We have previously shown that much of the TGF-β2 produced by rabbit corneal epithelial cells in culture is in the biologically active form suggesting that it could serve as an active autocrine cytokine. Therefore, it could be hypothesized that HCECs maintain a positive TGF-β2 autocrine feedback loop and that Matrigel acts to reduce TGF-β2 levels by interfering with this loop. In fact, the Matrigel component collagen IV has been reported to bind and sequester TGF-β2, providing a possible mechanism. We investigated this hypothesis in a set of experiments addressing the question of transfection efficiencies, HCECs were cotransfected with reporter constructs, empty-bla(M) or TGFβ2-bla(M), and a dsRed vector at a 5:1 ratio. Bla(M) activity (%) indicates the percentage of successfully transfected HCECs, red cells, which had positive TGF-β2 gene transcriptional promoter activity, blue cells. Bla(M) activity (%) is calculated for triplicate fields of view (average ± SD). *P < 0.05, GFR-Matrigel versus laminin.
When HCECs plated on GFR-Matrigel were treated with recombinant human TGF-β2, the mRNA level for TGF-β2 increased to the level observed in HCECs cultured on purified laminin (Fig. 5C). Treatment with exogenous TGF-β2 did not further increase TGF-β2 mRNA levels in HCECs plated on laminin, suggesting that the cells were already making the highest possible amount. Of note, addition of exogenous TGF-β2 did not rescue the reduction in TGF-β2 promoter activity that occurred when cells were plated on GFR-Matrigel (Fig. 5D).

These data indicate that Matrigel does not inhibit the ability of exogenous TGF-β2 to initiate a signal cascade that leads to increased levels of steady state TGF-β2 mRNA. Moreover, unlike Matrigel, exogenous TGF-β2 does not affect the activity of the TGF-β2 promoter, suggesting different control mechanisms. These results fail to support the hypothesis that Matrigel reduces TGF-β2 production by reducing activity of an autocrine TGF-β2 feedback loop.

**Effect of Basement Membrane–like Topography on TGF-β2 Production**

Failing to find evidence to support an indirect mechanism of Matrigel-mediated reduction of TGF-β2 production involving inhibition of a TGF-β2 autocrine loop and also failing to identify a direct mechanism involving specific molecular components of Matrigel, we considered more complex possibilities. Recent work has provided evidence that the highly porous nanotopography that results from the three-dimensional (3-D) associations between ECM molecules that compose basement membranes activates signal transduction cascades in cultured cells. Therefore, we hypothesized that the 3-D architecture of Matrigel, as polymerized on coated tissue culture plastic, is responsible for the inhibition of TGF-β2 production by HCECs plated on its surface.

One of our laboratories (Meiners) has developed 3-D synthetic nanofibrillar surfaces that mimic the porosity, geometry, and complexity of the extracellular matrix. Plating of cells on this surface activates Rac, a member of the Rho family of small GTPases. This signaling pathway is vital to cell morphology and cell-cell interactions and has been implicated in TGF-β signaling.

HCECs were plated on these surfaces, created on Aclar coverslips electrospun without (std) or with (std+) amines for 24 hours. However, we found that plating of HCECs on these nanofibrillar surfaces did not reduce TGF-β2 mRNA levels (Fig. 6A). HCECs were less spread and more rounded when plated on nanofibrillar surfaces versus plastic, reduced-Matrigel, or Aclar coverslips (Fig. 6B). This suggests that the cells were responding to the 3-D matrix, as described previously for other cells types.

The results do not support the idea that the 3-D topography of Matrigel is the controlling factor determining reduction in TGF-β2 production.

**Effect of Matrigel on Levels of the Cytoskeleton-Associated Protein Ezrin**

The results of the preceding experiments indicated that neither individual biochemical components nor 3-D structure alone was sufficient to mediate Matrigel’s ability to reduce production of TGF-β2. A re-evaluation of these findings sug-
FIGURE 6. Basement membrane–like topology does not reduce TGF-β2 mRNA levels in HCECs. HCECs were plated overnight in serum-free KSM at a density of 10^5 per well on a 12-well plastic plate (P), coated with laminin (LM) or GFR-Matrigel (rM), or on an Aclar coverslip (Aclar-std) or an Aclar coverslip electrospun with nanofibers linked without (Aclar-std) or with (Aclar-std+) amines. (A) Total RNA was harvested after 24 hours and subjected to qRT-PCR. The relative TGF-β2 mRNA is calculated as a ratio of the TGF-β2 to the loading control threshold cycles and represents triplicates (average ± SD) from one of three experiments. *P < 0.05. GFR-Matrigel versus plastic. (B) Representative micrographs of HCECs plated on different ECMs.

Immunostaining showed that many HCECs plated on laminin stained positive for ERM, whereas there were fewer positive HCECs when cells were plated on GFR-Matrigel (Fig. 7A). Cell counts revealed that there were 66% ± 6% ezrin-positive cells when plated on laminin versus 30% ± 2% ezrin-positive cells when plated on GFR-Matrigel. This difference is significant (P < 0.05). Using immunoblot analysis, we quantified total ezrin levels in HCECs plated on GFR-Matrigel versus laminin (Fig. 7B). We found a significantly reduced level of ezrin in HCECs plated on Matrigel. These results are consistent with a possible role for ezrin in determining Matrigel’s ability to reduce TGF-β2 production by corneal epithelial cells.

**Discussion**

Surgical procedures in cornea that result in penetration of the epithelial basement membrane and Bowman’s layer typically stimulate a fibrotic repair response that results in deposition of a hazy repair tissue, interfering with corneal clarity. Recently, our research group implicated epithelial TGF-β2 as a key modulator of fibrotic repair in the mouse cornea. We also found that rabbit epithelial cells produce much less TGF-β2 protein when plated on Matrigel, a complex basement membrane–like extracellular matrix extract. The goal of the present study was to explore this initial finding further to acquire information that might focus a pharmacologic development strategy.

To increase clinical relevance we moved into a human model, after determining that TGF-β2 is also the prominent human corneal epithelial isoform. We show that the effects of Matrigel cannot be explained by growth factor contaminants, reduction in heparin sulfate proteoglycans has no effect, and pure forms of the major ECM components laminin and collagen IV cannot alone reproduce the effect. Experiments also failed to implicate inhibition of a constitutive TGF-β2 autocrine feedback loop. Thus, we went on to consider the hypotheses that incorporates a requirement for complexity. TGF-β2 production was not inhibited by plating cells on a synthetic nanofiber matrix with a 3-D topography similar to Matrigel, previously shown to activate Rac signaling. However Matrigel caused a reduction in ezrin, a member of the ERM family that plays a role in establishing polarity of epithelial cells in tissues through the Rho signaling pathway. These findings indicate that Matrigel inhibits TGF-β2 gene expression and point to a mechanism requiring the complexity of Matrigel composition and structure.

Although not specifically investigated in this study, Matrigel may alter TGF-β2 protein processing. Like TGF-β1b, TGF-β2 is secreted in an inactive form as a complex composed of mature TGF-β2 covalently linked to a latency-associated proteoglycan. Although we have preliminary qRT-PCR data indicating that matrix does not change the level of LTBP-1 mRNA in human corneal epithelial cells (data not shown), therefore, cells plated on either laminin or Matrigel would be able to secrete TGF-β2 in an inactive form.

**Differences between the Rabbit and Human Cell Culture Models**

In the human model, we found that the decrease in intracellular TGF-β2 protein levels in cells plated on Matrigel takes several days, whereas we were able to see this difference within 24 hours in the rabbit model. The reason for this difference is likely to be a reflection of various differences between the cell culture methodologies rather than a species difference. Because we had an ample source of tissue for rabbit cell culture, we were able to isolate cells directly from the...
cells in culture before use in experiments. Corneal epithelial cells clearly change to some degree when expanded in culture—for example, their secreted gelatinolytic metalloproteinase signature shifts toward higher levels of gelatinase A (unpublished observations, 1990). Nevertheless, the HCECs used in this study retained the keratin differentiation marker for epithelium, as well as the preference for TGF-β2 expression over TGF-β1 that we saw in the rabbit model. Another change made necessary by the requirement for expanding the number of cells was use of a low-calcium medium to enhance the rate of cell replication. To maintain cell homeostasis, we used this same medium for experiments, as opposed to the standard calcium medium used in our previous rabbit study. Either of these differences could contribute to greater TGF-β2 protein stability and alter the time course by which Matrigel reduces the protein level. Indeed, there are many reports in the literature about regulation of TGF-β protein stability and the central role of this parameter in determining the ultimate level of TGF-β activity.46,47 We would not attempt to derive any conclusions about the relevance of this finding to the in vivo situation since the conditions are so different. Fortuitously, however, this longer time frame was useful for our ability to characterize the phenomenon as we were easily able to observe the earlier decrease in TGF-β2 mRNA levels and transcriptional promoter activity, which would have been more compressed in time in the rabbit model.

**Failure to Implicate an Autocrine TGF-β2 Feedback Loop**

TGF-βs can upregulate activity of the TGF-β genes in cells that produce them via a positive autocrine feedback loop.25 In fact, we have previously shown that much of the TGF-β2 produced by rabbit corneal epithelial cells in culture is in the biologically active form,14,15 which suggests a way that Matrigel acts to inhibit TGF-β2 production—by repressing this feedback loop. In fact, the Matrigel component collagen IV has been reported to bind and sequester TGF-β2,34 providing a possible mechanism for this inhibition. Our finding that purified collagen IV alone was not able to inhibit TGF-β2 production, however, did not support this possibility. Additional experiments performed in this study also failed to provide support. The presence of Matrigel failed to inhibit the capacity of exogenously added TGF-β2 to activate Smad3 signaling and stimulate TGF-β2 mRNA levels. A preliminary immunoblot analysis that we performed on conditioned media from the cells treated with exogenous TGF-β2 and plated on either laminin or Matrigel displayed relatively equal amounts of recombinant TGF-β2 (data not shown), supporting the idea that Matrigel does not selectively sequester TGF-β2.

In fact, the process that is used to create GFR-Matrigel does not reduce levels of naturally occurring TGF-β2. It could be argued that Matrigel is already saturated with TGF-βs and cannot absorb more; thus, exogenous TGF-β2 could act without restraint, but TGF-β2 produced endogenously by corneal epithelial cells should also be able to escape from this absorption. However, the strongest evidence against the autocrine inhibition mechanism is that exogenous TGF-β2 increases the levels of TGF-β2 mRNA without affecting TGF-β2 promoter activity. Because Matrigel decreases both TGF-β2 mRNA levels and TGF-β2 gene promoter activity, we infer that there are different mechanisms at play. For example, TGF-β2 mRNA levels could be increased in the absence of transcription by increasing mRNA stability, a well-known TGF-β regulatory mechanism.38
Evidence for Involvement of the Actin Cytoskeleton

In epithelial cells, the cortical actin cytoskeleton is a highly dynamic structure that controls the localization of protein complexes associated with the plasma membrane and the machinery that regulates the actin assembly (reviewed in Ref. 49). Reorganization of the actin cytoskeleton is linked to signaling via the Rho family of small GTPases (reviewed in Ref. 50). The epithelial basement membrane has a complex 3-D architecture, and much work has indicated that this nano-topography can influence organization of the cortical cytoskeleton in cells in culture.55 Growth of cells on 3-D nanofibrillar surfaces results in a preferential and sustained activation of the small GTPase Rac.57 Cell surface receptors can also transmit signals into a cell via structural changes that occur when they bind to their ECM ligands (reviewed in Ref. 51). The cytoplasmic face of cell contact sites comprises large macromolecular assemblies that link transmembrane cell adhesion molecules to the cytoskeleton. These assemblies are dynamic structures that are the targets of regulatory signals that control cell adhesiveness.52 The ERM family proteins are part of the cortical cytoskeleton. Immunofluorescence studies of cultured epithelial cells have revealed that ERM proteins are coexpressed and co-concentrated at cell-surface structures such as microvilli, filopodia, uropods, ruffling membranes, retraction fibers, and cell-adhesion sites where actin filaments are associated with plasma membranes.53 Signal transduction through ERM proteins has emerged as an important means of coordinating localized and dynamic cellular processes that require membrane cytoskeletal reorganization.49,54 They play an important role in the activation of members of the Rho family by recruiting these regulators of actin cytoskeleton.55 Our finding that levels of the ERM protein ezrin are regulated by plating of corneal epithelial cells on Matrigel suggests ERM proteins and the Rho pathway as a focus for future studies to understand how Matrigel controls TGF-β2 production.

It seems a paradox that the complex basement membrane-like ECM Matrigel should inhibit TGF-β2 production in cell culture, but that there should be so much TGF-β2 protein present in the normal uninjured corneal epithelium—a previous finding12 now confirmed in this study. How can we understand the cell culture phenomenon in terms of corneal biology? Cell culture bears much in common with wound healing, and our previous findings have suggested that epithelial cells migrating over basement membrane to close a superficial corneal abrasion downregulate TGF-β2 production, but cells migrating over a stromal wound bed upregulate TGF-β2 production. Once the epithelium is completely repaired, however, TGF-β2 synthesis may once again increase despite the presence of the basement membrane. Understanding basement membrane regulation of corneal epithelial TGF-β2 as a cytoskeletal-regulated process involving the complexity of basement membrane structure and biochemical composition may help make sense of this paradox. Migrating cells have much different cytoskeletal organization and ECM adhesions than cells in the normal differentiated epithelium, and the associating regulatory proteins are different.56 It is in these differences that we are likely to find the answers to how basement membrane regulates TGF-β2 production.

In the present study, our goal was to identify mechanisms by which Matrigel inhibits TGF-β2 production. We show that TGF-β2 protein production is controlled at multiple levels of gene expression by Matrigel, including protein accumulation, mRNA accumulation and activity of the gene’s transcriptional promoter. The effects of Matrigel cannot be explained by growth factor contaminants and cannot be reproduced by purified matrix components or by a structurally similar synthetic matrix that stimulates Rac signaling pathway. The capacity of Matrigel to reduce ezrin focuses future studies on the ERM/Rho signaling pathway.

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References

Inhibition of TGF-β2 Production by Matrix


