Corneal Epithelial Cell Attachment with Endogenous Laminin and Fibronectin

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Purpose. To evaluate the role of endogenously produced laminin and fibronectin as well as the effect of exogenous laminin and fibronectin in the attachment of human corneal epithelial cells in vitro.

Methods. Primary cultured human corneal epithelial cells labeled with $^3$H-thymidine were seeded onto plates coated with laminin or fibronectin, or onto uncoated bacteriologic plates. Attachment of cells was measured in the presence or absence of antisera against laminin or fibronectin, by counting radioactivity.

Results. Human corneal epithelial cells attached to plates coated with human laminin or human fibronectin in a dose-dependent manner, with 69% and 50% of cells attached to the wells coated with 40 ng/ml of laminin and fibronectin, respectively ($P < 0.001$). The percentage of attachment to uncoated bacteriologic plates increased from 1.2% at 45 min of incubation to 6.7% at 90 min, 22.2% at 3 hr, and 40.1% at 6 hr of incubation. Cycloheximide, a protein synthesis inhibitor, completely inhibited cell attachment. Rabbit antiserum against human fibronectin reduced cell attachment to the uncoated plates to 67% of the control value ($P < 0.01$), whereas rabbit antiserum against human laminin decreased the attachment to 52% of the control ($P < 0.01$). A combination of these two antisera reduced cell attachment to 46% of the control ($P < 0.01$).


Corneal epithelial cells rest on a thin layer of extracellular matrix (ECM) called basement membrane, which plays a crucial role in epithelial adhesion to underlying stroma. ECM have many effects on cell adhesion, proliferation, and differentiation, and the nature of these effects depend on types of ECM and associated cells.

Fibronectin is not detected on the basement membrane in normal rabbit cornea but appears beneath migrating epithelial cells during cornea wound healing. There are conflicting reports regarding the role of fibronectin in corneal epithelial healing. Nishida et al reported that exogenously added fibronectin promoted corneal wound healing in vivo and in vitro. However, a recent clinical study showed that topical fibronectin neither had no effect on human persistent epithelial defects. In addition, others have reported that fibronectin neither promotes wound healing in animals after epithelial scrape or keratectomy in vivo, nor does it promote wound closure.

Laminin, a glycoprotein that is one of the major components of basement membrane, is also thought to play significant roles in wound healing. Laminin is...
resynthesized within 48 hr in rabbit corneal wound healing.\textsuperscript{16} In addition, recent reports suggest correlation between early appearance of laminin and hemidesmosome formation or focal adhesion.\textsuperscript{17,18}

Epithelial cells not only react to exogenously added ECM but also produce endogenous ECM.\textsuperscript{15,19} However, information about the role of endogenously produced ECM in corneal wound healing is very limited, compared to a large number of reports describing effects of exogenously supplied ECM.

This report evaluates the role of endogenously produced fibronectin and laminin as well as the effects of exogenously supplied fibronectin and laminin, on human corneal epithelial cell attachment in vitro.

**MATERIALS AND METHODS**

**Corneal Epithelial Cells Preparation**

Human corneal cells were obtained by the method of Ebato et al.\textsuperscript{20} Briefly, four 2 x 2 mm limbal explants were placed on a 35-mm dish with the epithelial side up and were allowed to dry for approximately 5 min. One milliliter of modified supplemented hormonal epithelial medium,\textsuperscript{21} consisting of equal volumes of Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, Gaithersburg, MD) and Ham's F12 (Gibco-BRL) enriched with 15\% fetal bovine serum, 10 ng/ml epidermal growth factor (Earth Chemical, Hyogo, Japan), 0.1 \( \mu \text{g/ml} \) cholera toxin (Sigma, St. Louis, MO), 1 mM l-glutamine (Gibco-BRL), 5 \( \mu \text{g/ml} \) insulin (Collaborative Research, Bedford, MA), 0.5\% dimethyl sulfoxide (Fisher, Pittsburgh, PA) and 20 \( \mu \text{g/ml} \) gentamicin (Elkins-Sinn, Cherry Hill, NJ), was added. Culture medium was changed every 2 or 3 days until corneal epithelial cells had covered 80\% of the dish. Corneas were obtained from the Eye Bank of Western Pennsylvania.

**Adhesion Assay**

When corneal epithelial outgrowth had reached approximately 80\% of the dish, the explants were removed and corneal epithelial cells were labeled with 2 ml of supplemented hormonal epithelial medium containing 25 \( \mu \text{Ci/ml} \) of \( ^{3} \text{H}-\text{thymidine} \) (20 Ci/mmol, NEN, Boston, MA) for 24 hr.\textsuperscript{22} The cells were treated with 0.25\% trypsin/0.1 \% ethylenediaminetetraacetic acid for approximately 10 min then, 0.5 \( \mu \text{g/ml} \) soybean trypsin inhibitor in DMEM (Sigma) was added to stop trypsin digestion. Cells were suspended and washed with DMEM three times and a final suspension was prepared to a density of 1.0 \( \times 10^{5} \) cells/ml or 5.0 \( \times 10^{5} \) cells/ml in DMEM containing 0.2\% heat denatured bovine serum albumin (Sigma). In some assays, 25 \( \mu \text{g/ml} \) cycloheximide was added to the culture 2 hr before cells were harvested and it was included in the medium throughout the assays to determine the effect of inhibition of protein synthesis on cell attachment.

For assays of cell attachment to exogenously provided ECM, bacteriologic 24-well plates (Coaster, Cambridge, MA) were coated at 37\°C for 2 hr with 250 \( \mu \text{l} \) of human fibronectin (Gibco-BRL) or human laminin (Telios, San Diego, CA) diluted to 40 \( \mu \text{g/ml} \) with DMEM. The coated plates as well as noncoated plates used for studying endogenously produced laminin and fibronectin were incubated with 10 mg/ml of bovine serum albumin at 37\°C for 1 hr to block nonspecific binding sites. In each well, 5 \( \times 10^{4} \) cells labeled with \( ^{3} \text{H}-\text{thymidine} \) were seeded and the cells were allowed to attach to the plate for 45 min to 6 hr. After incubation, unattached cells were rinsed by washing three times with phosphate buffered saline with Ca\textsuperscript{++} and Mg\textsuperscript{++}. The cells that remained attached were lysed with 1 ml of 1\% sodium dodecyl sulfate (Bio-Rad, Richmond, CA) and radioactivity of each sample was measured by liquid scintillation counting (Beckman, Fullerton, CA). Attachment of cells was calculated by dividing the radioactivity in cells that remained attached by the total radioactivity in the original cell suspension.

In inhibition assays, cells were allowed to attach in the presence of rabbit antiserum against human laminin (Telios) or rabbit antiserum against human fibronectin (Telios), which had been diluted 1:20 with DMEM containing 0.2\% bovine serum albumin with or without cycloheximide. Percentages of attachment under these conditions were compared to control values obtained by allowing cells to attach in the absence of antisera.

**Detection of Laminin and Fibronectin Synthesized by Epithelial Cells**

Nonlabeled human corneal epithelial cells were seeded on 35-mm plastic dishes at a density of 3 \( \times 10^{5} \)/dish in the same manner as that described for the attachment assay. The cells were labeled with 200 \( \mu \text{Ci/ml} \) of \( ^{35} \text{S}-\text{methionine} \) in methionine-free medium for 24 hr. The medium was collected and immunoprecipitated with the rabbit antisera against human laminin or human fibronectin. Immunoprecipitates were subjected to sodium dodecyl sulfate 7\% polyacrylamide gel electrophoresis,\textsuperscript{23} then autoradiography.

**Statistics**

Differences between means were compared by \( t \) tests.

**RESULTS**

**Cell Attachment to Exogenously Provided ECM**

After 45 min of incubation, human corneal epithelial cells attached to plates coated with human laminin or human fibronectin in a dose-dependent manner, with 69\% and 50\% of cells attached to the wells coated with...
40 μg/ml of laminin and fibronectin, respectively ($P < 0.001$, Fig. 1). The attachment to the fibronectin- or laminin-coated plates did not increase between 45 min and 6 hr (data not shown).

Inhibition assays using rabbit antiserum against human laminin or human fibronectin were carried out to confirm that the attachments were promoted specifically by laminin or fibronectin. Rabbit antiserum against human laminin inhibited the attachment to the laminin-coated plates, yielding 24% of the control attachment, whereas it did not inhibit the attachment to the fibronectin-coated plates ($P < 0.001$, Figure 2). Conversely, antiserum against human fibronectin inhibited the attachment to the fibronectin-coated plates, yielding 6% of the control attachment but did not inhibit the attachment to the laminin-coated plates ($P < 0.01$, Fig. 2).

To evaluate the role of de novo synthesis of proteins in the adhesion to plates coated with ECM, cells were treated with cycloheximide to inhibit protein synthesis. Cycloheximide treatment did not change the pattern of cell attachment to fibronectin- or laminin-coated plates (Fig. 3).

**Cell Attachment to Bacteriologic Plates**

Corneal epithelial cells attached to the uncoated plates in a time-dependent manner. The percentage of attachment increased from 1.2% at 45 min incubation to 6.7% at 90 min, 22.2% at 3 hr, and 40.1% at 6 hr incubation (Figure 4). The attachment to the uncoated plates at 6 hr was approximately 80% of that seen on fibronectin-coated plates at 6 hr incubation. Treatment of the cells with 25 μg/ml cycloheximide, which inhibits de novo protein synthesis, completely inhibited their attachment to the uncoated plates (Fig. 4).

To evaluate the role of endogenously produced laminin or fibronectin in cell adhesion, inhibition assays were carried out using rabbit antiserum against human laminin or antiserum against human fibronectin.

**Figures**

- **Figure 1.** Cell attachment to bacteriologic plates coated with laminin or fibronectin at 45 min incubation. Human corneal epithelial cells attached more to the plate coated with 40 μg/ml of laminin than that coated with 40 μg/ml of fibronectin ($P < 0.001$). Data are presented as mean ± SD of values from four experiments, each done in triplicate.

- **Figure 2.** Inhibition of cell adhesion to bacteriologic plates coated with laminin or fibronectin with antiserum against laminin or fibronectin at 45 min incubation. Data are presented as mean ± SD ($**P < 0.01, ***P < 0.001$) of values from four experiments, each done in triplicate.

- **Figure 3.** Effect of 25 μg/ml cycloheximide treatment on cell attachment to bacteriologic plates coated with laminin or fibronectin at 45 min incubation. Error bars represent SEM from four assays, each assay done in triplicate.
tin. Rabbit antiserum against human fibronectin reduced cell attachment to the uncoated plates to 67% of the control value ($P < 0.01$, Figure 5), whereas rabbit antiserum against human laminin decreased the attachment to 52% of the control ($P < 0.01$) and a combination of these two antisera reduced cell attachment to 46% of the control ($P < 0.01$). Conversely, preimmune rabbit serum did not inhibit the cell attachment.

Detection of Laminin and Fibronectin Synthesized by Epithelial Cells

Biosynthesis of fibronectin and laminin by human corneal epithelial cells was biochemically assessed. The medium precipitated with rabbit serum against human laminin showed two bands corresponding to laminin on autoradiography by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 6; purified laminin showed two bands 190 kDa and 400 kDa). When culture supernatants were immunoprecipitated with rabbit antisera against human fibronectin, bands corresponding to human fibronectin were detected (purified fibronectin showed a 220-kDa band).

DISCUSSION

Corneal epithelial cell attachment to underlying connective tissue is important not only in normal eyes but also during wound healing. Extracellular matrix is believed to play crucial roles in the attachment. Current information about corneal epithelial attachment mainly comes from animal experiments that have examined the effects of exogenously added matrix components on the cell attachment. The extracellular matrix influence may vary not only depending on the responding cell type but also depending on the animal species. In the current study the role of matrix components in human corneal epithelial attachment was evaluated, which previously had not been elucidated well.

Results of the current study indicate that human corneal epithelial cells adhere to both fibronectin and laminin. Although human corneal epithelial cells can attach both to exogenous fibronectin and laminin, laminin is a better substrate for their attachment. Conversely rabbit corneal epithelial cells were previously shown to adhere better to fibronectin than to laminin. The discrepancy between that report and our results may be attributable to species-specific differences in the corneal epithelial cells. Rabbit and human corneal epithelial cells have been shown to differ in their rate of proliferation and differentiation as monitored by specific keratin expression. Sources (animal species) of the matrix components may cause different results in experimental findings. In fact, in our preliminary experiments, human corneal epithelial cells did not attach efficiently to mouse laminin obtained from two different sources.

The use of $^3$H-thymidine to monitor cell attachment in these experiments theoretically could have influenced the results, because this method would label proliferating cells preferentially, which might behave differently from cells that are more quiescent. However, in preliminary experiments, we used a method to measure cell attachment that involved electronic cell counting after attached cells had been released by trypsinization, and we achieved the same results. Because this method would not distinguish between proliferating and nonproliferating cells, we believe that the use of $^3$H-thymidine did not bias the results.

In the maintenance of healthy corneal epithelium in the normal eye or during wound healing, endogenously produced ECM may be as much as or more

![Graph](image-url)
Endogenous Corneal Epithelial Attachment Factors

FIGURE 6. Autoradiography after immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, culture supernatant immunoprecipitated with antiserum against human laminin; lane 2, culture supernatant immunoprecipitated with antiserum against human fibronectin; lane 3, culture supernatant immunoprecipitated with preimmune rabbit serum.

Important than exogenously provided ECM as observed in other cell types. However, influences of endogenously produced ECM in the attachment of human corneal epithelial cells had not been elucidated. To evaluate the role of endogenously produced ECM, we examined cell adhesion to bacteriologic plates that were not coated with matrix components (coated with bovine serum albumin to block nonspecific cell binding). The ability of corneal epithelial cells to attach to these bacteriologic dishes and its time dependency suggest that these cells de novo synthesize ECM, which are used for their attachment. This is also suggested by demonstrating that cell attachment does not occur if protein synthesis is inhibited by cycloheximide. Laminin and fibronectin are two of the matrix components that are newly synthesized and used by the cells for attachment. This is evident from the inhibition of cell attachment by antisera against laminin and fibronectin. Other endogenously produced ECM such as collagen are also likely to play a part in cell attachment, because a combination of antisera against laminin and fibronectin did not completely inhibit cell attachment to the uncoated dishes.

Although fibronectin is thought to play a key role in wound healing, there are conflicting reports about promotion of corneal wound healing by exogenously added fibronectin. In our experiments, attachment to the uncoated bacteriologic plates requires longer incubation than that with fibronectin-coated plates. However, the attachment to the uncoated plates is approximately 80% of that to fibronectin-coated plates when incubated for 6 hr. Therefore, endogenously produced ECM may be sufficient and exogenously added fibronectin may not be critical in cell adhesion.

In summary, we showed that endogenous laminin and fibronectin as well as exogenous laminin and fibronectin play significant roles in human corneal epithelial cell attachment in vitro. Factors stimulating endogenous laminin and fibronectin synthesis may be useful in treatment of ocular surface diseases characterized by poor adhesion of epithelium to substrate.

Key Words
corneal epithelium, attachment, endogenous, laminin, fibronectin

References
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