Epidermal Growth Factor Stimulates Integrin-Mediated Cell Migration of Cultured Human Corneal Epithelial Cells on Fibronectin and Arginine-Glycine-Aspartic Acid Peptide

Brian A. Maldonado* and Leo T. Furcht*†

Purpose. The aim of this work was to show epidermal growth factor (EGF)-dependent migration of human corneal epithelial cells to fibronectin and GRGDSP peptide. The authors assessed the role of cell surface integrin heterodimer α5β1 in mediating haptotactic cell migration to fibronectin by the use of specific function-blocking integrin antibodies.

Methods. A haptotactic cell migration assay in a Boyden chamber was used to compare the relative migration of the cultured human corneal epithelial cells in the presence of fibronectin and GRGDSP peptide-coated filters. Epithelial cells were incubated in the presence of function-blocking integrin antibodies or anti-EGF-receptor antibodies to determine their role in haptotactic cell migration.

Results. Human corneal epithelial cells grown as primary cultures migrated in the presence of fibronectin or GRGDSP peptide, but only on stimulation with EGF. Antibodies to the EGF receptor blocked the EGF-mediated stimulation of haptotactic cell migration. Anti-β1 and anti-α5 antibodies each inhibited haptotactic cell migration to fibronectin and GRGDSP peptide.

Conclusions. Epidermal growth factor provides an important stimulus of haptotactic cell migration of human corneal epithelial cells. Stimulation of cell migration by EGF was maximal in the range of 5 to 10 ng/ml; this response was completely blocked by incubation with an anti-EGF receptor antibody. Function-blocking integrin antibodies, specifically anti-β1 and anti-α5, inhibited integrin-mediated cell migration to fibronectin and GRGDSP peptide. These data suggest that EGF represents an essential initial stimulus for haptotactic cell migration of human corneal epithelial cells; furthermore, integrins are important in mediating cell migration to fibronectin and GRGDSP. Invest Ophthalmol Vis Sci. 1995;36:2120-2126.

The corneal epithelium imparts not only a layer of protection to the underlying cornea, it also plays an important role in the maintenance of the basement membrane and the optical transparency of the underlying stroma. The migration of corneal epithelial cells across the basement membrane is an early event in the reconstitution of a functional corneal epithelium; corneal epithelial cells surrounding a denuded area respond by migrating and covering the entire wound site.

The basement membrane is an intricate and specialized extracellular matrix. In the cornea, the basement membrane represents an important physiological barrier between the epithelial cell layer and the stroma. Three major types of molecules are found in basement membranes: type IV collagen, heparan sulfate proteoglycans, and noncollagenous proteins such as laminin, nidogen, and osteonectin. Fibronectin normally is not present in basement membrane; however, as a result of corneal injury, plasma fibronectin readily accumulates at this location. Fibronectin is thought to provide a provisional matrix to facilitate corneal epithelial cell spreading and migration.
during wound healing; once healing has been achieved, fibronectin disappears and is no longer detectable at the wound site. Our laboratory has shown that fibronectin is important not only in promoting cell adhesion but in promoting haptotactic cell migration of rabbit corneal epithelial cells.

Plasma fibronectin is a large dimeric glycoprotein composed of similar, but not identical, polypeptide subunits, linked by disulfide bonds. The central cell binding domain is one of two domains in fibronectin that mediate cell adhesion. The tripeptide arginine-glycine-aspartic acid (RGD) is the active peptide within the central cell binding domain; this domain of fibronectin is a strong promoter of cell adhesion and migration. Fibronectin, and the RGD sequence in particular, are specific ligands of the α5β1 integrin heterodimer; our laboratory and others have shown that this integrin is one among several integrins expressed in primary cultures of corneal epithelial cells and in corneal tissue.

The RGD sequence also is expressed in many adhesion-promoting proteins, among them type IV collagen. RGD is found several times in a1(IV) and α2(IV) chains. These RGD sequences appear to be largely conserved among species (human and mouse), suggesting the biologic importance of this sequence. The RGD sequence of type IV collagen may be important in the biologic activity of type IV collagen because this collagen mediates rabbit corneal epithelial cell adhesion and haptotactic cell migration.

The epidermal growth factor (EGF) has a significant role in wound healing in the cornea and is a potent corneal epithelial cell mitogen and stimulator of cell migration. Polymerase chain reaction analysis has demonstrated EGF mRNA in corneal epithelial cells, suggesting a possible autocrine role for this growth factor; additionally, the lacrimal gland may represent an important additional source of growth factor. Accelerated corneal surface wound healing has been observed when humans and several species, including primates and rabbits, were administered EGF. The receptor to EGF has been identified on corneal epithelial cells, intracellular signaling from this receptor may trigger cell migration, phosphorylation of intracellular protein substrates and cytoskeletal reorganization may also be important in mediating EGF stimulation of cell migration.

Cell surface integrins have been shown to be important not only as mediators of cell adhesion but also of cell migration. Our work and that of others has shown that corneal epithelial cells express the integrins required to enable these cells to adhere and migrate over the extracellular matrix and the basement membrane. Focal contacts are formed on interaction with the extracellular ligand. This interaction can trigger changes in cell shape that may be a prelude to cell migration. The importance of cell surface integrins in keratinocyte cell migration is emphasized further by the observation that the β1 integrin subunit is upregulated as a prelude to cell migration; this occurs in response to various changes in the basement membrane composition. The fibronectin receptor has been shown to be vital in the cell migration of tumor cells, emphasizing the important role of integrins in cell migration.

Fibronectin and EGF play an important role in wound healing of the human corneal epithelium; we examined the functional role of EGF and the α5β1 cell surface integrin in haptotactic cell migration of human corneal epithelial cells on fibronectin and GRGDSP peptide. Specific anti-integrin antibodies probe the role of the α5β1 integrin in mediating haptotactic cell migration toward fibronectin and GRGDSP peptide. An anti-EGF antibody is used to block the stimulation of haptotactic cell migration by EGF.

MATERIALS AND METHODS

Corneal Epithelial Cell Culture

Human donor corneas were obtained from the Minnesota Lions Eye Bank at the University of Minnesota (Minneapolis). Corneas were stored in McCarey-Kaufman or Dextran storage media at 4°C, with no limitations on the age of the donor. Informed consent was obtained from responsible relatives, and the tenets of the Declaration of Helsinki were followed. This research is in keeping with the standards set by our department, and all requirements for review and approval of this research according to the Committee on the Use of Human Subjects in Research have been met.

Primary cultures of human corneal epithelial cells were obtained as described. Donors generally were older than 60 years of age. Corneas were excised and incubated in Dispase (1 U/ml, grade II; Boehringer Mannheim, Indianapolis, IN) for 1 hour at 25°C. The corneal epithelium was separated from the underlying stroma with forceps and cut into 1 mm² to 2 mm² blocks. These were then transferred to 75-cm² Falcon tissue culture flasks (Becton, Dickinson, NJ) containing keratinocyte basal medium (Clonetics, San Diego, CA). Epithelial tissue dissected from one to two corneas was used to seed 75-cm² tissue culture flasks. The serum-free medium was supplemented with insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), and bovine pituitary extract (0.40% vol/vol), except as noted in the text (referred to as keratinocyte growth medium, or KGM). Cultures were incubated in a 5% CO₂-95% air atmosphere and were fed every other day.
Haptotactic migration assay was carried out essentially as previously described.26 Fibronectin was purified from human plasma.27 Fibronectin and GRGDSP−OVA were diluted as required into phosphate-buffered saline, pH 7.4, containing Gentamycin (Sigma, St. Louis, MO) 50 μg/ml. The polycarbonate filters (14 μm pore size; Porctics, Livermore, CA) were coated overnight at 37°C. These filters were washed in sterile distilled water and air dried before assembly into the haptotaxis chamber. The cultures were fed with keratinocyte basal medium for 24 hours before harvesting the cells for the migration assay. Primary human corneal epithelial cells were harvested with 5 ml of trypsin−ethylenediaminetetraacetic acid in Hanks’ balanced salt solution. The cells were resuspended in keratinocyte basal medium containing 2 mg/ml bovine serum albumin and were seeded at a final density of (5 × 10^4 cells/well). Epidermal growth factor was added to the cells at this time (in the concentrations indicated in the text) and were included in both upper and lower chambers. The upper and lower wells of the chamber contained equal concentrations of each reagent, except for the cells included only in the top well. The chambers were incubated for 16 to 20 hours at 37°C in 5% CO_2−95% air atmosphere. The filters were removed and then fixed and stained with Diff-Quik (Baxter, McGaw Park, IL) and mounted on a glass slide. Nonmigrated cells (on the top surface of the filter) were removed by gentle swabbing of that surface. Migrated cells on the undersurface were counted using a Zeiss (Thornwood, NY) microscope equipped with a ocular grid. Migration was quantitated in four fields for each well, and the results were expressed as the number of cells migrating per mm^2 in triplicates for each experimental condition.

The Boyden chamber assay is well suited to quantify motility of corneal epithelial cells because the effects of EGF-mediated cell division are separable from motility.29 Prolonged cell migration times for corneal epithelial cells from primary cultures have been used with success.26

**Inhibition of Haptotactic Cell Migration by Anti-Integrin and Anti-Epidermal Growth Factor Receptor Antibodies**

Human corneal epithelial cells were isolated as described. Cells were preincubated for 30 minutes at 37°C with either suitable dilutions of function-blocking integrin antibodies or anti-EGF receptor antibodies before loading on the Boyden chamber. The migration assay was carried out for 20 hours as described above. The following monoclonal antibodies were used: P3D10 (anti-α5) and P5D2 (anti-β1). Anti EGF-receptor was purchased from UBI (Lake Placid, NY).

**RESULTS**

**Dose Dependence of Epidermal Growth Factor Stimulation of Haptotactic Cell Migration of Human Corneal Epithelial Cells**

These studies examined the requirement of EGF by human corneal epithelial cells in the haptotactic migration to fibronectin and the synthetic peptide GRGDSP. Preliminary observations suggested that confluent cells could not be stimulated to migrate by EGF under the same conditions with which the subconfluent cells were able to respond.

Human corneal epithelial cell cultures were fed with keratinocyte growth medium as usual; 24 hours before the migration assay, the cultures were fed with keratinocyte basal medium. The day of the assay, EGF was added to the human corneal epithelial cells in the amounts indicated. For controls, we used cells with no further addition of EGF. The concentrations of EGF tested were in the range of 0.01 to 40 ng/ml; the migration response to EGF was dose dependent, and maximal stimulation was observed between 5 and 10 ng/ml for fibronectin and between 2 and 5 ng/ml for GRGDSP−OVA (Fig. 1). In general, haptotactic cell migration decreased with concentrations of EGF in excess of 10 ng/ml.

Cells from cultures fed with keratinocyte growth medium (as opposed to keratinocyte basal medium) up to the time of harvesting already migrated maximally, and no further effect by EGF was observed even if omitted for the duration of the migration assay (data not shown).

**Inhibition of EGF-Mediated Stimulation of Human Corneal Epithelial Cell Migration by Anti-EGF Receptor Antibody**

An antibody directed to the EGF receptor blocked the EGF-dependent stimulation of haptotactic cell migra-
EGF and Integrins in Corneal Epithelial Migration on FN

FIGURE 1. Dose dependence of epidermal growth factor (EGF) stimulation of haptotactic cell migration of human corneal epithelial cells to fibronectin and GRGDSP-OVA. Polycarbonate filters (14-μm pores) were coated with 5 μg/ml fibronectin or 25 μg/ml GRGDSP-OVA. Human corneal epithelial cells were incubated with EGF concentrations up to 40 ng/ml and were allowed to migrate for 20 hours at 37°C. Error bars represent the mean ± 1 SD; *n = 3.

Inhibition of Human Corneal Epithelial Haptotactic Cell Migration to Fibronectin and GRGDSP by Function-Blocking Integrin Antibodies

The anti-β1 integrin antibody was effective at inhibiting human corneal epithelial cell migration to fibronectin and GRGDSP-OVA. The inhibition of cell migration we observed was greater than 90% with this antibody (Fig. 3). The effect of the anti-α5 antibody on cell migration to fibronectin also was significant, but this inhibition was generally not greater than 44%. The anti-α5 antibody did inhibit haptotactic cell migration to GRGDSP-OVA greater than 90% (Fig. 3). The inhibition of cell migration with function-blocking integrin antibodies was shown to be dose dependent for both antibodies. Control experiments in the presence of normal mouse IgG, anti-α4, were unable to inhibit cell migration to fibronectin (data not shown). These data are consistent with observations suggesting that the α5β1 integrin is important in mediating cell adhesion and haptotactic cell migration to fibronectin. The tripeptide RGD represents a very important adhesion as well as a migration-promoting peptide for these cells (Fig. 3).

Nevertheless, given the available evidence, we would estimate that this may require at least 6 hours, this would suggest that protein synthesis may be required for stimulation of haptotactic cell migration.

Cells incubated with EGF alone and an uncoated filter did not exhibit any detectable migration. When cells were incubated with the anti-EGF-receptor antibody alone in the absence of EGF, no stimulation of cell migration was found. These data suggest that cell stimulation with EGF, or merely binding of the antibody to the receptor, was insufficient to stimulate cell migration.

Because of the duration of the haptotactic migration assay, we were unable to establish the precise length of time required for cell stimulation by EGF.

FIGURE 2. Inhibition of epidermal growth factor (EGF)-mediated stimulation of haptotactic cell migration of human corneal epithelial cells to fibronectin and GRGDSP-OVA by an anti-EGF receptor antibody. Polycarbonate filters (14-μm pores) were coated with 5 μg/ml fibronectin or 25 μg/ml GRGDSP-OVA. Corneal epithelial cells were incubated with 10 ng/ml of EGF and the indicated concentrations of anti-EGF receptor antibody for 30 minutes before loading the cells in the Boyden chamber. Cells were allowed to migrate for 20 hours at 37°C. Error bars represent the mean ± 1 SD; *n = 3.
FIGURE 3. Inhibition of haptotactic cell migration of human corneal epithelial cells to fibronectin and GRGDSP-OVA by function-blocking integrin antibodies to β1 and α5 integrin subunits. Polycarbonate filters (14-μm pores) were coated with 5 μg/ml fibronectin or 25 μg/ml GRGDSP-OVA. The corneal epithelial cells were incubated with 10 ng/ml of epidermal growth factor (EGF) and the indicated dilutions of anti integrin antibodies for 30 minutes before inclusion in the Boyden chamber. Cells were allowed to migrate for 20 hours at 37°C. Error bars represent the mean ± 1 SD; n = 3.

DISCUSSION

The human corneal epithelium heals by a number of biologic processes that involve cell spreading and migration, cell division, and matrix synthesis. Recapitulation is an important and critical event by which the wound is resurfaced by migrating epithelial cells. Our laboratory has shown that fibronectin may have an important role in reepithelialization because it promotes haptotactic cell migration of rabbit corneal epithelial cells.

Epidermal growth factor specifically potentiated haptotactic cell migration of human corneal epithelial cells. These cells required stimulation with EGF to permit cell migration when grown for 24 hours in keratinocyte basal medium only. This effect was shown to be dose dependent, revealing that the migration of these cells is directly related to functional integrins on the cell surface. In agreement with previous reports in which chemotactic migration of corneal epithelial cells in response to EGF were studied, haptotactic cell migration declined when EGF concentrations exceeded optimal values; these effects may be ascribed to downregulation of the EGF receptor in response to excess ligand. These data support the in vivo wound repair studies that have shown enhancement of reepithelialization by the exogenous application of EGF and of fibronectin. These results point to an important relationship between EGF cell stimulation and migration to fibronectin and to the RGD-bearing peptide. Unlike results of a previous report, fibronectin alone was unable to stimulate haptotactic cell migration of human corneal epithelial cells. These differences may be attributed to species differences in cell migration.

The EGF-dependent stimulation of haptotactic cell migration was blocked specifically with an anti-EGF receptor antibody. These data suggest that EGF in particular is responsible for stimulating human corneal epithelial cell migration to fibronectin and GRGDSP peptide. Addition of the neutralizing anti-EGF receptor antibody in the presence of EGF significantly decreased cell migration to levels just above those of the unstimulated controls.

Antibodies directed specifically to the β1 integrin subunit blocked human corneal epithelial cell migration to fibronectin in a dose-dependent fashion. These data suggest that members of the β1 subfamily of integrins are important not only in cell adhesive events but also in haptotactic cell migration. The role of the α5β1 integrin in cell migration of the human corneal epithelial cells may be restricted to the expression of this integrin when the tissue undergoes repair because it is not clear whether this heterodimer is expressed in the normal corneal epithelium. In this study, we have shown that human corneal epithelial cells undergo haptotactic migration in the presence of fibronectin; haptotactic cell migration occurred only when cells were stimulated by the soluble growth factor EGF. Our data show that the migration of human corneal epithelial cells in response to fibronectin requires functional cell surface integrins. These data as a whole suggest that EGF may exert its role at the level of the integrin receptors at the cell surface. Whether EGF affects the affinity of specific integrins, interactions of the integrin with the cytoskeleton, or cell surface expression is a matter of speculation.

Human corneal epithelial cells most likely derive positional information, as well as migratory and growth cues, mediated through a recognition system that involves RGD and selected peptides from basement membrane components. These specific signals may be important in directing certain stages of cellular differentiation and the reestablishment of the normal corneal epithelium morphology and function. Our data point to a possible role for EGF in enabling human corneal epithelial cells to interact with the extracellular matrix and the basement membrane.
EGF and Integrins in Corneal Epithelial Migration on FN

Key Words
epidermal growth factor (EGF), fibronectin, haptotactic cell migration, human corneal epithelia, integrin

Acknowledgments
The authors thank Dr. E. Wayner for the monoclonal antibodies, and they thank Xiaoiling Wang for her expert technical assistance with the tissue culture and cell migration assays.

References
28. Grant MB, Khaw PT, Schultz GS, Adams JL, Shimizu RW. Effects of epidermal growth factor, fibroblast growth factor, and transforming growth factor-β on


