Insulin-like Growth Factor-1 Induces Migration and Expression of Laminin-5 in Cultured Human Corneal Epithelial Cells

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Purpose. The effects of insulin-like growth factor (IGF)-1 on laminin (Ln)-5 and the associated integrins during in vitro HCEC migration were examined. Also investigated were the effects of IGF-1 on the migration of human corneal epithelial cells (HCECs).

Methods. HCEC migration was examined by wound-healing and chemoattraction assays. For migration inhibition assays, HCECs were pretreated with inhibitors of the IGF-1 receptor (αIR3), the PI3-K/AKT pathway (LY294002), and the MEK-ERK pathway (PD98059). The expression levels of Ln-5 and fibronectin (Fn) were determined by Western blot analysis, and the expression levels of the β1 and α3 integrins were determined by confocal microscopy and Western blot analysis. The migration inhibition with anti-integrin α3 and β1 antibodies was also determined.

Results. HCEC migration was significantly increased in the presence of IGF-1 and Ln-5. IGF-1 enhanced the production of Ln-5 in both a dose- and time-dependent manner, and this upregulation was blocked by pretreatment with αIR3 or LY294002. IGF-1 treatment upregulated expression of β1 integrin, but not α3 integrin. The HCEC migration facilitated by IGF-1 was inhibited with the anti-integrin antibody for β1. However, there was no cross-talk between Ln-5 and integrin β1 production.

Conclusions. The results reveal that IGF-1 induces HCEC migration through the independent production of Ln-5 and β1 integrin, which are directed at least in part by activation of the PI3-K/AKT pathway, but are not affected by the MEK-ERK pathway. (*Invest Ophthalmol Vis Sci.* 2006;47:873–882) DOI: 10.1167/iovs.05-0826

The corneal epithelium is a self-renewing, stratified epithelium that provides the first line of defense against invading microorganisms. The integrity of the corneal surface depends on a delicate balance among the forces of corneal epithelial cell proliferation, migration, differentiation, and desquamation.[1] Disruption of the corneal epithelial barrier activates the corneal healing and remodeling processes, which can predispose the tissue to stromal ulceration and cause corneal opacification, ultimately leading to irreversible visual deficits. Epithelial healing is achieved by migration of the epithelial cells to cover the denuded area and enhanced cell proliferation to reestablish the epithelial structure. Corneal epithelial cell migration is modulated by various humoral and extracellular matrix (ECM) proteins.[1] Growth factors, such as insulin-like growth factor (IGF)-1,[2] epidermal growth factor (EGF),[3] interleukin 6,[4] fibroblast growth factor-2 (FGF-2),[5] transforming growth factor-β (TGF-β),[6] keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF)[7,8] have been shown to stimulate corneal epithelial cell migration both in vivo and in vitro. Glycoproteins of the ECM, such as fibronectin (Fn), laminin (Ln), and collagen IV, also facilitate cell migration.[9,10] However, the precise relationship between these motogenic cytokines and ECM protein production in the corneal epithelium is not well understood.

The Ln proteins are a family of ECM molecules that exist as cross-shaped heterotrimers of the α-, β- and γ-chains and are mainly localized in the basement membranes of various tissues. The five α-, three β-, and three γ-chains give rise to at least 15 Ln isoforms.[11] Of them, Ln-1 and -5 are known to be components of the corneal basement membrane. Ln-1 is the universal isoform of Ln and comprises almost all the tissues in the basement membrane,[11] whereas Ln-5, which is composed of the α3-, β3-, and γ2-chains, is a component of skin and corneal basement membranes and is known to be involved in epithelial cell migration and adhesion.[12,13] Genetic defects in any of the three Ln-5 subunits cause junctional epidermolysis bullosa,[14] and Ln-5 has been shown to be overexpressed at the sites of epithelial wounds[15] and the leading edges of invading carcinoma cells.[16] Taken together, these findings suggest that Ln-5 may play an important role in epithelial cell homeostasis, cell migration, and cancer cell invasion.

IGF-1 is a multifunctional regulatory peptide that shares structural homology with proinsulin. It has been shown to mediate proliferation, differentiation, and survival effects, depending on the target cell and the presence of other hormones and growth factors.[17] Accumulating evidence indicates that IGF-1 promotes cell motility in a variety of normal and malignant cell types, including the corneal epithelial cells of some species. Nishida et al.[18] reported that substance P and IGF-1 synergistically stimulated corneal epithelial migration in an organ culture of the cornea, and found that although addition of SP or IGF-1 alone did not affect epithelial migration, cotreatment with SP and IGF-1 significantly stimulated it.[19] Of note, no previous work has examined the migratory effects of IGF-1 in primary human corneal epithelial cells (HCECs), or the possibility of cross-talk between ECM components and migration-associated cytokines in this context.

In the current study, we investigated whether IGF-1 stimulates HCEC migration and whether the expression levels of Ln-5 and/or the Ln-5-associated integrins are altered during IGF-1-induced HCEC migration in vitro.

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Materials and Methods

Reagents

Human recombinant Ln-5, which consists of the 160-kDa α3-chain, the 135-kDa β3-chain, and the 150- and 105-kDa forms of the γ2-chain, was purified as previously described.[20] The monoclonal human Ln-5 antibody (P3H9–2) was purchased from Chemicon (Temecula, CA), polyclonal Ln-β3 (H-300) and -γ2 (H-183) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and human Fn and type IV collagen from Sigma-Aldrich Chemical Co. (St. Louis, MO). The monoclonal anti-α3 integrin antibody (Gi9) was purchased from Beckman Coulter (Fullerton, CA) and monoclonal anti-integrin β1 (CD29; clone JB1A) from Chemicon. Anti-phospho-AKT (Ser473) and anti-phospho-ERK (Thr980) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-JNK (SAPK; Thr183/Tyr185) and anti-human p38/SAPK2, a monoclonal antibody (clone 2F11) were purchased from Biosource International (Camarillo, CA). Neutralizing human anti-IGF-1R mAb αIR3 antibodies were obtained from Oncogene Research Products (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture, fetal bovine serum (FBS), HEPES-buffer, amphotericin B, and gentamicin were purchased from Invitrogen-Gibco (Grand Island, NY). Other reagents and chemicals, including mouse-derived epidermal growth factor (EGF), cholera toxin (subunit A), dimethyl sulfoxide (DMSO), hydrocortisone, transferrin, and human insulin were purchased from Sigma-Aldrich. Dispase II was purchased from Roche Applied Science (Mannheim, Germany). Affinity-purified goat polyclonal antibodies against human IGF-1 and IGF-1 receptor (IGF-1R) were purchased from R&D Systems, Inc. (Minneapolis, MN). Horseradish peroxidase–conjugated secondary antibodies were purchased from Dako (Glostrup, Denmark), and LY294002 and PD98059 were purchased from Calbiochem (Darmstadt, Germany).

Preparation of Human Corneal Limbal Tissue for Primary Epithelial Cell Culture

In accordance with the tenets of the Declaration of Helsinki and with the permission of the institutional review board, human corneal limbal tissue was harvested from donor corneal buttons after keratoplasty. The corneal limbal tissues were washed three times with DMEM containing 50 g/mL gentamicin and 1.25 μg/mL amphotericin B. After removal of excessive conjunctiva, sclera, and iris tissues, the remaining tissue samples were placed in culture dishes and exposed for 1 hour to 1.2 U/mL dispase II in a Mg^{2+}- and Ca^{2+}-free solution at 37°C under 95% humidity and 5% CO_2. In each case, the epithelial tissue was separated from the rest of the tissue and cut into 2.0-mm^2 tissue sections with a no. 15 blade and scissors. The segmented epithelial tissue was placed on the upper chamber of a cell migration apparatus (Transwell; diameter, 6.5 mm; pore size, 0.4 μm, Corning-Costar, Acton, MA) containing 3T3 fibroblasts in the lower chamber, and cells were cultured in supplemented hormonal epithelial medium (SHEM) made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F-12 medium supplemented with 10% FBS, 0.5% DMSO, 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B, 2 ng/mL mouse EGF, 5 μg/mL insulin, 5 μg/mL transferrin, 0.5 mg/mL hydrocortisone, and 30 ng/mL cholera toxin. Human corneal limbal epithelial cells were maintained at 37°C under 95% humidity and 5% CO_2. The medium was changed every other day, and cell outgrowth was monitored daily for 3 weeks by inverted phase microscopy (model IX70; Olympus, Tokyo, Japan). When the cultured corneal epithelium was ~80% to 90% confluent, the cells were subcultured with 0.25% trypsin and 5.0 mM EDTA with a 1:3 split. Second- and third-passage cells were used for all experiments involving cultured human corneal epithelium.

Migration Assay

HCEC migration was determined by monolayer wound-healing and chemoattraction assays. During these experiments, we omitted both serum and growth factors for 12 hours from the SHEM to mitigate the effect of migration-related cytokines. All the experiments were performed three times in a minimum of three samples, and at least three different areas were observed in each sample.

For monolayer scratch assays, 2×10^6 cells were plated onto a chamber slide and incubated in SHEM. When a confluent monolayer was formed, cells were incubated in serum- and growth factor-free SHEM medium for 12 hours. Then, a scratch wound was made with a fire-polished glass pipette, and the cells were further incubated in the presence or absence of various concentrations of IGF-1, Ln-5, type IV collagen, and Fn. Migration was examined at various time points up to 48 hours. The plates were washed three times with PBS, and the migratory cells were counted and photographed with a microscope (model IX70; 50× and 100× magnifications; Olympus, Tokyo, Japan,).

The chemotaxis assays were performed with a modified Boyden chamber assay. Briefly, cell-migration chambers (six-well plate, Transwell; Corning-Costar) were used, containing 6.5-mm, 8.0-μm pore size polycarbonate membrane inserts that were coated with 500 ng/mL human type IV collagen, Fn, or various concentrations of recombinant Ln-5 (10, 50, 100, or 1000 ng/mL) in PBS for 2 hours at 37°C. Then, the inserters were rinsed twice with PBS and blocked with 1% heat-inactivated BSA in PBS for 60 minutes at room temperature. The upper chamber was loaded with HCECs at 1000 μL of 2×10^6cells/mL, and the apparatus was incubated for 24 hours. Before counting the migratory cells on the lower surface of the inserter, the lower surface of the membrane was washed twice with PBS, fixed with 4% paraformaldehyde with PBS for 15 minutes, and stained with 0.1% crystal violet for 15 minutes. The migratory cells were quantified by counting three fields of view (100×) with the same microscope used in the monolayer scratch assay. Experiments were performed in triplicate and repeated at least twice.

Migratory Function Blocking Study

For inhibitory studies, cells were incubated for 2 hours with one of the following: 25 or 50 μM of LY294002 inhibitor of phosphoinositide-3 kinase (PI3-K)/AKT) and PD98059 (inhibitor of ERK), blocking or neutralizing antibodies against IGF-1 receptor (αIR3, 10 μg/mL), β1 integrin (10 μg/mL), α3 integrin (10 μg/mL), or an IgG matched control mAb, and migratory assays were performed as just described.

Western Blot Analysis

Cells were washed with ice-cold PBS and then lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% glycerol, 10 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% Triton X-100) on ice for 30 minutes. Lysates were sonicated, and the cell homogenates were centrifuged at 15,000*g* for 10 minutes. The protein concentrations in the resultant supernatants were determined with the Bradford reagent, and equal amounts of protein (30 μg) were boiled in Laemmli sample buffer and resolved by 8% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Billerica, MA) and probed overnight with antibodies against Fn; Ln α3-, -β3-, and -γ2-chains; and integrins α3 and -β1 (diluted 1:500–1:3000). The immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibodies and visualized by enhanced chemiluminescence.

Immunocytochemical Staining

Cells were fixed for 5 minutes in 3.7% formaldehyde and then permeabilized in 0.5% Triton X-100 for 8 minutes. Single- or double-label immunofluorescence was performed. In control experiments, samples were run without primary antibodies or with irrelevant IgG to determine nonspecific binding of secondary antibodies. For the experiments, the samples were incubated with anti-integrin α3, -integrin β1, and -Ln-5 for 2 hours at room temperature, followed by incubation for 1 hour with FITC-conjugated secondary antibodies. The samples were then rinsed with PBS and observed under a fluorescence microscope (Eclipse TE200 equipped with a digital camera DXM 1200; Nikon,

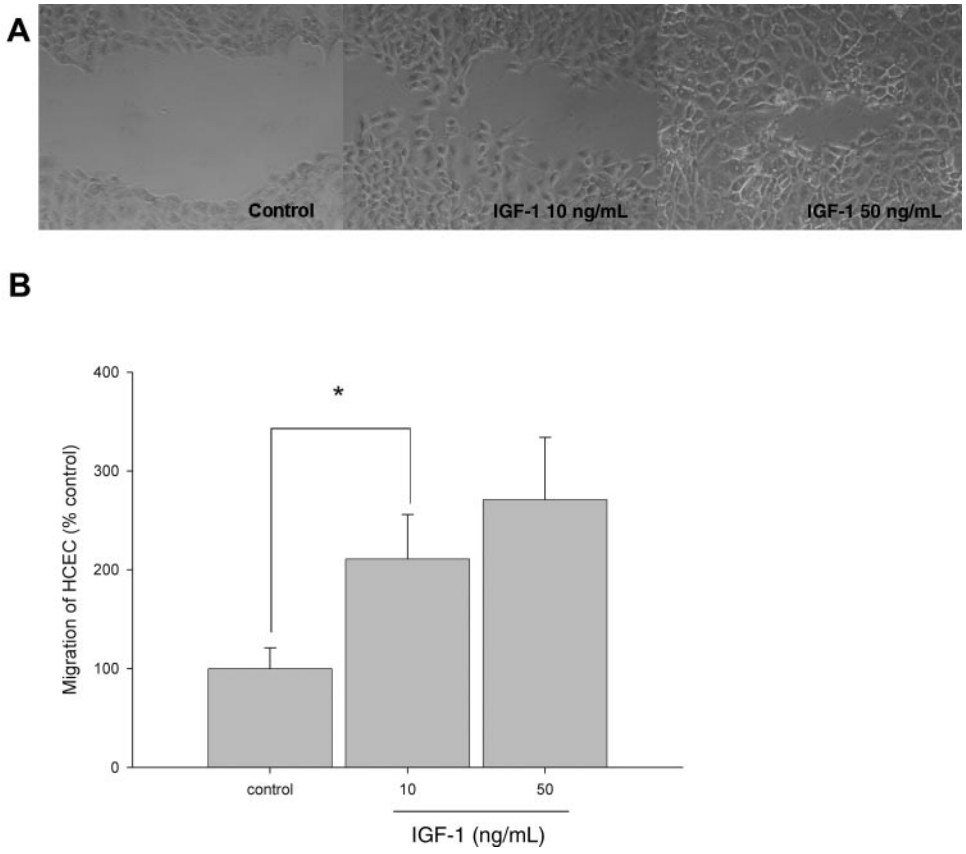

FIGURE 1. Cell restitution assay of the effect of IGF-1 on migration of HCECs. Monolayers were scratched and treated with or without 10 or 50 ng/mL IGF-1. Twelve hours later, the plates were washed three times with phosphate buffered saline (PBS), and the migratory cells were counted and photographed with an inverted microscope. (**A**, **B**) *Significant difference ($P < 0.05$; one-way ANOVA). Bars, ±SE.

Tokyo, Japan) using appropriate filters for visualization of the fluorescein.

Statistics

All data are expressed as the mean ± SD. Multivariate analysis of variance with post hoc Newman-Keuls tests determined significant differences between the treatment groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of IGF-1 on HCEC Migration

HCEC monolayers were wounded, and migration was assessed after treatment with various concentrations of IGF-1. Twelve hours after wounding, serum- and growth factor–free control cells demonstrated minimal movement into the denuded area. In contrast, cells treated with 10 and 50 ng/mL IGF-1 substantially migrated into the denuded area and showed a dose-dependent relationship with the concentration of applied IGF-1 (Figs. 1A, B) Migrating cells were flattened, with ruffling borders and lamellipodia (Fig. 1A). These morphologic changes were more clearly obvious in IGF-1-treated cells than in control cells. These results indicate that IGF-1 treatment dose dependently increases migration of HCECs.

Effect of IGF-1 on PI3-K and MAPK Signaling in HCECs

To examine IGF-1 signaling in human corneal epithelial cells, serum- and growth factor-starved cells were treated with 10 ng/mL IGF-1 for up to 3 hours and analyzed for activation of the PI3-K/AKT and mitogen-activated protein kinase (MAPK) pathways (Fig. 2). Phosphorylation of AKT was detectable within 5 minutes and persisted for at least 180 minutes after IGF-1 treatment, whereas phosphorylation of ERK1/2 was increased at 10 minutes and remained above basal level for at least 180 minutes. In contrast, there was no evidence of activating phosphorylation of JNK or p38. These data suggest that in HCECs, IGF-1 induces activation of PI3-K/AKT and ERK 1/2, but not JNK or p38.

Effect of Inhibition of IGF-1 Signaling Components on IGF-1-Induced HCEC Migration

Because our results revealed that PI3-K and ERK 1/2 were involved in IGF-1-mediated intracellular signaling in HCECs, and a previous study had shown that $\alpha 3$ and $\beta 1$ integrins

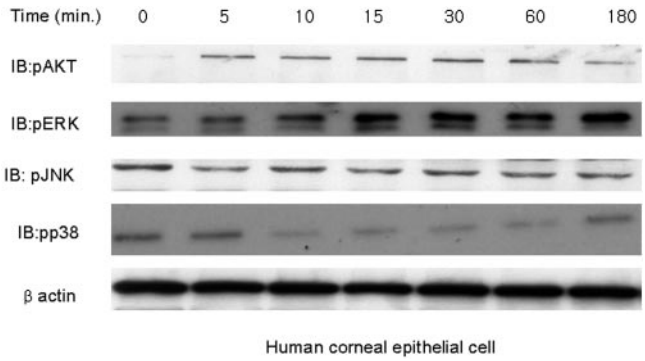

FIGURE 2. Western blot analysis of phospho-AKT, -ERK 1/2, -JNK, and -p38 in HCECs treated with IGF-1 (10 ng/mL). Serum- and growth factor–starved HCECs were treated with IGF-1 for the indicated times, and cell lysates (30 μg) were resolved by 8% SDS-PAGE and immunoblotted with anti-pAKT, -pERK1/2, -phospho-JNK, and -phospho-p38 antibodies. β-Actin served as the loading control.

mediate epithelial cell migration in other cell types,[21] we next investigated the role of these factors in IGF-1-induced cell migration. Wound-healing assays were conducted as described earlier for 12 hours, and cell migration was compared between cells treated with IGF-1 (10 ng/mL) alone and those pretreated with neutralizing antibodies and chemical inhibitors. As shown in Figure 3, LY294002 (25 μM) and anti-β1 integrin antibodies (10 μg/mL) completely blocked the IGF-1-induced HCEC migration, whereas an ERK inhibitor (PD98059; 25 μM) and the anti-α3 integrin antibodies (10 μg/mL) did not. These data confirm that PI3-K and β1 integrins are associated with IGF-1-related HCEC migration.

Effect of IGF-1 Treatment on Ln-5 Subchain Expression

As Ln-5 and Fn are known to be important modulators of HCEC migration,[1,10] we next used Western blot analysis to investigate the expression of Ln-5 and Fn after IGF-1 treatment of HCECs. Ln-5 γ2 subchain production was increased at 6 hours after treatment of IGF-1 (10 ng/mL), but no change in Fn production was seen after IGF-1 treatment (Fig. 4A). Treatment with the ERK inhibitor (PD98059) had no effect on the IGF-1-induced production of the γ2 subchain, whereas the specific PI3-K inhibitor (LY294002) decreased the expression of the Ln-5 γ2 subchain (Fig. 4B).

To determine the expression of Ln-5 within a time sequence, immunoblot analysis for Ln-5 was performed after IGF-1 treatment. In this experiment, some cells were pretreated with 25 and 50 μM LY294002, 2 hours before IGF-1 treatment. The expression levels of the Ln-5 α3-, β3-, and γ2-subchains were significantly upregulated within 3 hours after treatment, compared with the serum- and growth factor-free control cultures (Fig. 4C). Both the processed (160 kDa) and unprocessed (190 kDa) forms of the α3 subchain were increased at 3 hours, but these levels had returned to control levels at 6 and 12 hours. The expression levels of the β3 (155 kDa) and γ2 (150 kDa: unprocessed, 105 kDa: processed) subchains were significantly increased at 3 and 6 hours after IGF-1 treatment and had returned to basal levels by 12 hours after treatment. The γ2 subchain expression levels peaked at 6 hours after treatment and showed the largest IGF-1-induced change. Pretreatment of LY294002 blocked IGF-1-mediated production of the β3- and γ2-subchains in a dose-dependent manner (Fig. 4C, lanes 3, 4).

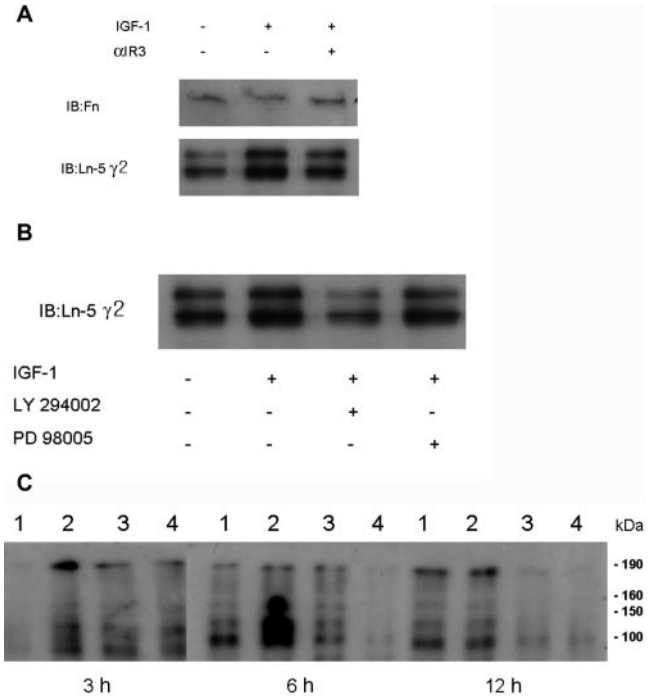

FIGURE 4. IGF-1-induced Ln-5 expression was determined by Western immunoblot. Serum- and growth factor-starved cells were treated with IGF-1 (10 ng/mL) and incubated for 6 hours. Cell lysates were examined for production of Ln-5 subchain-γ2 and Fn by Western blot analysis (**A**, **B**). Two hours before the IGF-1 treatment, some cells were pretreated with a neutralizing antibody against the αIR3 IGF-1 receptor, or inhibitors LY294002 (25 μM) and PD98059 (25 μM). Ln-5 expression was compared over time. *Lane 1*: serum and growth factor free; *lane 2*: 10 ng/mL IGF-1 treatment; *lane 3*: 10 ng/mL IGF-1+25 μM LY294002; and *lane 4*: 10 ng/mL IGF-1+50 μM LY294002 (**C**).

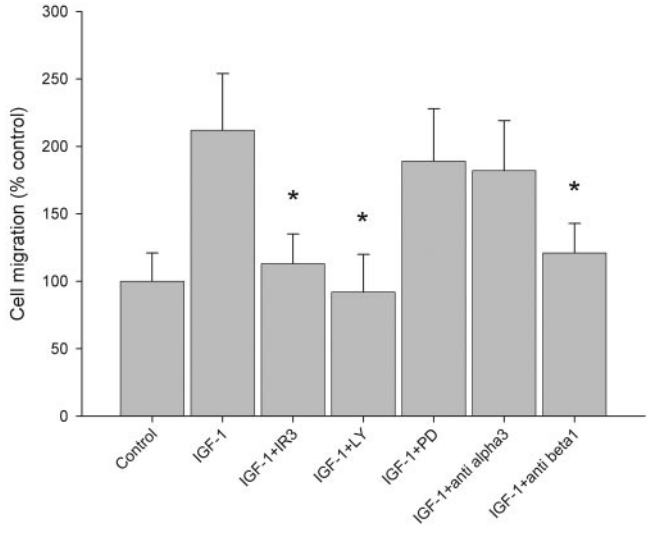

FIGURE 3. HCEC were pretreated with neutralizing antibodies against the αIR3 IGF-1 receptor (10 μg/mL) and integrin β1 (10 μg/mL) and α3 (10 μg/mL), as well as inhibitors against PI3 kinase (LY294002; 25 μM) and ERK (PD98059; 25 μM). The cells were then treated with IGF-1 (10 ng/mL) and migration was assessed in a wound-healing assay at 12 hours by microscope. *Significant ($P < 0.05$; one-way ANOVA) inhibition compared with IGF-1 treatment alone. Bars, ±SE. Magnification, ×50.

Localization of Ln-5 during IGF-1-Induced HCEC Migration

As the translocation of Ln-5 has been associated with cell migration or attachment,[13,16] indirect fluorescein microscopy was used to examine the localization patterns of Ln-5 during a monolayer scratch assay with 10 ng/mL IGF-1 treatment of HCECs. In serum-starved control cells, fewer migratory cells were noted, and Ln-5 was not exactly localized (Fig. 5A). Three hours after IGF-1 treatment, significant Ln-5 expression was observed in the migratory leading cells (Fig. 5B, arrows), compared with the trailing cells (Fig. 5B, arrowheads). Time course analysis revealed that in IGF-1-treated cells, Ln-5 expression progressively formed continuous immunoreactive lines along the borders of migratory cells (Figs. 5B, 5C). In cells pretreated with LY294002 before IGF-1 treatment, Ln-5 expression did not form continuous lines along the borders of cells (Fig. 5D). In sharp contrast, Ln-5 was also expressed within the marginal membrane protrusions of cells treated with IGF-1 (Fig. 5E).

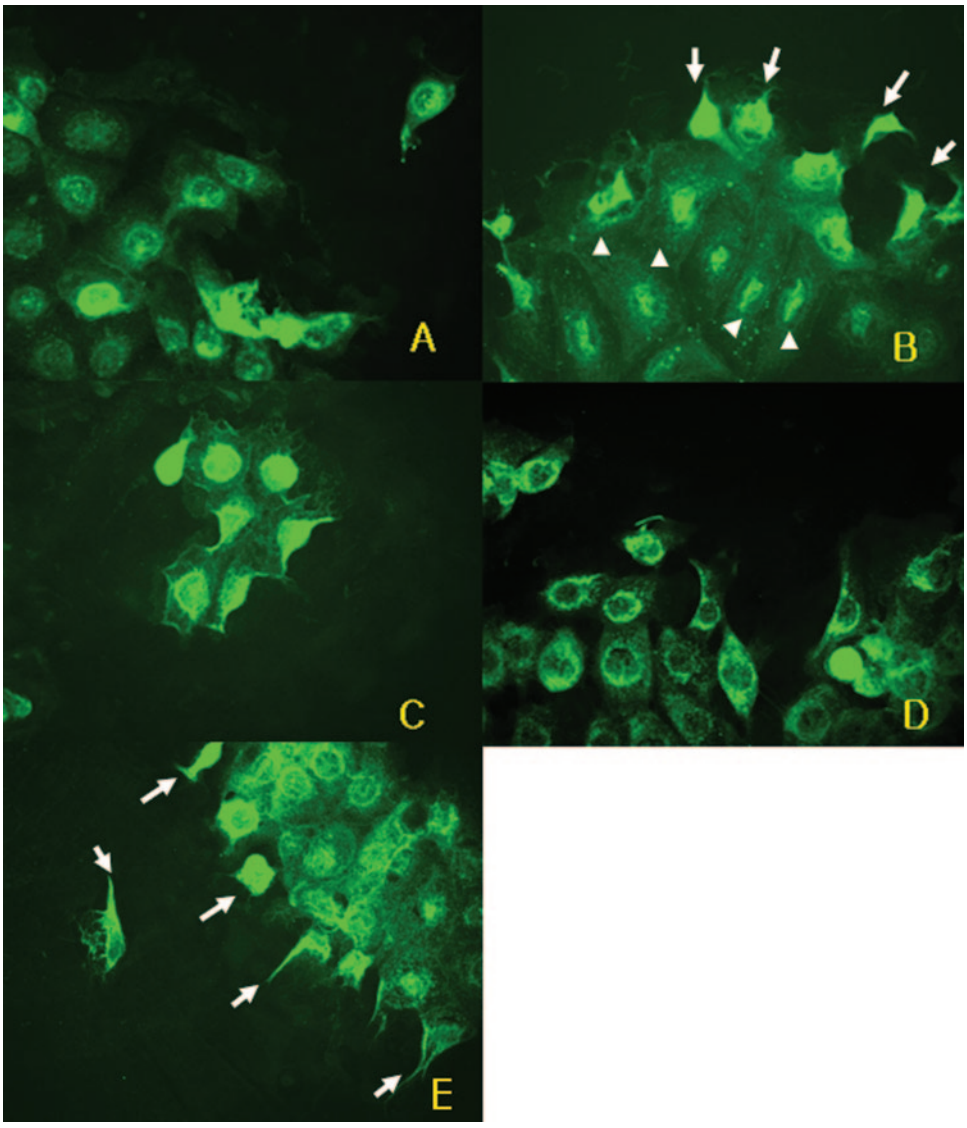

FIGURE 5. Localization of laminin-5 in HCECs in the absence or presence of IGF-1. An indirect immunofluorescence method was used to examine Ln-5 localization in serum- and growth factor–free control cells (**A**), as well as those treated with 10 ng/mL IGF-1 for 3 (**B**) and 6 (**C**) hours, and those pretreated with 25 μM LY294002 for 2 hours before treatment with IGF-1 (**D**). *Arrows*: cytoplasmic processes and localization of laminin-5 (*arrowheads*) in migrating cells 3 hours after treatment with IGF-1 (**E**).

HCEC Migration on Matrix Glycoproteins, Fn, Ln-5, and Type IV Collagen

As our results suggested that IGF-1 can induce HCEC migration and upregulation of Ln-5, we next investigated the effects of Ln-5 on human corneal epithelial cells in early epithelial wound healing. To compare the role of Ln-5 in cell migration with that of other basement membrane glycoproteins, including Fn and type IV collagen, cell migration was determined by chemoattraction assays on glycoprotein-precoated chamber slide inserts. As shown in Figure 6, HCEC migration increased on all the tested matrix glycoproteins over 24 hours, compared with the serum- and growth factor-free control cultures. The Ln-5-coated inserts showed the highest levels of cell migration. These levels began to show a statistically significant difference from 6 hours after treatment, compared with the type IV collagen- and Fn-coated slides, which showed increased cell migration up to 24 hours after treatment.

To confirm these findings, another migration assay was performed with a monolayer scratch injury, using cells incubated in medium containing the various basement membrane glycoproteins. The wounded monolayers were treated with or without 500 ng/mL type IV collagen, Fn, or Ln-5, and migration was observed. At 12 hours after assay, the Ln-5-treated HCEC monolayer condition showed the highest levels of migration compared with Fn- or type IV collagen–treated cells (Fig. 6B).

Next, the dose–response effect of Ln-5 on HCEC migration was examined in additional chemoattraction assays. HCEC migration was not induced by Ln-5 concentrations below 100 ng/mL, whereas Ln-5 concentrations between 100 and 1000 ng/mL induced a significant increase in HCEC migration (Fig. 7A). With the time sequence, from 6 hours after the assay, HCEC migration was facilitated by Ln-5, compared with the control (Fig. 7B).

Effect of IGF-1 Treatment on Expression of β1 Integrin and α3 Integrin

In mammary epithelial cells, motility and adhesive interactions with the matrix may be mediated by integrin expression levels.[22] Thus, we tested whether IGF-1-treated HCECs showed changes in the expression levels of α3 and β1 integrins, which are known to be related to Ln-5-associated cell migration.[21] Western blot analysis revealed that β1 integrin expression was increased at 6 and 12 hours after cells were treated with IGF-1, and that this increase could be blocked by pretreatment with LY294002 (Fig. 8).

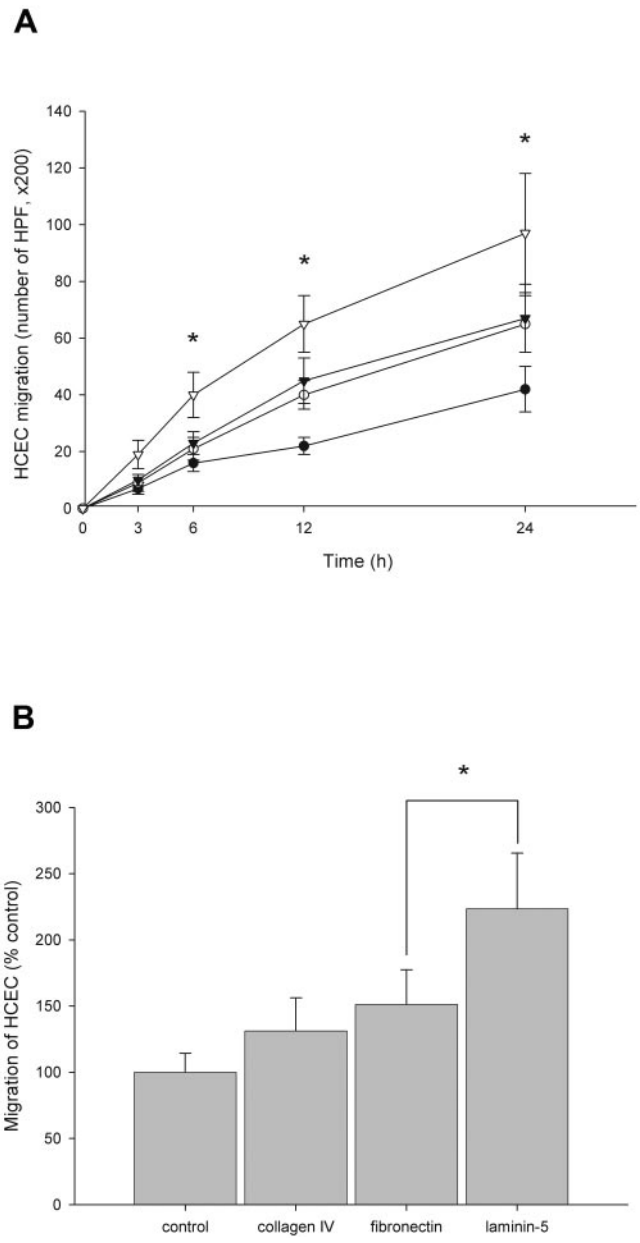

FIGURE 6. Comparison of HCEC migration on inserts coated with collagen type 4 IV (○; 0.5 μg/mL), Fn (▼; 0.5 μg/mL), or Ln-5- (▽; 0.5 μg/mL), and serum- and growth factor-free controls (●). HCEC migration was determined with chemoattractant assays for 24 hours (**A**). Wound-healing experiments were conducted with or without 500 ng/mL of type IV collagen, Fn, or Ln-5, and migratory cells were counted 12 hours after injury (**B**). *Significant difference ($P < 0.05$; one-way ANOVA) compared with cells treated with 0.5 μg/mL Fn. Bars, ±SE.

Effect of Neutralizing Anti-IGF-1 and Anti-integrin Antibodies on HCEC Migration

To determine the relationships among Ln-5, integrin α3, and integrin β1, HCEC migration on Ln-5 was examined in the presence of anti-α3 and -β1 antibodies. As shown in Figure 9A, at 12 hours, the anti-integrin β1 neutralizing antibodies significantly and dose dependently decreased cell migration on Ln-5, whereas the anti-integrin α3 antibodies had a less-pronounced inhibitory effect. Compared with the untreated control, cell migration was inhibited by 45% in cultures treated with 20 μg/mL anti-integrin β1, but only by 11% in cultures treated with 20 μg/mL anti-integrin α3. These inhibitory effects continued for 48 hours (Fig. 9B). Treatment with neutralizing antibodies against both α3 and β1 had an inhibitory effect on cell migration similar to that in cells treated with the anti-β1 antibody alone (Fig. 9).

As these data indicate that the functional upregulation of both Ln-5 and integrin β1 were essential for IGF-1-induced HCEC migration, we investigated the production of integrin β1 in HCECs grown on Ln-5-coated matrices, to determine whether cross-talk exists between Ln-5 and integrin β1. The IGF-1-induced production of integrin α3 or β1 was similar in the presence or absence of Ln-5 (Fig. 10), indicating no evidence of cross-talk.

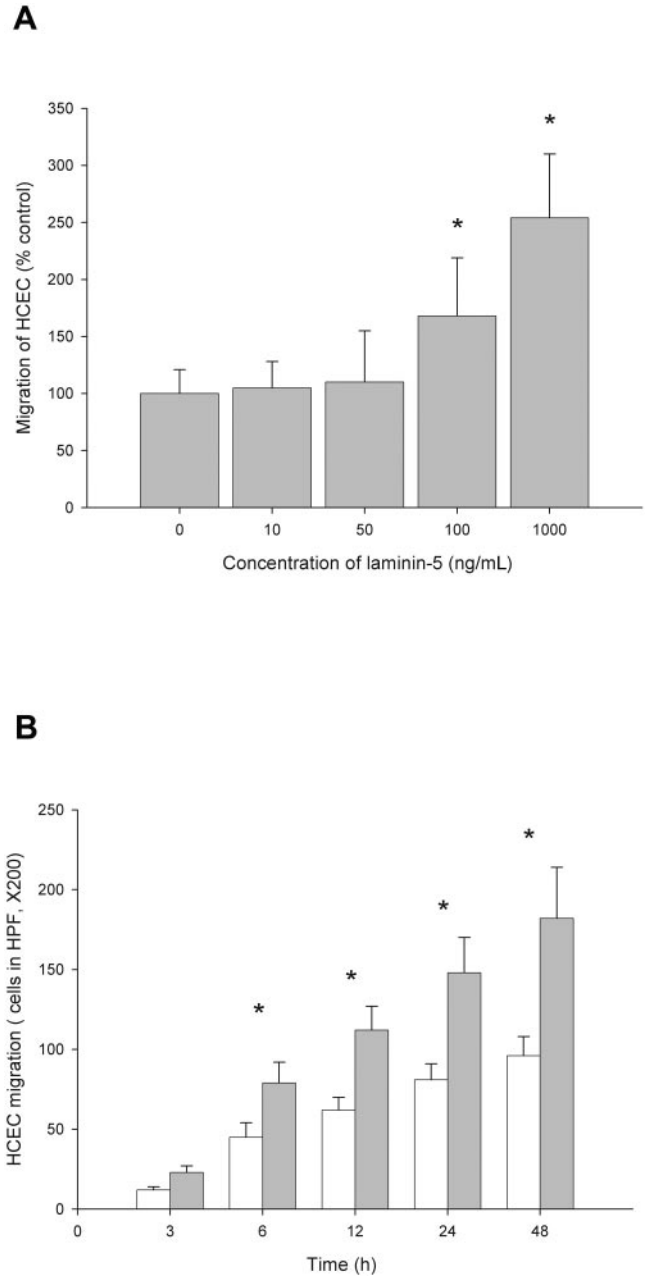

FIGURE 7. HCEC migration on Ln-5-coated inserts of a cell-migration chamber over different doses (**A**) and time points (**B**). (□) Without Ln-5; (▨) with Ln-5 (100 ng/mL). *Significant difference ($P < 0.05$; one-way ANOVA) compared with serum- and growth factor–free controls.

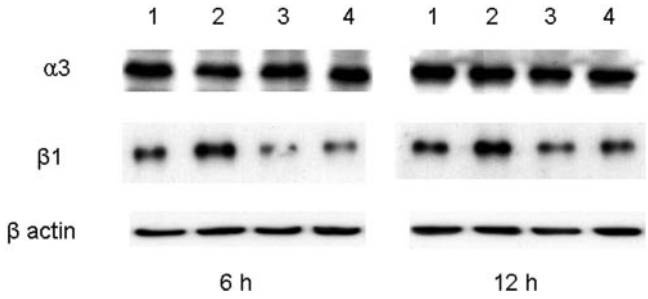

FIGURE 8. The expression levels of integrin $\alpha 3$ and $\beta 1$ in IGF-1-treated HCECs. Cells were pretreated with LY294002 or left untreated for 2 hours, then treated with or without IGF-1 for up to 12 hours. HCEC lysates were subjected to Western blot analysis with anti-integrin $\alpha 3$ and $\beta 1$ antibodies. *Lane 1*: serum- and growth factor–free control; *lane 2*: IGF-1 10 ng/mL; *lane 3*: 25 μM LY294002 pretreatment; and *lane 4*: 50 μM LY294002 pretreatment.

DISCUSSION

The present study demonstrates that IGF-1 treatment of HCECs can induce the expression of the matrix protein Ln-5 and its cell surface receptor integrin $\beta 1$, and that these effects are associated with increased HCEC migration. The induction of Ln-5 and integrin $\beta 1$ by IGF-1 was inhibited by LY294002, indicating the involvement of PI3-K/AKT signaling in this response.

IGF-1 is a multifunctional peptide capable of promoting migration in a wide range of normal and tumor cell types,[23] including corneal epithelial cells. Although IGF-1 expression has not yet been shown in the cornel epithelium in vivo, expression of IGF-1R has been found in corneal epithelial cells in vivo.[24] Furthermore, we identified IGF-1 receptor expression in HCECs from a paraffin-embedded human corneal tissue block, particularly in the limbal area, and noted IGF-1 production in cultured HCECs (data not shown). These results suggest that IGF-1 may be a key cytokine involved in processes central to maintenance of the corneal epithelial surface (i.e., migration and attachment). Recently, Shanley et al.[25] reported that insulin, a peptide that is closely related to IGF-1 and shares similar mechanisms of signal transduction, promotes HCEC migration through PI3 kinase signaling. PI3 kinase is an essential molecule in IGF-1-induced cell migration and has anti-apoptosis signaling functions in various cell types, including corneal epithelial cells.[26] IGF-1 is also known to promote corneal epithelial cell (CEC) migration via substance P (SP).[18,19] However, the migratory effect of IGF-1 is thought to be species- and cell type–specific. Several studies have reported that IGF-1 stimulated the migration of CECs with a synergistic effect of SP in rabbit[2,18,27] and rat models.[27,28] However, Murphy et al.[29] reported that in dogs with spontaneous chronic corneal epithelial defects, IGF-1 did not play a critical role in corneal wound healing. Furthermore, McDermott et al.[30] reported that topical application of SP and/or IGF-1 did not modulate corneal epithelial wound healing in galactosemic rats. However, few prior studies have examined corneal epithelial cell migration in primary human cells. In this study, it was demonstrated that IGF-1 has a migratory effect on HCECs, and that this effect involves signaling via the PI3 kinase pathway, but not the ERK, JNK, or p38 pathways.

Cell migration to cover the defective area is one of the most important events in wound repair. Although many signaling pathways related to cell migration have been identified, the induction of signals that allow cells to migrate is not well understood. Also, the pathways are different for each cytokine or in each cell type. Andre et al.[31] reported that in a wound-healing assay, IGF-1-induced colonic epithelial cell migration occurred through multiple signaling pathways, including PI3-K, MAP kinase, and protein kinase C (PKC)-γ and -δ. However, in MCK-7 cells, the PI3-K pathway was important for cellular motility, but the MAP kinase pathway was not.[32] The function of MAP kinase was found to be essential for the motile response to MDCK canine epithelial cells,[33] whereas pretreatment with the MEK1 inhibitor, PD98059, was essential for the chemotactic response of MCF-7 cells to IGF-1.[34] Moreover, IGF-1-induced migration has been associated with focal adhesion kinase

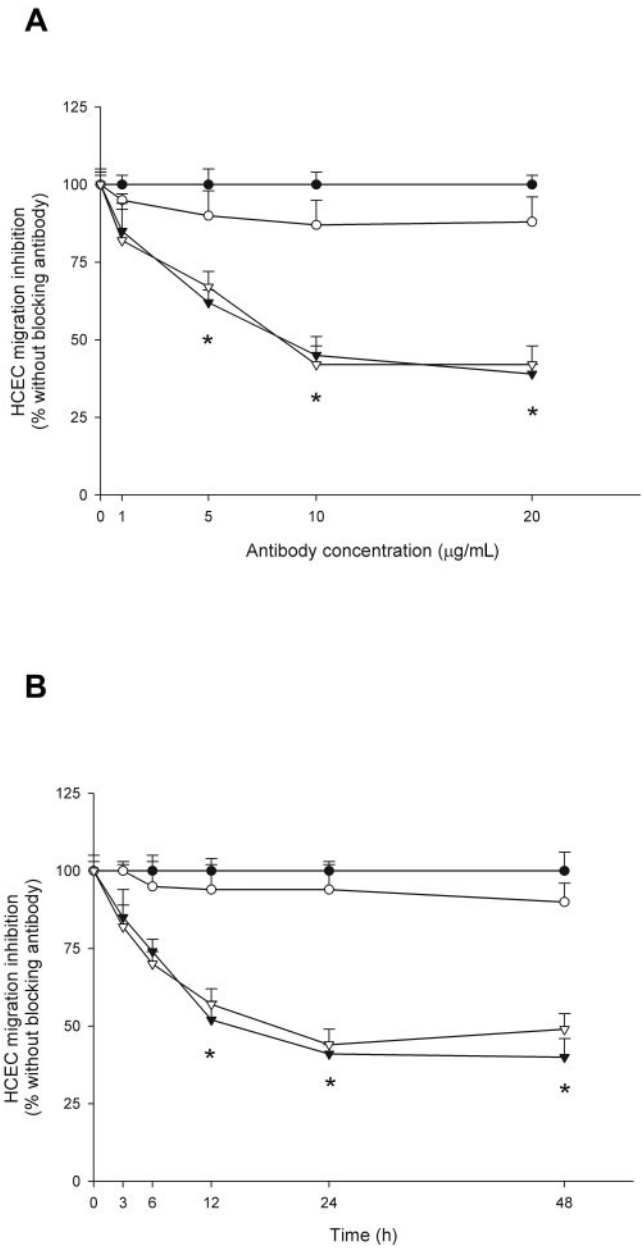

FIGURE 9. Effect of neutralizing antibodies against integrin $\alpha 3$ (○) and $\beta 1$(▼) on HCEC migration on 100 ng/mL Ln-5. HCECs were cultured with or without 10 μg/mL anti-$\alpha 3$- and -$\beta 1$-antibodies for 48 hours in the Ln-5-coated cell migration chamber. The results were compared in terms of antibody concentration at 12 hours (**A**) and over time (**B**). (○) Anti-integrin $\alpha 3$ alone; (▼) anti-integrin $\beta 1$ alone; (▽) both together; and (●) control. *Significant ($P < 0.05$; one-way ANOVA) difference compared with the control.

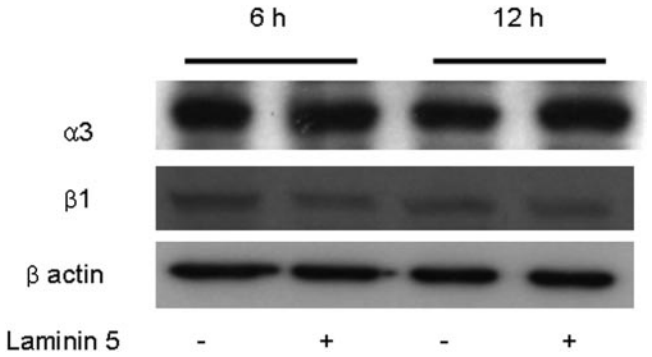

FIGURE 10. Integrin $\alpha 3$ and $\beta 1$ production on Ln-5. HCECs (2.2×10^6/mL) were seeded on 24-well plates coated or not coated with 200 ng/mL ln-5. After 12 hours, cell lysates were assayed by immunoblot for the presence of $\alpha 3$ or $\beta 1$ integrins.

(FAK), PKC, c-Src, and integrins and was also shown to be cross-regulated by ECM- and integrin-related signals, including paxillin and $p130^{CAS}$.[35–37] In CEC wound healing, Saika et al.[6] reported that TGF-β enhances CEC migration through the activation of p38MAPK in C57BL/6J mice. And Sharma et al.[7] and Wilson et al.[8] reported that cross-talk between p38 and ERK1/2 activation is essential in KGF- and HGF-induced CEC migration. Taken together, our findings and those in the previous reports indicate that activation of intracellular signal pathways for cell migration differs among various cell types and species, and that these differences may be responsible for the variations in activation of intracellular signal pathways.

The cells of the basement membrane lay down matrix and influence cellular activities such as adhesion, motility, cytoskeletal organization, and gene expression.[38] Ln-5, as a component of the basement membrane, facilitates epithelial cell migration and wound healing, and its expression may be related to the spreading of corneal epithelial cells.[39,40] Previous studies have reported that some tumor cell lines overexpress Ln-5 via migration-associated cytokines such as TGF-β[41] and IL-6.[42] However, this is the first study to report the induction of Ln-5 by a cytokine in HCECs. Although Ln-5 was expressed in migrating HCECs in the absence of IGF-1 treatment, addition of IGF-1 triggered further upregulation of Ln-5. Western blot analysis revealed that although IGF-1 treatment induced all three components of Ln-5 in HCECs, the $\beta 3$- and $\gamma 2$-chains were more prominently increased than the $\alpha 3$-subchain, which is generally believed to induce hemidesmosome assembly and cell attachment through integrin interactions. In contrast, the $\gamma 2$-subchain has been shown to be a central factor in cell migration.[43] Thus, the stronger upregulation of the $\gamma 2$-subchain is consistent with the effect of IGF-1 on migration, rather than adhesion.

A single matrix molecule may demonstrate opposite effects on cell migration and adhesion when it adheres to different integrins, due to differing effects on matrix protein processing and assembly.[1,11,43,44] For example, the matrix molecule Ln-5 supports cell migration and is enriched at the front of migrating cells, where it indirectly associates with the reorganization of the actin cytoskeleton.[44] However, Ln-5 also plays an essential role in attachment of human corneal epithelial cells.[40] The mechanisms by which Ln-5 exerts two apparently opposite activities are poorly understood. Some studies have suggested that stable adhesion is mediated by interactions between Ln-5 and integrin $\alpha 6\beta 4$,[45,46] whereas cell migration is supported by interactions between Ln-5 and integrin $\alpha 3\beta 1$.[35,47] the $\alpha 3\beta 1$ integrin has been reported to form complexes with the transmembrane-4 superfamily (TM4SF) proteins. These different complexes have been suggested to play different roles in various cell types.[48,49] Tsuji et al.[50] reported that A431 epidermoid cells produce a factor that potentiates the motility of melanoma cells in a $\alpha 3\beta 1$-integrin-dependent manner. Also, recent studies have shown that interactions between Ln-5 and integrin $\alpha 7\beta 1$ or integrin $\alpha 2\beta 1$ may be involved in epithelial cell migration.[51,52] Furthermore, Tai et al.[53] reported that IGF-1-induced cell migration is mediated by $\beta 1$ integrin activation and PI3-K/AKT signaling. In the present study, we found that $\beta 1$ integrin was also an important migration-associated factor in HCECs, but $\alpha 3$ integrin was not, and the $\beta 1$ integrin was also induced by PI3-K, as was Ln-5.

There is significant evidence of cross-talk between IGF-1-associated PI3 kinase and ECM proteins in several cell types. Nguyen et al.[54] reported that deposition of Ln-5 and ligation by integrin activated PI3 kinase signaling promoted cell adhesion and spreading. Gu et al.[55] reported that Fn and some Ln isoforms could modulate PI3 kinase/Akt activity by altering integrin expression. Our results did not provide evidence of cross-talk between Ln-5 and $\beta 1$ integrin, but it remains possible that the induced Ln-5 proteins affect PI3-K/AKT signaling via the cross-regulated integrin-dependent signaling pathways in HCECs. Also, although Ln-5 did not affect the production of $\beta 1$ integrin, $\beta 1$ integrin may be activated by cross-linking with Ln-5 during HCEC migration.

In summary, we show for the first time that IGF-1 induces HCEC migration by upregulation of Ln-5 and $\beta 1$ integrin protein expression via the PI3-K/AKT pathway, but not the MEK-ERK pathway. The inductions of Ln-5 and $\beta 1$ integrin by IGF-1 appeared to occur independently, and most likely contributed to IGF-1-induced cell migration. Future work is needed to understand better the IGF-1-induced cytokine signaling within the matrix.

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