Effect of the cell migration and expression of basement membrane laminin-5 by insulin- like growth factor-1(IGF-1) in cultured corneal epithelial cell

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Directed by Professor Kim Eung Kweon

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This certifies that the Doctoral Dissertation of Lee Hyung Keun is approved

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We examined the effects of insulin-like growth factor-1 (IGF-1) on laminin-5 (Ln 5) and the associated integrins during *in vitro* HCEC migration. And we investigated the effects of IGF-1 on the migration of human corneal epithelial cells (HCECs). HCEC migration was examined by wound healing and chemoattractant assays. For migration inhibition assays, HCECs were pretreated with inhibitors of the IGF-1 receptor (αIR3), PI3-K/AKT pathway (LY 294002) and the

MEK-ERK pathway (PD 98059). The expression levels of laminin-5 and fibronectin (Fn) were determined by Western blot analysis, while 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. HCEC migration was significantly increased in the presence of IGF-1 and Ln-5. IGF-1 enhanced the production of laminin-5 in both a dose- and time-dependent manner, and this up-regulation could be blocked by pretreatment with αIR3 or LY 294002. IGF-1 treatment up-regulates expression of β1 integrin, but not α3 integrin. And the facilitated HCEC migration by IGF-1 was inhibited with anti-integrin antibody for β1. However, there was no cross-talk between Ln-5 and integrin β1 production. Our results reveal that IGF-1 induces HCEC migration through the independent productions of laminin-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.

Key Words : corneal epithelium, cell migration, insulin-like growth factor, laminin-5

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I. INTRODUCTION

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 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-1 (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, laminin, 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 laminin (Ln) proteins are a family of extracellular matrix molecules that exist as cross-shaped heterotrimers of 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 of the tissues in the basement membrane,[11] while 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 was shown to be over-expressed 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. IGF-1 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. reported that substance P and IGF-1 synergistically stimulated corneal epithelial migration in an organ culture of the cornea,[18] and found that while addition of either SP or IGF-1 alone did not affect epithelial migration, co-treatment with SP and IGF-1 significantly stimulated epithelial migration.[19] Interestingly, 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.

Here, we investigated whether IGF-1 could stimulate HCEC migration, and whether the expression levels of Ln-5 and/or the Ln-5 associated integrins were altered during IGF-1-induced HCEC migration *in vitro*.

II. MATERIALS AND METHODS

1. 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), γ2 (H-183) was from SantaCruz Biotechnology, Inc. (SantaCruz, CA) and human fibronectin (Fn) and type IV collagen were 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) was purchased 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 antibodies (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 medium (DMEM), F-12 nutrient mixture, fetal bovine serum (FBS), HEPES-buffer, amphotericin B and gentamicin were purchased from Gibco-BRL (Grand Island, NY). Other reagents and

chemicals, including mouse-derived epidermal growth factor, 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 LY 294002 and PD 98059 were purchased from Calbiochem (Darmstadt, Germany).

2. Preparation of human corneal limbal tissue for primary epithelial cell culture

A. Isolation of human corneal limbal tissue

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 following 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 Mg^{++}- and

Ca^{++}-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 X 2.0 mm tissue sections with a #15 blade and scissors.

B. Primary corneal limbal cell culture

The segmented epithelial tissue was placed on the upper chamber of a transwell apparatus(diameter, 6.5mm; pore size 0.4 μm, Corning, Acton, MA) containing 3T3 fibroblasts in the lower chamber, and cells were cultured in supplemental hormonal epithelial medium (SHEM) made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F12 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 three weeks by inverted phase microscopy (Olympus, IX70, Tokyo, Japan). When the cultured corneal epithelium was ~80-90% 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.

3. Migration assay

HCEC migration was determined by monolayer wound healing and chemoattractant 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 with minimum triplicate samples and at least three different areas were observed in each sample.

A. Cell restitution assay (Monolayer wound closure assay)

For monolayer scratch assays, $2x10^6$ 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 using 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 using a microscope (Olympus, IX70, Tokyo, Japan, 50X and 100X magnifications).

B. Boyden chamber chemotaxis assay

The chemotaxis assays were performed using a modified Boyden chamber assay. Briefly, transwell chambers (6well plate, Corning, Acton, MA)

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, fibronectin or various concentrations of recombinant Ln-5 (10, 50, 100 1000 ng/ml) in PBS for 2 hours at 37^{o}C. Then, the inserters were rinsed twice with PBS, blocked with 1% heat-inactivated BSA in PBS for 60 minutes at room temperature. The upper chamber was loaded HCEC with 1000 μl of $2x10^{6}$cells/ml and the apparatus was incubated for 24 hours. To count 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 (100X) with the same microscope used in the monolayer scratch assay. Experiments were performed in triplicate and repeated at least twice.

4. Migratory function blocking study

For inhibitory studies, cells were incubated for 2 hours with one of the following: 25 or 50 μM of LY 294002 inhibitor of phosphoinositide-3 kinase (PI3-K)/AKT) and PD 98059 (inhibitor of ERK), blocking or neutralizing antibodies against IGF-1 receptor (αIR3, 10μg/ml), β1 integrin (10μg/ml), α3 integrin (10μg/ml), or an IgG matched control mAb and migratory assays

were performed as above.

5. 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 x g for 10 minutes. The protein concentrations in the resultant supernatants were determined using 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 fibronectin, Ln α3, -β3, and -γ2 chains, and integrins α3 and -β1 (diluted 1:500 to 1:3000). The immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

6. Immunocytochemical staining

Cells were fixed for five minutes in 3.7% formaldehyde and then

permeabilized in 0.5% Triton X-100 for eight minutes. Single or double-label immunofluorescence was performed. As controls, samples were run without primary antibodies or with irrelevant IgG to determine non-specific binding of secondary antibodies. For experiments, 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 (Nikon Eclipse TE200 equipped with Nikon digital camera DXM 1200) using appropriate filters for visualization of the fluorescein.

7. Statistics

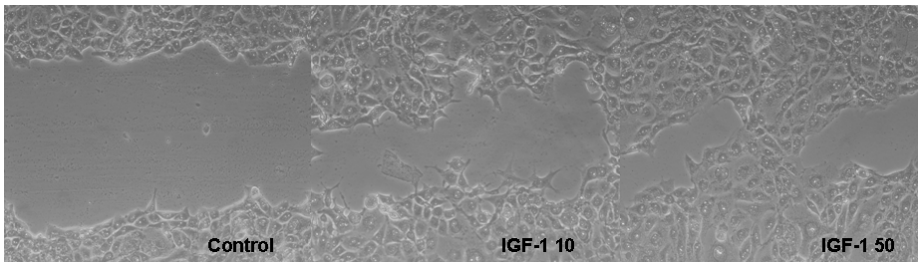
All data are expressed as the mean±SD. Multivariate analysis of variance with post hoc Newman-Keuls tests determined significant differences between the treatment groups. $P < 0.05$ was considered statistically significant.

III. RESULTS

1. Insulin-like growth factor-1 increases human corneal epithelial cell migration

HCEC monolayers were wounded, and migration was assessed following treatments 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 (Fig. 1A, B) Migrating cells were flattened, with ruffling borders and lamellipoda (Fig. 1A); these morphological changes could be seen more clearly in IGF-1-treated cells than in controls. These results indicate that IGF-1 treatment dose-dependently increases migration of HCECs.

A.



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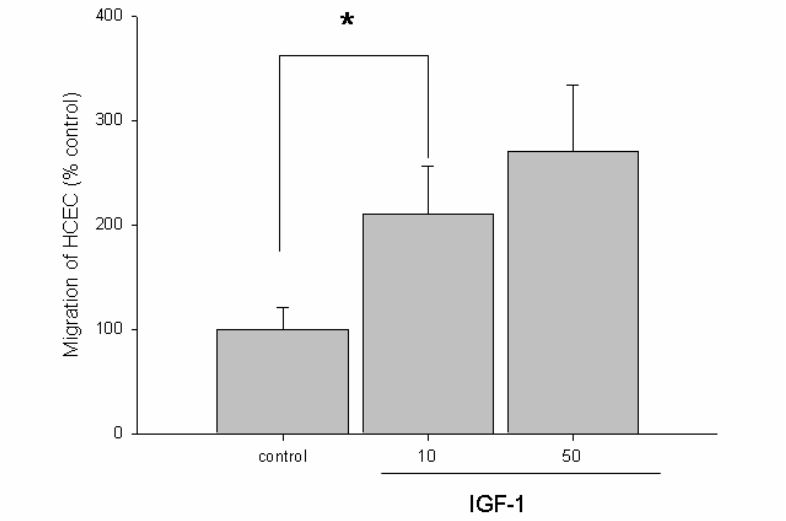


Figure 1. Cell restitution assay of the effect of IGF-1 on migration of human corneal epithelial cells (HCECs). Monolayers were scratched and treated with or without 10ng/ml, 50 ng/ml IGF-1. Twelve hours later, the plates were washed three times with phosphate buffered saline (PBS), and the migratory cells were counted and photographed using a inverted microscope (Olympus, IX70, Tokyo, Japan; X100, magnification). (A, B) *, significant difference (p<0.05, One way ANOVA); bars, ±SE.

2. **IGF-1 induces PI3-K and MAPK signaling in human corneal epithelial cells**

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, while 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.

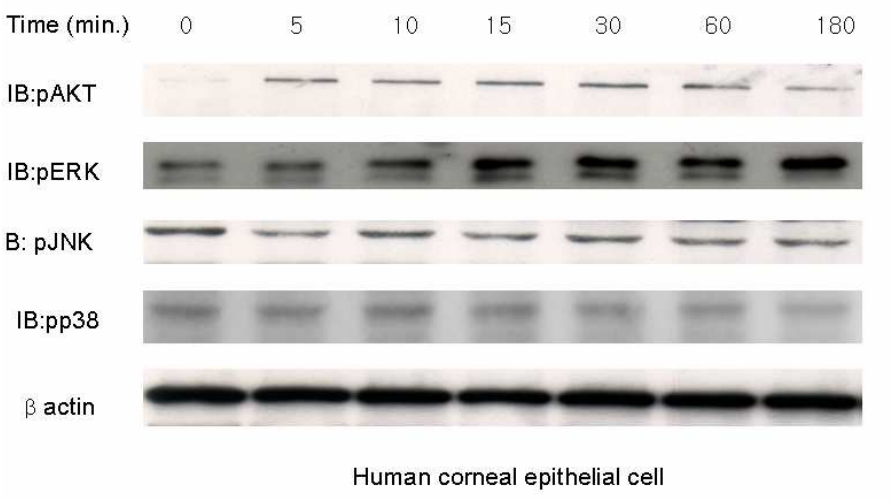


Figure 2. Western blot analysis of phospho-AKT, -ERK 1/2, -JNK, and -p38

in human corneal epithelial cells (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, anti-pERK1/2, anti-phospho-JNK, and anti-phospho-p38 antibodies. β-actin served as the loading control.

3. Inhibition of some IGF-1 signaling components blocks IGF-1-induced HCEC migration

As our results revealed that PI3-K and ERK 1/2 are involved in IGF-1-mediated intracellular signaling in HCECs, and a previous study had shown that α3 and β1 integrins 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 above for twelve 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, LY 294002 (25 μM) and anti β1 integrin antibodies (10 μg/ml) completely blocked the IGF-1-induced HCEC migration, whereas an ERK inhibitor (PD 98059; 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.

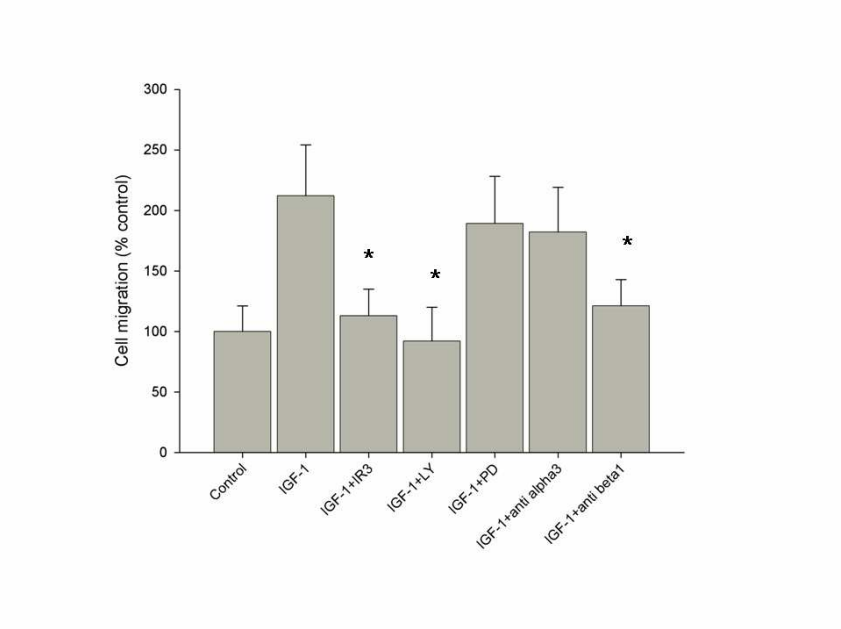


Figure 3. Human corneal epithelial cells (HCECs) migration were pretreated with neutralizing antibodies against the αIR3 IGF-1 receptor (10 μg/ml), integrin β1 (10 μg/ml) and α3 (10 μg/ml), as well as inhibitors against PI3 kinase (LY 294002; 25 μM) and ERK (PD 98059; 25 μM). The cells were then treated with IGF-1 (10ng/ml) and migration was assessed in a wound-healing assay at twelve hours, using Olympus IX 70 microscope (X50, magnification). *, significant (p<0.05, One way ANOVA) inhibition compared with IGF-1 treatment alone; bars, ±SE.

4. IGF-1 treatment induces laminin-5 subchain expression

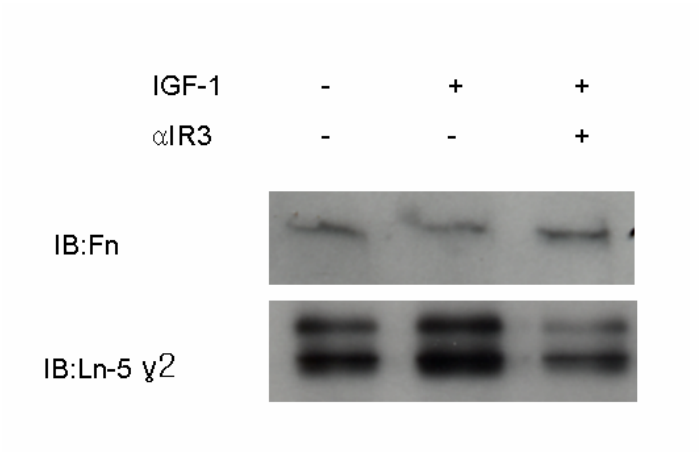
As Ln-5 and fibronectin are known to be important modulators of

HCEC migration,[1,10] we next used Western blot analysis to investigate the expression of Ln-5 and fibronectin following IGF-1 treatment of HCECs. Ln-5 γ2 subchain production was increased at 6 hours after treatment of IGF-1 (10ng/ml), but no change in fibronectin production was seen following IGF-1 treatment (Fig. 4A). Interestingly, treatment with the ERK inhibitor (PD 98059) had no effect on the IGF-1-induced production of the γ2 subchain, while the specific PI3-K inhibitor (LY 294002) decreased the expression of Ln-5 γ2 subchain (Figure 4B).

To determine the expression of Ln-5 with the time sequence, the immunobloting for Ln-5 was performed after IGF-1 treatment. In this experiment, some cells were pretreated with 25 and 50 μM LY 294002 2 hours before IGF-1 treatment. The expression levels of the Ln-5 α3, β3, and γ2 subchains were significantly up-regulated within 3 hours post-treatment, as compared with the serum- and growth factor-free controls (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 values at 6 and 12 hours. The expression levels of the β3 (155 kDa) and γ2 (150kDa: unprocessed, 105kDa: processed) subchains were significantly increased at 3 and 6 hours after IGF-1 treatment, and had returned to basal levels by 12 hours post-treatment. The γ2 subchain expression levels peaked at 6 hours

post-treatment and showed the largest IGF-1-induced change. Pretreatment of LY 294002 blocked IGF-1-mediated production of the β3 and γ2 subchains in a dose-dependent manner (Fig. 4C, lanes 3 and 4).

A.



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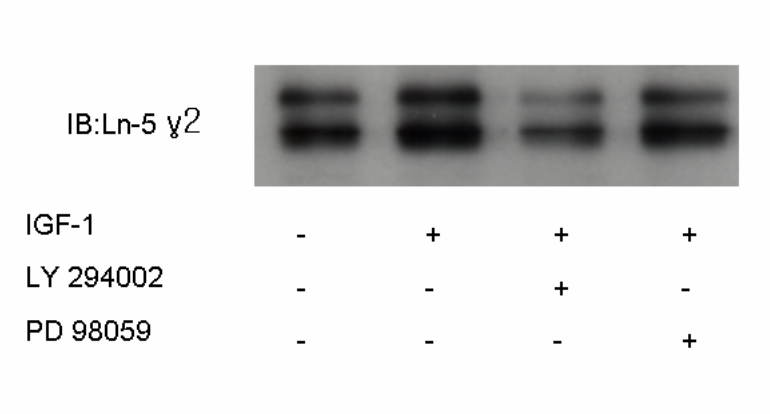


C..

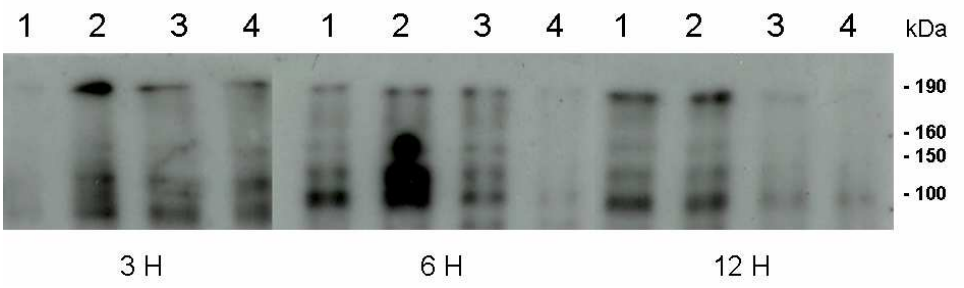


Figure 4. IGF-1-induced laminin-5 (Ln-5) expression was determined by Western immunoblot. Serum- and growth factor-starved cells were treated with IGF-1 (10 ng/ml) and incubated for 6 hours. Cell lysates were examined for production of Ln-5 subchains γ2 and fibronectin using Western blot analysis (A, B). Two hours before the IGF-1 treatment, some cells were pretreated with a neutralizing antibody against the αIR3 IGF-1 receptor, or inhibitors LY 294002 (25 μM) and PD 98059 (25 μM). (A). Ln-5 expression was compared over time. Lane 1, serum and growth factor free; lane 2, 10 ng/ml IGF-1 treatment; lane 3, 10ng/ml IGF-1+25 μM LY 294002; lane 4, 10ng/ml IGF-1+50 μM LY 294002 (c).

5. Localization of laminin-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 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 (white arrow), as compared with the following trailing cells (arrowhead) (Fig. 5B). Time course analysis revealed that in IGF-1 treated cells, Ln-5 expression progressively formed continuous immunoreactive lines along the borders of migratory cells (Fig. 5B, C). In cells pretreated with LY 294002 prior to IGF-1 treatment, Ln-5 expression did not form continuous lines along the borders of cells (Fig. 5D). In sharp contrast, the Ln-5 was also expressed within the marginal membrane protrusions of cells treated with IGF-1 (Fig. 5E).

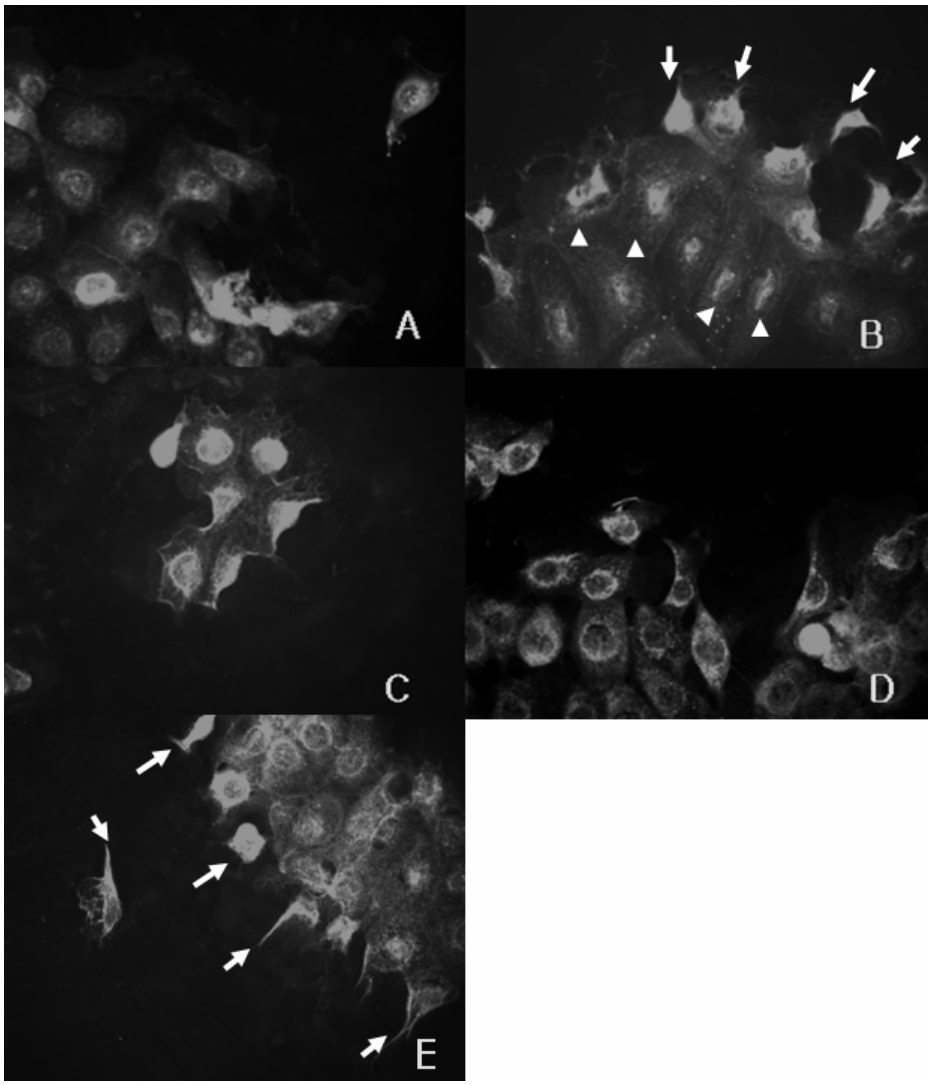


Figure 5. Localization of laminin-5 in human corneal epithelial cells (HCECs) in the absence or presence of insulin-like growth factor 1 (IGF-1). Indirect immunofluorescein method was used to examine Ln-5 localization in serum- and growth factor-free control cells (A), as well as those treated with

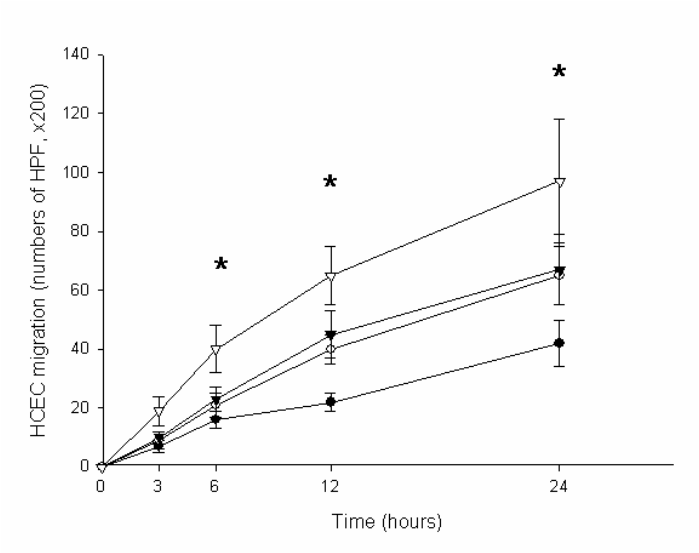
10 ng/ml IGF-1 for three (B) and six (C) hours, and those pretreated with 25 μM LY 294002 for 2 hours before treatment with IGF-1 (D). The arrows indicate the cytoplasmic processes and localization of laminin-5 in migrating cells 3 hours after treatment with IGF-1 (E).

6. HCEC migration on matrix glycoproteins, fibronectin, Ln-5, and type IV collagen

As our results suggested that IGF-1 can induce HCEC migration and up-regulation 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 fibronectin and type IV collagen, cell migration was determined by chemoattractant assays on glycoprotein pre-coated chamber slide inserts. As shown in Figure 6, HCEC migration was increased on all of the tested matrix glycoproteins over 24 hours, as compared with the serum- and growth factor-free controls. The Ln-5 coated inserts showed the highest levels of cell migration; these levels began to show statistically significant difference from 6 hours post-treatment, as compared to the type IV collagen- and fibronectin-coated slides, which showed increased cell migration upto 24 hours post-treatment.

To confirm these findings, another migration assay was performed with monolayer scratch using cells incubated in media containing the various basement membrane glycoproteins. The wounded monolayers were treated with or without 100 ng/ml of type IV collagen, fibronectin or Ln-5, and migration was observed. At 12 hours after assay, Ln-5-treated HCEC monolayer condition showed the highest levels of migration compared to fibronectin or type IV collagen treated condition (Fig. 6B).

A.



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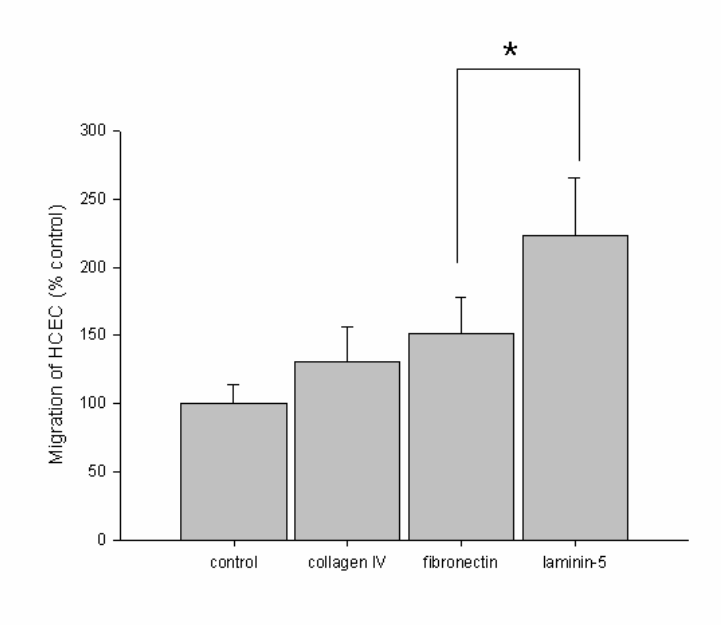
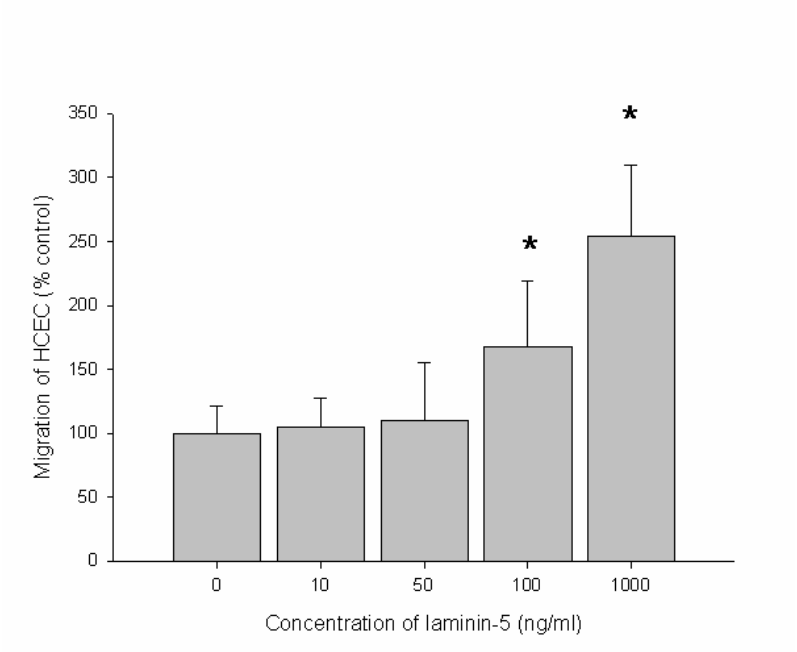


Figure 6. Comparison of human corneal epithelial cell (HCEC) migration on collagen type IV- (○; 0.5 μg/ml), fibronectin- (Fn) (▼; 0.5 μg/ml), and laminin-5- (Ln-5) (▽; 0.5 μg/ml) coated inserts, and serum- and growth factor-free controls (●). HCEC migration was determined using chemoattractant assays for 24 hours (A). Wound healing assays were conducted with or without 500 ng/ml of type IV collagen, fibronectin, or laminin-5 and migratory cells were counted 12 hours after assay (B). *, significant difference (p<0.05, One way ANOVA) compared with cells treated with 100 μg/ml Fn; bars, ±SE.

Next, the dose-response effect of Ln-5 on HCEC migration was examined in additional chemoattractant 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 significant increase in HCEC migration (Fig. 7A). With the time sequence, from 6 hours after the assay, HCEC migration was facilitated by Ln-5, as compared with the control (Fig. 7B).

A.



B.

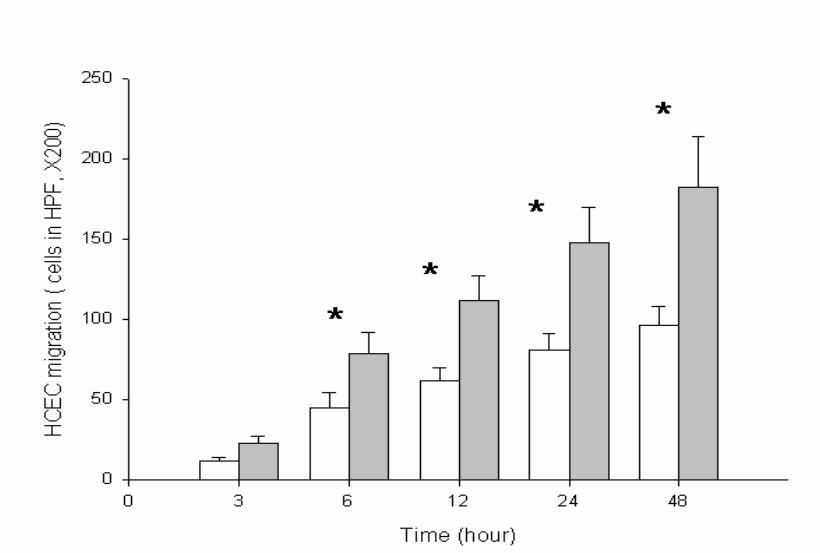


Figure 7. Human corneal epithelial cell (HCEC) migration on Ln-5 coated inserts of a transwell chamber over different doses (A) and time points (B). □, without Ln-5; ■, with Ln-5 (100 ng/ml); *, significant difference (p<0.05, One way ANOVA) compared with serum- and growth factor free control.

7. IGF-1 treatment up-regulates expression of β1 integrin, but not 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 the

α3 and β1 integrins, which are known to be related with Ln-5-associated cell migration.[21] Western blotting 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 LY 294002 (Fig. 8).

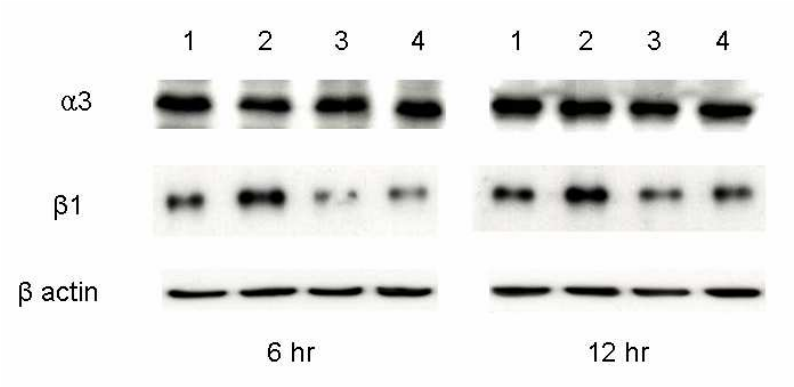
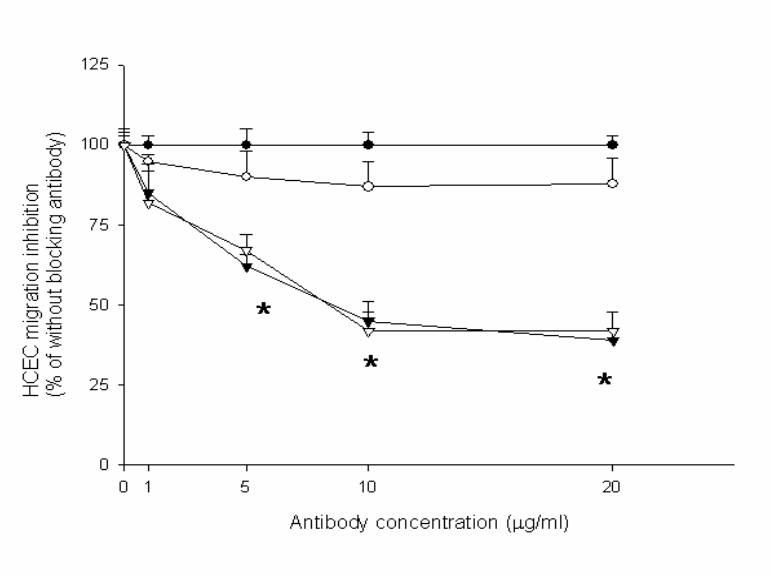


Figure 8. The expression levels of integrin α3 and β1 in IGF-1-treated human corneal epithelial cells (HCECs). Cells were pretreated with or without LY 294002 for 2 hours, then treated with or without IGF-1 for upto 12 hours. HCEC lysates were subjected to Western blot analysis with anti-integrin α3 and β1 antibodies; lane 1, serum and growth factor free control; lane 2, IGF-1 10 ng/ml; lane 3, 25 μM LY 294002 pretreatment; lane 4, 50 μM LY294002 pretreatment.

8. Neutralizing anti-IGF-1 and anti-integrin antibodies inhibits 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 after assay, 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 after the assay (Fig. 9B). Treatment with neutralizing antibodies against both α3 and β1 had an inhibitory effect on cell migration similar to that seen in cells treated with the anti-β1 antibody alone (Fig. 9A, B).

A.



B.

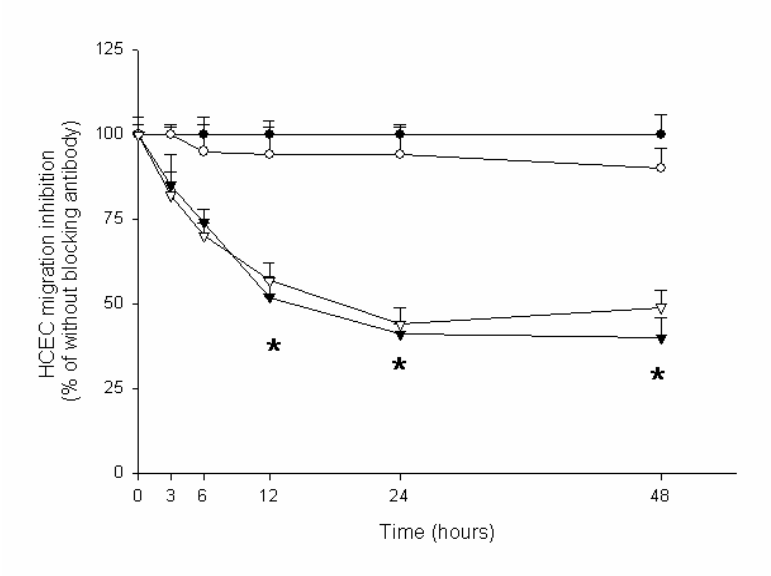


Figure 9. Effect of neutralizing antibodies against integrin α3 (○) and β1(▼)

on human corneal epithelial cell (HCEC) migration on 100 ng/ml laminin-5 (Ln-5). HCEC were co-culured with or without 20 μg/ml anti α3- and β1-antibodies for 48 hours in the Ln-5 coated transwell. The results were compared in terms of antibody concentration at 12 hours after assay (A) and time (B). ○, anti-integrin α3 alone; ▼ anti-integrin β1alone; ▽, both together; ● control; *, significant (p<0.05, One way ANOVA) difference compared with the control.

As these data indicate that the functional up-regulations of both Ln-5 and integrin β1 are important for IGF-1-induced HCEC migration, we lastly investigated the production of integrin β1 in HCECs grown on Ln-5-coated matrices, to determine whether cross-talk might exist between Ln-5 and integrin β1. Interestingly, 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.

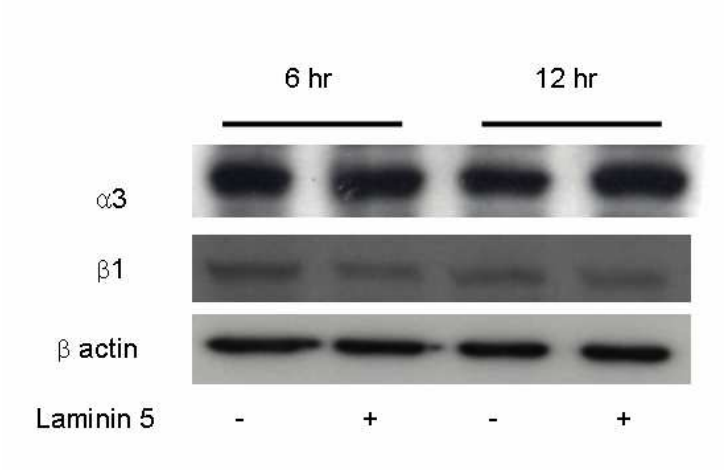


Figure 10. Integrin α3 and β1 production in human corneal epithelial cells on laminin-5. HCECs ($2.2x10^6$/ml) were seeded on 24-well plates coated with or without 200 ng/ml laminin-5. After 12 hours, cell lysates were immunoblotted for the presence of α3 or β1 integrins.

IV. DISCUSSION

The present study demonstrates that IGF-1 treatment of HCECs can induce the expression of matrix protein, Ln-5, and its cell surface receptor, integrin β1, and that these effects are associated with increased HCEC migration. The induction of Ln-5 and integrin β1 by IGF-1 could be inhibited by LY 294002, 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 cell. 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. 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.[25] 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. Nishida et al. 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. reported that in dogs with spontaneous chronic corneal epithelial defects, IGF-1 did not play a critical role in corneal wound healing.[29] Furthermore, McDermott et al. reported that topical application of SP and/or IGF-1 did not modulate corneal epithelial wound healing in galactosemic rats.[30] 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 from each cytokines or cell types. Andre et al. 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.[31] 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, PD 98059, was dispensable for the chemotactic response of MCF-7 cells to IGF-1.[34] Moreover, IGF-1-induced migration has been associated with focal adhesion kinase (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 reported that TGF-β enhances CEC migration through the activation of p38MAPK in C57BL/6J mice.[6] And Sharma et al reported that cross-talk between p38 and ERK1/2 activation is important in KGF and HGF induced CEC migration.[7,8] Taken together, our findings and 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 over-express 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 up-regulation of Ln-5. Western blotting revealed that although IGF-1 treatment induced all three components of Ln-5 in HCECs, the β3 and γ2 chains were more prominently increased than the α3 subchain. The α3 subchain is generally believed to induce hemidesmosome assembly and cell attachment through integrin interactions. In contrast, the γ2 subchain has been proven to be an important factor in cell migration.[43] Thus, the stronger up-regulation of the γ2 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 adhered 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 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 α6β4,[45,46] while cell migration is supported by interactions between Ln-5 and integrin α3β1.[35,47] α3β1 integrin was reported to form complexes with the transmembrane-4 superfamily (TM4SF) proteins; these different complexes were suggested to play different roles in various cell types.[48, 49] Tsuji et al. reported that A431 epidermoid cells produce a factor that potentiates the motility of melanoma cells in a α3β1 integrin-dependent manner.[50] Also, recent studies have shown that interactions between Ln-5 and integrin α7β1 or integrin α2β1 may be involved in epithelial cell migration.[51,52] Furthermore, Tai et al. reported that IGF-1-induced cell migration was mediated by β1 integrin activation and PI3-K/AKT signaling.[53] In the present study, we found that β1 integrin is also an important migration associated factor in HCEC, but α3 integrin is not. And the β1 integrin is also induced by PI3-K like Ln-5.

There is significant evidence of cross-talk between IGF-1-associated PI3 kinase and extracellular matrix 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 fibronectin 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 β1 integrin, but it remains possible that the induced Ln-5 proteins may affect PI3-K/AKT signaling via the cross-regulated integrin-dependent signaling pathways in HCECs. Also, although the Ln-5 did not affect the production of β1 integrin, β1 integrin may be activated by cross-linking with Ln-5 during the HCEC migration.

V. CONCLUSION

In summary, we herein show for the first time that IGF-1 induces HCEC migration by up-regulation of Ln-5 and β1 integrin protein expression via the PI3-K/AKT pathway, but not the MEK-ERK pathway. The inductions of Ln-5 and β1 integrin by IGF-1 appeared to occur independently, and likely contributed to IGF-1-induced cell migration. Future work will be required to better understand the IGF-1-induced cytokine signaling within the matrix.

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Abstract (in Korean)

배양된 각막상피세포에서 insulin-like growth factor-1 (IGF-1)이 세포의 이동과 바닥막 laminin-5의 발현에 미치는 영향

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이 형 근

본 연구는 insulin-like growth factor-1 (IGF-1)이 사람 각막상피세포의 이동과 laminin-5의 형성에 어떤 영향을 주는지 알아보고자 하였다. 먼저 배양된 각막상피세포의 이동이 IGF-1에 의하여 어떻게 변하는가를 cell restitution assay 와 chemoattractant assay를 통해 확인해 본다. 또, migration inhibiton assay를 위해 사람 각막상피세포를 IGF-1 receptor (IR3), PI3-K/AKT, MEK-ERK pathway 에 대한 각각의 억제제를 (αIR3, LY 294002, PD98059) 처리하여 세포의 이동을 관찰해 보고자 하였다. 또, Western blot 을 이용하여 IGF-1이 바닥막 단백질 (Laminin-5, fibronectin) 과 laminin-5와 결합하는 Integrin α3, β1 의 형

성에 어떠한 영향을 주는가 알아보고자 하였다. 마지막으로 α3, β1 integrin은 사람 각막상피세포의 이동에 어떠한 영향을 주는지 확인하여 보며 또 이러한 integrin은 바닥막 laminin-5와 어떤 상호관계가 있는지 확인해 보고자 하였다. 사람 각막상피세포의 이동은 IGF-1과 laminin-5가 있는 조건 하에서 각각 의미 있게 증가하였으며 IGF-1 은 laminin-5를 dose- and time- dependant manner로 증가시켰다. 이러한 IGF-1에 의해 촉진된 세포의 이동과 laminin-5의 형성은 αIR3이나 LY 294002 처리로 억제되었다. IGF-1 은 β1 integrin 의 형성을 증가시켰으나 α3 integrin 의 생성증가는 확인할 수 없었다. 또, IGF-1에 의해 촉진된 세포의 이동은 β1 integrin 의 중화항체로 인해 억제되는 것을 확인할 수 있었다. 그러나 laminin-5 와 β1 integrin간에 있어 cross-talk 은 확인할 수 없었다. 이러한 결과로 IGF-1은 사람 각막상피세포의 이동을 증가시키며 이러한 과정은 laminin-5, β1 integrin의 형성과 관련이 있음을 확인할 수 있었다. 또, 이러한 과정은 PI3-K/AKT pathway 가 영향을 주는 것을 확인할 수 있었다.

핵심되는 말: 각막상피세포, 세포이동, insulin-like growth factor-1, laminin-5