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Lumican induces human corneal epithelial cell migration and integrin expression via ERK 1/2 signaling

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ABSTRACT

Lumican is a major proteoglycans of the human cornea. Lumican knock-out mice have been shown to lose corneal transparency and to display delayed wound healing. The purpose of this study was to define the role of lumican in corneal epithelial cell migration. Over-expression of lumican in human corneal epithelial (HCE-T) cells increased both cell migration and proliferation, and increased levels of integrins α2 and β1. ERK 1/2 was also activated in lumican over-expressed cells. When we treated HCE-T cells with the ERK-specific inhibitor U0126, cell migration and the expression of integrin β1 were completely blocked. These data provide evidence that lumican stimulates cell migration in the corneal epithelium by activating ERK 1/2, and point to a novel signaling pathway implicated in corneal epithelial cell migration.

The cornea is a transparent structure which consists of a stratified epithelial cell layer, a thick stroma, and an endothelial cell layer. Both the epithelial and endothelial cell layers are separated from the stroma by basement membranes. About 90% of the corneal depth consists of stroma, which is organized as an extracellular matrix, including collagen fibrils surrounded by proteoglycans [1].

The small leucine-rich proteoglycans (SLRPs) are thought to regulate the assembly of the collagen matrix [2,3]. Proteoglycans are composed of a protein core with glycosaminoglycan side chains [4]. It seems that although they are expressed in other tissues, the predominant corneal glycosaminoglycans are dermatan sulfate and keratan sulfate [5]. The keratan sulfate chain is attached to the protein by a bi-antennary type *N*-acetylglucosamine of an oligosaccharide containing a mannose N-linked to an asparagine residue of the proteoglycan [6]. Lumican with a keratan sulfated side chain is a major component of the cornea [7]. In addition to lumican, two other keratan sulfate proteoglycans, named keratocan and mimecan (or osteoglycin) have been identified in the cornea [5,8].

There are reports of a connection between proteoglycans and corneal diseases. Funderburgh et al. [9] demonstrated the involvement of decorin and biglycan in diseased human cornea, and there is evidence that congenital stromal dystrophy is caused by a mutation of the decorin gene [10].

The role of lumican in corneal stroma formation during development has been intensively investigated. Lumican-null mice display skin laxity, connective tissue defects, and corneal opacity [11]. In addition stromal thickness is reduced, there are collagen fibril abnormalities in the posterior stroma [12] and corneal transparency is affected during development [13]. Lumican has been shown to play essential roles in wound healing by modulating epithelial cell migration [14], and human lumican purified from amniotic membranes enhances re-epithelialization and the proliferation of mouse corneal epithelial cells [15].

The mechanism of lumican-induced cell migration and cell proliferation is still unclear. Here, we demonstrate that lumican induces cell migration by activating ERK 1/2 signaling. We show that ERK 1/2 also stimulates expression of integrin β1.

Methods

Cell culture and reagents. Human corneal epithelial cells (HCE-T) were kindly provided by Dr. Kaoru Araki-Sasaki (Osaka University, Osaka, Japan). Cells were cultured in DMEM/F-12 medium (Welgene, Korea) containing 5% FBS, 5 μg/ml insulin (Sigma, St. Louis, MO), 10 ng/ml human EGF (Sigma), 100 ng/ml cholera toxin (Biomol, Plymouth, PA), and 0.5% DMSO (Sigma). U0126, SP600125, and SB203580 were purchased from EMD Chemicals (Gibbstown, NJ).

Human lumican cloning and stable transfection. The full-length cDNA for human Lumican (1079 bp, Gene Accession No. NM_002345) was amplified by PCR using primers (forward:

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GAAAGCAGTGTCAAGACAGTAA, reverse: TGTTCCAGGATCCAGATATT) and subcloned into EcoRV/BamHI digested pIRES-neo vector (Clontech, Mountain View, CA). Dashed line in reverse primer indicated the modification for BamHI digestion. 1 μg of mock and lumican plasmid were transfected using the Wellfect-EX Plus and Enhancer-Gold transfection reagent (Welgene). At 2 days after transfection, medium containing 800 μg/ml of G418 was applied to select the transfectants for 2 weeks with media changed every day. The selected cells were grown and expanded for further experiments and maintained containing 400 μg/ml of G418.

RT-PCR. Total RNA was extracted from HCE-T cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two micrograms of total RNA was reverse-transcribed with AMV reverse transcriptase (Promega, Madison, WI), and PCR was performed with specific primers. The primers used for RT-PCR were as followed: lumican (forward: TAA ACC ACA ACA ACC TGA CA, reverse: CAA GTT GAT TGA CCT CCA GG, 492 bp); Actin (forward: CAC TCT TCC AGC CTT CCT TC, reverse: CTC GTC ATA CTC CTG CTT GC, 314 bp); integrin α2 (forward: GGC GAC GAA GTG CTA CGA AAG, reverse: TTA GTA TCA AGG GCG TTT CTG, 160 bp); integrin α3 (forward: CAA CCC TCT CAA CCT CAC T, reverse: GGT CAG CCT CTC TGT CTC TG, 586 bp); integrin αV (forward: GCT GGT CTT CGT TTC AGT GT, reverse: GAG CAA CTC CAC AAC CCA, 550 bp); integrin β1 (forward: AAC CTT CAG TGG AAA GCC A, reverse: TGA ATA CAC AAA GGC CAA CA, 621 bp); integrin β4 (forward: GCC TTC ACT TTG AGC ACT CC, reverse: ATG TGG GTG CTA AGG GTT CC, 543 bp).

Western blot analysis. For Western blot analysis, the HCE-T cells were harvested in RIPA buffer (25 mM Tris–HCl, pH 7.4, 1% Tween-20, 0.1% SDS, 0.5% sodium deoxycholate, 10% Glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml of aprotinin, leupeptin, and pepstatin). Protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL). The lysates were boiled for 5 min in 1X SDS sample buffer, loaded and separated on 10% or 12% SDS–PAGE gel, and transferred to a nitrocellulose membrane (Amersham Life Science, Cleveland, OH) using electrotransfer apparatus (Amersham). About 5% skim milk in TBS–0.1% Tween-20 was used for blocking and antibody dilution buffer. The membrane was developed by ECL solution (Santa Cruz, Santa Cruz, CA). Pre-stained molecular weight standards were purchased from Elpis-Biotech (Korea). For lumican, cell culture medium was collected and concentrated by Amicon Ultra-4 (Millipore, Beverly, MA). Antibodies used in this study were obtained as follows: integrins β1, β4, and αV were from Millipore. Integrins α2 and α5 were from BD bioscience (San jose, CA). Phospho-ERK, phospho-p38, phospho-Akt, phospho-JNK, phospho-Src, Akt, and p38 were from Cell signaling (Denvers, MA). ERK, Src, and JNK were from Santa Cruz. Actin was from Sigma. Lumican was from R&D systems (Minneapolis, MN).

Cell migration assay. For the cell migration assay, sterile 8 μm polycarbonate membrane inserts (#137443, Nunc, Denmark) were hydrated with serum-free medium just before use. The outer chambers in a 24-well plates were filled with 0.5 ml medium and 0.3 ml DMEM/F12 containing 1×10^5 cells was added to inner chambers and incubated at 37 °C for 24 h. Cells on the upper side of the chamber were removed gently with a cotton swab and the cells on the lower side of the chamber were fixed with 4% paraformaldehyde, then stained with 0.2% crystal violet. The stained cells were photographed under a microscope. Each group was assayed in triplicate. For inhibitors, the cells and inhibitors (SP600125, SB203580, and U0126) were treated just before plating. In Fig. 1B upper panel, confluent cells in 6-well plates were wounded by the manual scraping with a yellow pipette tip. Assessment of cell migration was performed under microscopy after 24 h of scraping followed by methanol fixation, and 0.2% crystal violet staining.

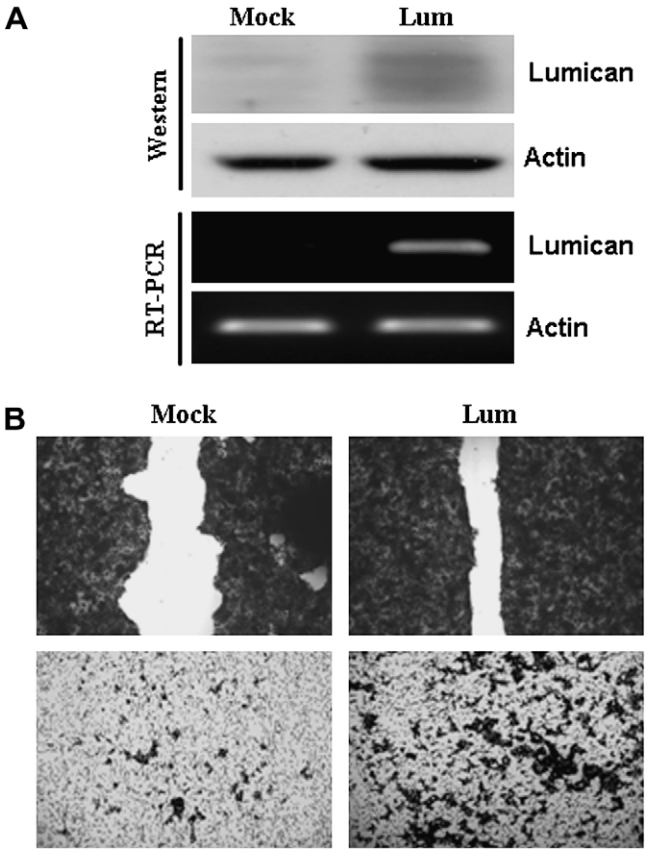

Fig. 1. Over-expression of lumican induces corneal epithelial cell migration. (A) Western blot analysis and RT-PCR showing over-expression of lumican in HCE-T cells. (B) Migration of mock and Lum cells. Migration was assayed by manually damaging confluent cells (upper panel), and by polycarbonate membrane insert migration assay (lower panel). Original magnification, 100×. The results shown are from one of four separate experiments.

Results

Over-expression of lumican induces HCE-T cell migration

To examine the effect of lumican on human corneal epithelial cells, we used HCE-T cells over-expressing lumican. First, we cloned a full-length human lumican cDNA by RT-PCR and inserted it into pIRES-neo vector. We then, transfected the purified pIRES-lumican plasmid into HCE-T cells (Lum cells). After colony selection for two weeks, over-expression of lumican in the Lum cells was confirmed by Western blotting and RT-PCR (Fig. 1A). Expression of lumican was barely detectible in control HCE-T cells, and over-expression of lumican did not influence the expression of other keratan sulfate proteoglycans such as keratocan or mimecan (data not shown).

Next, we compared cell migration between mock-transfected and Lum cells by two methods. The upper panel in Fig. 1B compares wound width closure by mock and Lum cells. The wound width of the Lum cells was narrower than that of the mock cells. In addition, polycarbonate membrane inserts experiment in the lower panel reveals increased migration by the Lum cells. We also examined the proliferation of mock and Lum cells in serum-free culture; after 9 days of culture, there were twice as many Lum cells as mock cells (data not shown).

Integrins α2 and β1 are induced in lumican over-expressing cells

Cell migration is usually mediated by integrins and associated extracellular matrix proteins (ECMs). We therefore examined

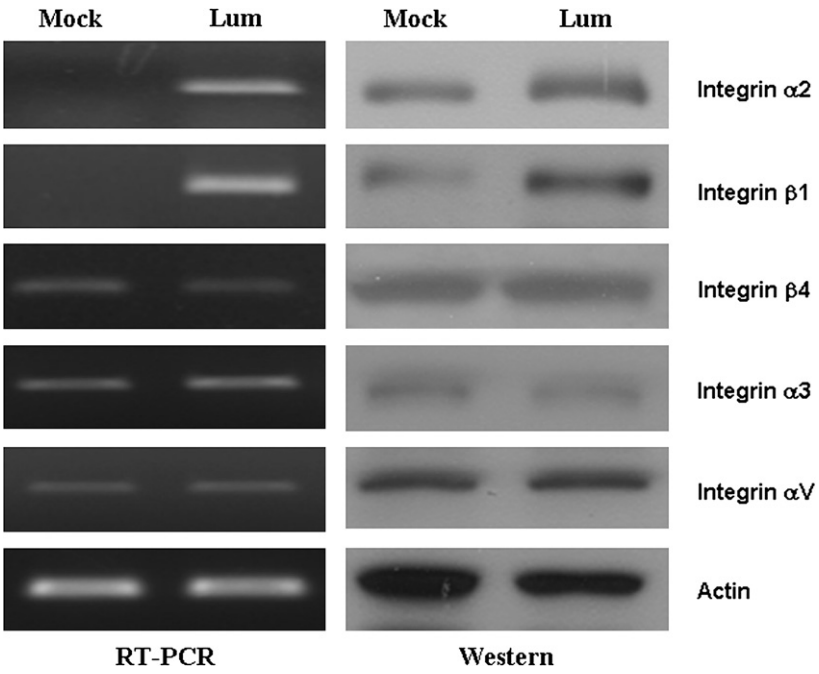

Fig. 2. Lumican enhances expression of integrins α2 and β1 in HCE-T cells. The expression patterns of integrins were analyzed by RT-PCR (left panel) and Western blotting (right panel) of mock and Lum cells. The cells were plated in 6-well plates, transferred the next day to serum-free medium, and harvested after a further 24 h. The results are representative of three independent experiments.

which integrins and ECM proteins might be involved in lumican-induced HCE-T cell migration. RT-PCR and Western blot results showed significant increases of integrins α2 and β1, whereas expression of integrins α3, αV, and β4 was unchanged (Fig. 2). No expression of integrins α5, α6, and β2 was detected by RT-PCR or Western blotting (data not shown).

Next, we investigated the expression of ECM molecules. We detected no changes in the expression of collagens types I–IV and VII, fibronectin, or laminin by either RT-PCR or Western blotting (data not shown).

ERK mediated HCE-T cell migration and integrin expression

We previously showed that insulin-induced HCE-T cell migration is mediated by ERK and Akt signaling [16]. In this study, we investigated the signaling pathways involved in lumican-induced cell migration. Mock and Lum cells were plated and cultured for 24 h in culture medium without serum. Fig. 3 shows the phosphorylation of Akt, Src, p38 MAPK, JNK, and ERK 1/2. Interestingly, only phosphorylation of ERK 1/2 increased in the Lum cells. Next, we tested the inhibitory effect of specific inhibitors on lumican-induced cell migration and integrin expression, using the MAPK-specific inhibitors SP600125 for JNK, SB203580 for p38 MAPK and U0126 for ERK 1/2.

Migration of the Lum cells was only inhibited in the U0126 pretreated cells (Fig. 4A). The Western blot results in Fig. 4B show that the activation and increased expression of ERK 1/2 and integrin β1, respectively, were completely blocked by U0126 in the Lum cells. The increased expression of integrin α2 was not inhibited by U0126, SP600125, or SB203580.

Discussion

The role of lumican has been investigated using lumican knock-out mice. Chakravarti et al. [11] demonstrated abnormal skin collagen fibril assembly, skin fragility and laxity in these lumican deficient mice. In addition to the skin, lumican deficient mice also displayed disruption of the corneal stroma, loss of stroma thickness and light-scattering, increased corneal opacity, and abnormalities of collagen fibrils in the posterior stroma [11–13]. The regulatory role of lumican in the growth and metastasis of cancers [17,18], and tumor-promoting or tumor-suppressing processes, has been well studied [19,20].

The involvement of small leucine-rich proteoglycans (SLRPs) in pathological conditions of the cornea has been suggested. A failure to synthesize a mature keratan sulfate proteoglycan is observed in corneal dystrophy [21], and mutations of SLRP genes have been identified in high myopia [22]. Lumican deficient mice also show delayed corneal wound healing, suggesting that lumican may modulate epithelial cell migration or adhesion [14]. Yeh et al. [15] provided direct evidence for this idea using a lumican purified from human amniotic membrane. Healing of wounded mouse corneas was accelerated by lumican even in lumican deficient mice, implying that lumican promotes corneal epithelial cell migration and proliferation. Our present results also revealed increased migration (Fig. 1B) and cell division (data not shown) in lumican over-expressing human corneal epithelial cells.

Our previous study revealed that, Akt and ERK 1/2 are activated during insulin-induced HCE-T cell migration [16]. ERK 1/2 (Fig. 3) and its upstream activator, MEK 1/2 (data not shown), but not Akt, were also activated in the lumican over-expressing HCE-T cells. When we inhibited ERK 1/2 signaling with U0126, cell migration was strongly blocked (Fig. 4A). We therefore suggest that HCE-T cell migration in response to lumican is regulated by the activity of ERK 1/2. Further study is necessary to define more clearly the lumican-induced signaling pathways that promote HCE-T cell migration.

It was reported recently that melanoma cell adhesion and cell migration is dependent on lumican and its ligand, integrin [23]. We also found that lumican over-expression induced expression of integrins α2 and β1 (Fig. 2). Integrin β1 levels were decreased by the ERK 1/2 inhibitor U0126, but the expression of integrin α2 was not affected by inhibitors of ERK 1/2, p38 MAPK, or JNK. It may be that integrin α2 expression is induced by some unknown signaling pathway.

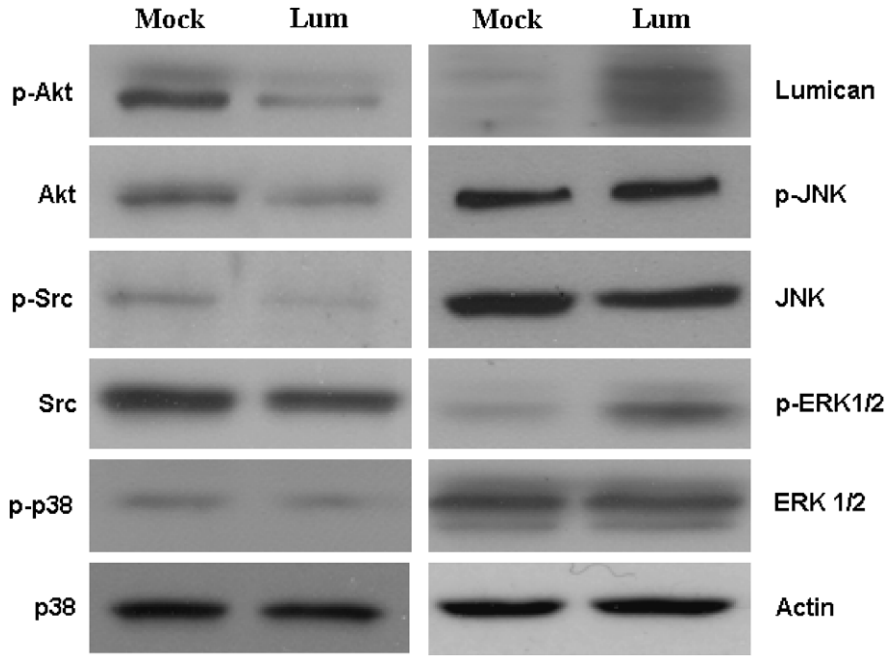

Fig. 3. Activation of ERK 1/2 is increased by lumican in HCE-T cells. Activation of Akt, Src, p38, JNK, and ERK 1/2 was analyzed by Western blot. The cells were plated on 6-well plates in DMEM/F12 + 1% FBS. After 24 h, they were collected and total lysates were loaded on a 10% PAGE gel. The results are representative of three independent experiments.

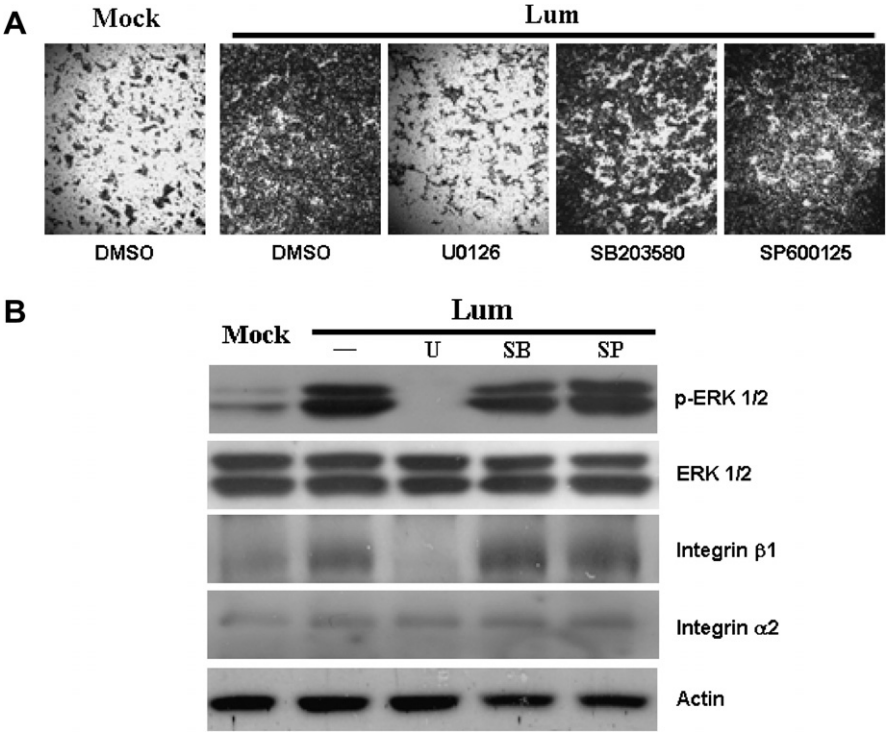

Fig. 4. ERK 1/2 inhibitor U0126 blocks the cell migration and integrin expression induced by lumican. (A) Mock and Lum cells were plated in a polycarbonate membrane inserts with U0126 (10 μM), SB203580 (20 μM), or SP600125 (10 μM). After 24 h, the cells were fixed, stained with crystal violet and photographed under a microscope. Original magnification, 100×. (B) Activation of ERK 1/2 and expression of integrins α2, and β1 was analyzed by Western blotting in cells treated with DMSO (—), U0126 (U), SB203580 (SB), and SP600125 (SP).

In conclusion, the over-expression of lumican in HCE-T cells stimulates cell migration and expression of integrins α2 and β1. The cell migration and expression of integrin β1 are regulated by ERK 1/2 signaling in response to lumican. Our results should help to clarify the mechanism of corneal epithelial cell migration involved in corneal wound healing.

Acknowledgments

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