Lumican is also widely present as a non- or low-sulfated glycoprotein in connective tissues of many other organ systems, e.g. skeleton, heart, kidney, and lung (14–18). During mouse embryonic ocular development, lumican is synthesized by keratocytes; detected as a glycoprotein, not as a KSPG (19); and also transiently expressed by the corneal epithelium, neural retina, and epidermis (14). These observations suggest that epithelial tissues possess the capacity to express lumican under certain conditions.

Several studies have demonstrated that SLRP proteins can modulate cellular behaviors, i.e. cell migration and proliferation during embryonic development, tissue repair, and tumor growth, in addition to their extracellular matrix functions as regulators of tissue hydration and collagen fibrillogenesis (20–22). For example, decorin is one of the SLRP proteins with well characterized functions for modulating cellular behaviors. Decorin protein alters the cell cycle process in neoplastic cells both by modulating the activities of growth factors and by direct interaction with a cell-surface receptor (23, 24). Cultured vascular endothelial cells start to express decorin after the formation of tube-like structures and also up-regulate biglycan expression during repair after a damage (25, 26). Under some pathological conditions, the corneal epithelium shows an increase in decorin immunoreactivity compared with normal corneal epithelium (27). Macrophages, on the other hand, express the cell-surface receptor for lumican. These cells bind the low-sulfated glycoprotein form of lumican, but not lumican modified with keratan sulfate chains, suggesting that non- or low-sulfated lumican might provide a scaffold for macrophages invading injured corneal stroma (28). These observations prompted us to hypothesize that lumican, like decorin, may have biological functions for modulating corneal cell behavior such as adhesion, migration, or proliferation during tissue morphogenesis or wound healing.

In this study, employing in situ hybridization and immunocytochemistry, we first showed that migrating corneal epithelial cells ectopically and transiently express lumican during wound healing. To examine the hypothesis that lumican actively modulates epithelial wound healing, we then examined the effects of an anti-lumican antibody on closure of a corneal epithelial defect and the healing of corneal epithelial defects in lumican-null mice. Our results suggest that lumican may play a role in epithelial cell migration or adhesion, thus contributing to corneal epithelial wound healing.
MATERIALS AND METHODS

Animal Experiments for Histology—Experimental mice \( (n = 52) \) were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg of body weight). The central corneal epithelium (3 mm in diameter) was demarcated with a trephine and subsequently removed using a No. 69 Beaver Blade™ under a stereomicroscope as previously reported (29, 30). Neomycin ointment was topically applied to prevent bacterial infection. The animals were then killed at specific intervals of healing (1, 2, 4, or 8 h and 1, 2, 3, 5, 7, 14, 21, or 28 days). Each eye was removed, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h, embedded in paraffin, and processed for histology.

Preparation of an Epitope-specific Polyclonal Anti-lumican Antibody—To prepare the polyclonal antibody, a synthetic oligopeptide sequence \((\text{YYDYIDPLFMYGQISPNC})\) deduced from mouse lumican cDNA was conjugated to keyhole limpet hemocyanin (32). The polyclonal antibodies were raised in rabbits as described previously (32). Anti-lumican antibodies were purified with an affinity column prepared by conjugating the oligopeptide to Sulfolink™ (Pierce) using the procedures recommended by the manufacturer.

Western Blotting to Characterize the Anti-lumican Antibody—Mouse corneal KSPGs and recombinant mouse lumican expressed in Escherichia coli were prepared as described previously (33), and the core protein of the KSPGs were deglycosylated by treatment with \(N\)-glycanase (9). Two \( \mu \)g of total protein was subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (9). Lumican was detected by immunostaining with the anti-lumican antibody (10 \( \mu \)g/ml) using an indirect method as described previously (32).

Immunohistochemistry—To locate lumican protein, paraffin sections from normal and injured corneas were incubated with the epitope-specific anti-lumican antibody (10 \( \mu \)g/ml) and an affinity-purified polyclonal anti-lumican antibody as described previously (34). Anti-lumican antibodies were prepared in rabbits as described above.

Transmission Electron Microscopy—Corneas were fixed in 2.0% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4 °C. The samples were then post-fixed in 2.0% osmium tetroxide, rinsed in 0.1 M phosphate buffer, and embedded in Epon 812 (Quetol 812, Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and observed by transmission electron microscopy (35).

In Vitro Wound Healing of the Corneal Epithelium of Organ-cultured Mouse Eyes—To examine whether the anti-lumican antibody inhibits corneal epithelial wound healing, we developed an in vitro wound healing model using organ-cultured mouse eyes. A central corneal epithelial defect (2 mm in diameter) was produced in both eyes of 38 anesthetized mice (32). The animals were then post-fixed in 2.0% osmium tetroxide, rinsed in 0.1 M phosphate buffer for 24 h at 4 °C. The samples were then post-fixed in 2.0% osmium tetroxide, rinsed in 0.1 M phosphate buffer, and embedded in Epon 812 (Quetol 812, Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and observed by transmission electron microscopy (35).

In Vivo Wound Healing of the Corneal Epithelium of Organ-cultured Mouse Eyes—To examine whether the anti-lumican antibody inhibits corneal epithelial wound healing, we developed an in vivo wound healing model using organ-cultured mouse eyes. A central corneal epithelial defect (2 mm in diameter) was produced in both eyes of 38 anesthetized wild-type mice under a stereomicroscope. Our preliminary immunohistochemical examination with a rat monoclonal anti-laminin antibody (X50, BIORDESIGN International, Kennebunkport, ME) showed the presence of the uninterrupted epithelial basement membrane immediately after the epithelial débridement (data not shown). The animals were killed immediately after the epithelial débridement. Each eyeball was enucleated and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 1.4% fetal calf serum and 50 \( \mu \)g/ml gentamycin in 10% CO\(_2\) and 90% air at 37 °C with the anti-lumican antibody (40 \( \mu \)g/ml) or normal rabbit IgG (control) as described previously (34). The antibody reacted to recombinant lumican (lane 1) and deglycosylated mouse KSPG core protein (lane 2). Lumican in Epithelial Wound Healing

FIG. 1. Western blot characterization of the anti-lumican antibody. Recombinant mouse lumican and mouse KSPG core protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was immunostained with the affinity-purified polyclonal anti-lumican antibody (10 \( \mu \)g/ml). The antibody reacted to recombinant lumican (lane 1) and deglycosylated mouse KSPG core protein (~41 kDa) (lane 2).

Derived from mouse strain 129/Sv by electroporation using a Bio-Rad Gene-Pulser. A targeted ES cell clone (frequency of 1:186) was identified by Southern blot hybridization and used to generate chimeric mice in our Gene Targeting Core Facility (University of Cincinnati). C57BL/6J blastocysts injected with 10–12 ES cells were implanted in pseudopregnant F1(CBA × C57BL6) foster mothers (Jackson Immunoresearch Laboratories, Inc.) Chimeric mice, identified by agouti coat color, were mated with C57BL/6J mice. Agouti coat-colored offspring were tested for the presence of the targeted locus by polymerase chain reaction (PCR) and Southern hybridization as described previously (32). To distinguish between individuals with none, one, or two copies of the mutant gene, we designed three primers in a PCR for detection of the wild type (primer 1, 5'-TACTTCAACGGCTCTTCAG-3'; and primer 2, antisense 5'-CAAGTCTAGGATCTCAAGG-3'; 190 base pairs) and mutant (primers 2 and 3, antisense 5'-CGAGAATGAGAGCTG-3' of the hprt minigene; 381 base pairs), respectively. Northern hybridization, in situ hybridization, and immunohistochemistry were used to determine the phenotypes of littermates using the procedures described above.

Healing of Corneal Epithelial Defects in Lumican-deficient Mice—Age-matched littermates were used as controls. Two-month-old Lum\(^{-/-}\) \((n = 22)\) and Lum\(^{+/-}\) \((n = 16)\) mice were anesthetized and subjected to 2-mm corneal epithelial débridement as described above. Our preliminary immunohistochemistry results showed the presence of the non-interrupted epithelial basement membrane immediately after epithelial scraping in both Lum\(^{-/-}\) and Lum\(^{+/-}\) mice as described above. The injured corneas were stained with fluorescein and photographed with a stereomicroscope every 24 h, beginning immediately after wounding, to evaluate the epithelialization and to detect any sign of infection. Healing of epithelial defects was graded and statistically analyzed in a manner similar to the in vitro wound healing experiment described above.

RESULTS

Characterization of Polyclonal Anti-lumican Antibody—We prepared epitope-specific anti-lumican antibody as described under “Methods and Materials.” Western blot analysis was used to characterize an affinity-purified rabbit polyclonal antibody directed against the N-terminal oligopeptide of mouse lumican (YYDYIDPLFMYGQISPNC). This sequence is not found in keratan or other members of the SLRP family (12, 27). Fig. 1 demonstrates that the antibodies reacted with recombinant lumican prepared from the expression clone of E. coli (lane 1) and a 41-kDa KSPG core protein isolated from
mouse corneas after deglycosylation with N-glycanase (lane 2).

**Phenotypic Changes in Mice Lacking Lumican**—Fig. 2A summarizes the strategy used to ablate the lumican gene in mice via gene-targeting techniques. A targeting construct containing the human hypoxanthine phosphoribosyltransferase and herpes simplex virus thymidine kinase genes was prepared as described under “Materials and Methods.” The genotypes of the lumican knockout mice were determined by PCR and Southern blot analysis (Fig. 2B and C). Northern hybridization, in situ hybridization, and immunohistochemistry revealed no expression of lumican mRNA and the absence of lumican protein antigens in Lum−/− mice (Fig. 2D and E). This phenotype of the lumican-null mice is indicative of a loss of lumican expression rather than the presence of a dominant-negative mutation. The homozygous mutant mice were born alive in the expected mendelian ratio and were fertile. The skin of adult Lum−/− mice was fragile as evidenced by the disruption of skin when experimental animals were killed by cervical dislocation. The back skin hairs of adult Lum−/− mice were disarranged. It is of interest to note that the male lumican-null mice appear to produce a smaller number of offspring compared with female Lum−/− mice when they are mated with wild-type mice. The reason for the phenomenon is not known. However, it could be secondary to the fragile skin phenotype.

**Clinical and Histological Observations of Eyes after Epithelial Debridement in Wild-type Mice**—A corneal epithelial defect (3 mm in diameter) was created as described under “Materials and Methods.” One day after injury, the central corneal stroma was still exposed, and a healing epithelium was observed in the peripheral. Polymorphonuclear leukocytes were seen in the healing stroma at day 1 after injury. By day 3, the defect was covered by regenerated epithelium in all corneas examined (data not shown).

**In Situ Hybridization Detection of Lumican mRNA**—As shown in Fig. 4A, lumican mRNA was detected in the stromal keratocytes, but not in epithelial cells of uninjured corneas. Injured corneal epithelium expressed lumican mRNA from 8 h until 3 days after the epithelial debridement (Fig. 4C–E and G–I). Seven days after injury, corneal epithelial defects were healed with a stratified multicellular epithelium that did not
yield positive hybridization signals (Fig. 4). No signals were seen with sense probes (Fig. 4, B and F). Neither lens epithelial cells nor neural retina hybridized to the lumican antisense riboprobes (data not shown). Keratocan mRNA was not detected in either normal or migrating epithelium, but was present in keratocytes (data not shown).

**Immunohistochemistry**—In tissue sections, the anti-lumican antibody stained normal corneal stroma (Fig. 5A) as well as sclera and dermal connective tissue of the eyelid (data not shown). Lumican staining in the corneal stroma was more intense in the posterior than in the anterior stroma. The anti-lumican antibody did not react to the epithelium or basement membrane of uninjured cornea (Fig. 5A) or other ocular tissues, e.g. neural retina, lens epithelial cells, and the epidermis of the eyelid (data not shown). After wounding, faint lumican immunoreactivity was seen in the cytoplasm of basal cells of regenerated epithelium. The antibodies also reacted to the basement membrane underlying the basal epithelial cells of injured corneas healed for 2 and 3 days. After day 7, the epithelium was negative for lumican protein (Fig. 5F).

**Effect of the Anti-lumican Antibody on Epithelial Healing**—Epithelial defects (2 mm in diameter) closed in ~48 h in the in vitro wound healing model. As shown in Table I, the anti-lumican antibody (40 μg/ml) significantly inhibited epithelial healing as compared with control normal rabbit IgG (40 μg/ml). Preliminary observations revealed that the addition of mitomycin C (0.1 μg/ml) to the culture medium did not retard the closure of the epithelial defect, but did inhibit epithelial proliferation as judged by incorporation of bromodeoxyuridine (data not shown). These observations are consistent with the notion that lumican may have a role in epithelial cell migration during wound healing.

**Healing of Epithelial Defects in Lumican-deficient Mice**—Fig. 6 and Table II summarize the wound healing of a 2-mm-diameter central corneal epithelial defect in lumican-null mice. One day after injury, the number of corneas completely re-epithelialized was significantly higher in Lum\(^{-/-}\) mice than in Lum\(^{+/+}\) mice. Two and 4 h after injury, no signals for lumican mRNA were observed in injured epithelium (C and D). Signals for lumican mRNAs were detected in injured corneal epithelium (open arrows) and in keratocytes (arrowheads) at 8 h (E) and at 1 (G), 2 (H), and 3 (I) day(s) after epithelial debridement, whereas no signals were seen with sense probes (F). At day 7, regenerating epithelium was negative for lumican mRNA, whereas keratocytes were positive (J). A–F, without counterstaining; G–J, counterstained with 0.5% neutral red. The arrows indicate the edge of the injured or healing epithelium. Bar = 100 μm.

**DISCUSSION**

The evidence available suggests that SLRP proteins are key regulatory molecules of collagen fibrillogenesis (36). Null mutants of SLRP family proteins, i.e. decorin, biglycan, fibromodulin, and lumican, are manifested by malfunctions of connective tissues associated with abnormal extracellular matrix, i.e. fragile skin, cloudy cornea, and thick collagen fibrils (15, 37–39). Our lumican-null mice have phenotypes that closely resemble...
Indicates the edge of the healing epithelium.

Bar protein.

An epithelial defect (3 mm in diameter) was created in corneas of anesthetized mice. The animals were killed after specific intervals of healing, and corneas were processed for immunohistochemistry with the anti-lumican antibody (10 μg/ml). In a normal mouse cornea, lumican protein was detected in the corneal stroma (A). No staining was seen in the control (B). Localization of lumican protein in healing mouse corneas after an epithelial debridement is shown in C–F. Very faint lumican immunoreactivity was detected in healing corneal epithelium (thick arrow) at day 1 (C) and was obviously observed in healing epithelia (open arrows) at days 2 (D) and 3 (E). Regenerated epithelium at day 7 (F) was negative for lumican protein. A–F were counterstained with hematoxylia. The thin arrow indicates the edge of the healing epithelium. Bar = 20 μm.

**TABLE I**

| Wound healing of a corneal epithelial defect in organ-cultured mouse eyes |
|---------------------------------------------------------------|
| Results are from organ cultures of mouse eyes with a 2-mm-diameter epithelial defect in the presence of the anti-lumican antibody (40 μg/ml) or normal rabbit IgG (40 μg/ml) for 48 h. Healed indicates completely resurfaced; punctate, resurfaced by regenerated epithelium with punctate staining; and defect, not healed with remaining defects. |
| Healed | Punctate | Defect | Total |
|--------|----------|--------|-------|
| Control IgG | 12 | 24 | 2 | 38 |
| Anti-lumican antibody | 7 | 15 | 16 | 38 |

* Percent defect area remaining (ranges): #, averages of 1.63% (1.0–2.3) and 14.1% (1.0–64.0), respectively.  
  * p < 0.005 compared with control IgG using the χ² test.

Those described by Chakravarti et al. (15). It is of interest that the results of the present study imply that lumican, in addition to serving as a regulator of collagen fibrillogenesis, may modulate epithelial cell migration during corneal wound healing. We observed an ectopic and transient expression of lumican mRNA in healing mouse corneal epithelium as early as 8 h after an epithelial debridement (Fig. 4). An obvious accumulation of lumican protein occurred in the basal epithelial cells and the basement membrane of injured epithelia after 2–3 days of healing (Fig. 5). The observation is consistent with the notion that lumican is secreted by the epithelial cells. The up-regulated lumican synthesis can account for the intracellular immunoreactivity observed in the healing epithelium. The addition of anti-lumican antibodies to the culture medium retarded closure of an epithelial defect in healing wounds in vitro (Table I), and Lum⁻/⁻ mice lacking lumican showed delayed re-epithelialization of corneal epithelial defects in vivo (Fig. 6 and Table II). These observations are consistent with the hypothesis that lumican expressed in injured corneal epithelium modulates corneal epithelial wound healing. Interestingly, keratocan, another member of SLRP family closely related to lumican, was not expressed by injured corneal epithelium, but only by stromal keratocytes. This finding indicates that these two KSPG proteins are transcriptionally regulated differently, albeit both are major KSPG constituents of the corneal stroma extracellular matrix.

Several other studies have presented results consistent with epithelial expression of lumican. In chick, KSPG precursor protein synthesis by organ culture of corneal epithelia amounts to 7.2% of the protein synthesis of organ-cultured whole corneas (40). Moreover, the basal and suprabasal cells of the hyperproliferative corneal epithelium of a Corn1 mouse (41), a mutant mouse characterized by hyperplasia of the central corneal epithelium associated with corneal neovascularization, express lumican mRNA. In the adult cornea, lumican exists as KSPG; however, in non-corneal tissues as well as in embryonic and wounded corneas, lumican is found as a low- or non-sulfated glycoprotein (14, 18, 42). Rat corneal epithelium was previously shown to transiently up-regulate glycoprotein synthesis as a result of wounding (43, 44). This glycoprotein may represent the lumican induction described in the present study. Our data do not directly elucidate the mechanism by which lumican may modulate epithelial cell adhesion or migration. Recently, a novel bone KSPG core protein of the SLRP family, osteoadherin, was found to be distributed in bovine fetal rib growth plate and in newly deposited bone in vivo (45). It has been demonstrated that osteoadherin can mediate cell attachment via binding by αvβ3 integrin in vitro (45). A divalent cation-dependent lumican cell-surface receptor has been identified in macrophages, implying possible binding of integrin to lumican. Binding of cells to lumican, however, is strongly inhibited by the presence of keratan sulfate

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![Image](image-url)
Lumican in Epithelial Wound Healing

A 2-mm epithelial defect was created at the center of the cornea of a Lum<sup>+/−</sup> or Lum<sup>−/−</sup> mouse with a No. 69 Beaver Blade<sup>®</sup> and was allowed to heal as described under “Materials and Methods.”

**TABLE II**

| Genotype | No. of corneas examined | Outcome | Genotype | No. of corneas | Outcome |
|----------|-------------------------|---------|----------|----------------|---------|
| +/-      | 22                      | Healed  | +/-      | 14             | Healed  |
|          |                         | Punctate|          | 3              | 0       |
|          |                         | Defect  |          | 6<sup>a</sup>  | 0       |
| -/-      | 16                      | Healed  | -/-      | 15<sup>d</sup> | 0       |
|          |                         | Punctate|          | 8              | 3       |
|          |                         | Defect  |          | 11             | 2       |
|          |                         |         |          | 1              | 5       |

<sup>a</sup> Healed indicates completely resurfaced; punctate, resurfaced by regenerated epithelium with punctate staining; defect, not healed with remaining defects. All injured corneas of Lum<sup>−/−</sup> mice healed within 5 days, whereas those of heterozygous Lum<sup>+/−</sup> healed in 3 days.

<sup>b</sup><sup>c</sup> Percent defect area remaining in defect group corneas (ranges): averages of 5.9% (0.25–25), 18.7% (1.0–66.3), 7.5% (4.0–12.3), and 3.3% (0.3–6.3), respectively.

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<sup>3</sup> S. Saika, C.-Y. Lin, and W.-W.-Y. Kao, unpublished observations.