Galectin-7 as a Potential Mediator of Corneal Epithelial Cell Migration

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Objective: To assess the role of a carbohydrate-binding protein, galectin-7, in reepithelialization of corneal wounds.

Methods: Transepithelial excimer laser ablations were performed on mouse corneas, and the wounds were allowed to partially heal in vivo for 18 to 22 hours. At the end of the healing period, expression levels of galectin-7 messenger RNA and protein were analyzed using semiquantitative reverse transcriptase–polymerase chain reaction, Western blot analysis, and immunohistochemical localization studies. To determine the effect of exogenous galectin-7 on reepithelialization of corneal wounds, corneas with 2-mm alkali burn wounds were allowed to partially heal in vitro for 20 to 24 hours in serum-free media in the presence or absence of recombinant galectin-7. At the end of the healing period, the wound areas were photographed and quantified.

Results: Expression of galectin-7 messenger RNA and protein was markedly up-regulated in the corneal epithelium after injury. Exogenous galectin-7 stimulated reepithelialization of corneal wounds. The stimulatory effect of galectin-7 on corneal epithelial wound closure was specifically inhibited by a competing sugar, β-lactose, but not by an irrelevant disaccharide, sucrose.

Conclusions: Galectin-7 has the potential to mediate corneal epithelial cell migration and reepithelialization of wounds.

Clinical Relevance: These findings have broad implications for developing novel, galectin-based, therapeutic strategies for treatment of nonhealing corneal epithelial defects.

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The failure of reepithelialization of wounds is usually due to a reduced potential of the adjacent epithelial cells to migrate across the wound bed. The failure of epithelial cells to migrate or of migrated epithelia to remain adhered to the surface of corneal wounds leads to worsening clinical conditions, such as nonhealing epithelial defects and corneal ulceration. Cell migration, a complex biological process entailing sequential adhesion and release from the substrate, is mediated, at least in part, by cell-matrix interactions. Although transmembrane integrin receptors are thought to be important players in cell-matrix interactions, recent studies have provided evidence that members of a class of carbohydrate-binding proteins, galectins, may also facilitate cell-matrix adhesion by mediating recognition between extracellular matrix (ECM) and cell surface glycoconjugate receptors.

Galectins constitute a gene family of widely distributed animal lectins characterized by their affinity for β-galactoside-containing glycans. They are found on the cell surface, in ECM, and in the cytoplasm and nucleus of cells. Of the 14 known members identified in mammalian tissues, galectin-1 and galectin-3 are well characterized. Both lectins are expressed in inflammatory cells and in epithelia and fibroblasts of various tissues and have been shown to bind to distinct isoforms of laminin, fibronectin, vitronectin, and integrins. They are therefore thought to have the potential to mediate cell-matrix interactions. Despite the fact that all galectins bind to β-galactose residues, different members of the galectin family may bind distinct glycoconjugate receptors and may mediate specific biological functions because each galectin has unique, fine specificity for more complex oligosaccharides.

In a recent study, Cao et al demonstrated that galectin-3, but not galectin-1, plays a role in corneal epithelial cell migration. Specifically, it was found that the rate of reepithelialization of corneal wounds is significantly slower in galectin-3–deficient mice compared with wild-type mice. In con-
trast, there was no difference in the rate of reepithelialization of corneal wounds between galectin-1 knockout mice and wild-type mice. Cao and coauthors further demonstrated that exogenous galectin-3, but not galectin-1, stimulates reepithelialization of corneal wounds.

In the present study, we provide evidence that another galactose-binding protein, galectin-7, has the potential to play a role in corneal epithelial cell migration and reepithelialization of corneal wounds. Galectin-7 was first reported in 1994.\textsuperscript{10} It is also known as PIG1 (p53-induced gene 1) and is thought to play a role in apoptosis.\textsuperscript{17,18} Unlike galectin-1 and galectin-3, which are widely expressed in a variety of cell types, expression of galectin-7 is restricted to epithelia that are stratified or are destined to become stratified.\textsuperscript{19,20} We demonstrate herein that the expression level of galectin-7 is markedly upregulated in mouse corneas after injury and that exogenous galectin-7 stimulates reepithelialization of corneal wounds in organ culture specimens.

### METHODS

#### PREPARATION OF NORMAL AND HEALING CORNEAS

Throughout this study, 2- to 4-month-old mice (C57BL/6 and 129 mixed genetic background) were used. All animal treatments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Vision Research and to the recommendations of the National Institutes of Health for the Care and Use of Laboratory Animals. Transepithelial excimer laser ablations (2-mm optical zone; 42- to 44-µm ablation depth, phototherapeutic keratectomy mode) were performed on the right eye of 5 to 10 mice using the Summit Apex Plus Excimer Laser (Summit Technology Inc, Waltham, Mass). Corneas were allowed to partially heal in vivo for 18 to 22 hours. At the end of the healing period, animals were humanely killed, and the corneas of both eyes were processed for Western blot analysis, immunohistochemical localization studies, and semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR).

#### WESTERN BLOT ANALYSIS

Briefly, protein extracts of normal and healing corneas were prepared in radioimmunoprecipitation assay buffer (50mM Tris-hydrochloride, pH 8.0, containing 150mM sodium chloride, 0.1% Nonidet P-40, and 0.5% deoxycholic acid) and were electrophoresed (20 µg) on 12.5% sodium dodecyl sulfate–polyacrylamide gels. Recombinant galectin-7 produced in Escherichia coli\textsuperscript{21} served as a positive control. The proteins were transferred onto polyvinyl difluoride membranes (Millipore Corporation, Bedford, Mass), and the blots were sequentially incubated with polyclonal rabbit anti-human galectin-7\textsuperscript{7,18} (1:250 dilution, overnight, 4°C) and horseradish peroxidase–conjugated goat anti–rabbit IgG secondary antibody (1:200 dilution, 1 hour, room temperature) (Vector Laboratories Inc, Burlingame, Calif).\textsuperscript{21} Immune complexes were detected using a chemiluminescence detection system (PerkinElmer Life Sciences, Boston, Mass). The blots were subsequently stripped in a stripping buffer (62.5mM Tris-hydrochloride, pH 6.8, 2% sodium dodecyl sulfate, 100mM β-mercaptoethanol) for 30 minutes at 50°C and reprobed with monoclonal mouse anti–α-tubulin antibody (Sigma-Aldrich Corp, St Louis, Mo) diluted 1:100,000. Control blots were processed in the same way except that the step involving incubation with the primary antibody was omitted.

#### IMMUNOHISTOCHEMICAL LOCALIZATION STUDIES

For immunohistochemical staining, the eyes (4 normal and 4 healing corneas) were fixed in 10% buffered formalin for 2 hours, processed, and embedded in paraffin wax. Five-millimeter thick sections of normal and healing corneas were dewaxed in xylene and hydrated by successive immersions in a graded series of ethanol and water. Sections were rinsed with phosphate-buffered saline solution and were sequentially incubated with 3% hydrogen peroxide to inhibit endogenous peroxidases, 2.5% goat serum in phosphate-buffered saline solution to block nonspecific binding, polyclonal rabbit anti–human galectin-7 (1:250 dilution),\textsuperscript{18} biotinylated goat anti–rabbit IgG (Vector Laboratories Inc) (1:200 dilution), a freshly prepared complex of avidin D and biotin peroxidase (Vector Laboratories Inc), and the diaminobenzidine reagent (KPL Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md). Control sections were treated with nonimmune rabbit IgG serum instead of the immune serum.

#### SEMIQUANTITATIVE RT-PCR

The total RNA of normal and healing corneas was isolated using the reagents provided in the Atlas Pure Total RNA Labeling System kit (BD Biosciences Clontech, Palo Alto, Calif) (yield: ≈11 µg for normal corneas and ≈4 µg for healing corneas). Semiquantitative RT-PCR was performed using the total RNA (0.5 µg) of the corneas and the gene-specific custom primers for galectin-7, ribosomal protein S29 and ornithine decarboxylase. Housekeeping genes ribosomal protein S29 and ornithine decarboxylase served as controls. Gene-specific primers were purchased from BD Biosciences Clontech, and other reagents used for PCR were from the Advantage RT for PCR kit and the Advantage 2 PCR kit (BD Biosciences Clontech). The annealing temperature was 68°C, and reactions were subjected to 18 to 38 cycles of PCR amplification. Amplified products collected at various cycles were analyzed by electrophoresis in 1.3% agarose–ethidium bromide gels. Intensity comparisons of the appropriate amplified products were made during the initial, exponential phase of DNA synthesis. Control reactions were incubated in the absence of RT.

#### EXPERIMENTS TO TEST THE THERAPEUTIC POTENTIAL OF GALECTIN-7

Recombinant full-length human galectin-7 was produced in E coli and purified as described previously.\textsuperscript{18} We produced 2-mm corneal wounds on both eyes of each animal by placing alkaline-soaked (0.5N sodium hydroxide) filter disks on the surface of the cornea for 2 minutes. The eyes were excised after injury, pinned down on paraplast wax in 24-well plates using 1-cm-long tips of 20-gauge needles (1 eye per well), and then incubated in serum-free media\textsuperscript{22} (0.5 mL/well) in a tissue culture incubator for 20 to 24 hours. The left eyes of animals served as controls and were incubated in the media alone. Right eyes were incubated in media containing various test reagents, including (1) galectin-1 or galectin-7 (20 µg/mL), (2) galectin-7 plus 0.1M β-lactose, and (3) galectin-7 plus 0.1M sucrose. At the end of the healing period, wound areas were visualized by staining with methylene blue and were quantitated using Sigma Scan software (SPSS Inc, Chicago, Ill).\textsuperscript{23} Each group contained a minimum of 3 eyes. All experiments were performed at least twice. An unpaired, 2-group t test was used to test for the significance of the data.
Figure 1. Western blot analysis showing that healing corneas express an increased level of galectin-7 protein compared with normal corneas. Radioimmunoprecipitation assay buffer extracts of normal and healing corneas representing 20 µg of protein were loaded in each lane and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels. After immunostaining with antibody to galectin-7, the blot was stripped and subsequently reprobed with antibodies to tubulin. The healing corneas contain an increased level of galectin-7 (14 kd) compared with normal corneas. Western blot analysis was performed at least twice on each sample with reproducible results. Recombinant galectin-7 (R-Gal7) (50 ng) served as a positive control.

RESULTS

HEALING CORNEAS EXPRESS INCREASED LEVELS OF GALECTIN-7

Western blot analysis using detergent extracts of normal and healing corneas revealed that healing corneas contain markedly increased levels of galectin-7 protein compared with normal corneas (Figure 1). In extracts of healing corneas, an intensely stained 14-kd component comigrating with recombinant galectin-7 was detected (Figure 1). In contrast, this component was detected only in trace amounts in extracts of normal corneas (Figure 1). Stripping and restaining of the same immunoblot with antitubulin revealed that extracts of normal and healing corneas contain nearly equal levels of tubulin protein (Figure 1). This ensured that samples representing equal amounts of protein of normal and healing corneas were used for electrophoreses. No components were detected in the control blots incubated with the secondary antibody alone (data not shown).

In immunohistochemical localization studies, the galectin-7 staining intensity was similar in the leading edge of the migrating epithelium (Figure 2A) and in the peripheral epithelium (Figure 2B) of healing corneas. In normal and healing corneas, the galectin-7 immunoreactivity was pronounced at sites of cell-matrix interactions (Figure 2). Corneal stromal matrix and keratocytes of normal and healing corneas did not react with anti–galectin-7 antibody. No immunoreactivity was detected in the control sections incubated with the nonimmune serum (data not shown).

Semiquantitative RT-PCR using the total RNA of normal and healing corneas revealed that healing corneas also contain an increased level of galectin-7 gene transcripts. The differences in the expression level of galectin-7 transcripts between normal and healing corneas were readily visible during the initial, exponential phase of DNA synthesis (Figure 3, lanes 1-3). However, the extent of up-regulation of galectin-7 messenger RNA (mRNA) transcripts in the healing corneas was lower than that of galectin-7 protein. Gene transcripts of 2 housekeeping genes, ribosomal protein S29 and ornithine decarboxylase, were expressed in nearly equal amounts in normal and healing corneas (Figure 3), ensuring that samples representing equal amounts of the total RNA of normal and healing corneas were used for amplification. No PCR products were amplified in the control reactions carried out in the absence of RT (data not shown). Semiquantitative RT-PCR and Western blot analysis were performed at least twice on each sample with reproducible results.

EXOGENOUS GALECTIN-7 STIMULATES CORNEAL EPITHELIAL WOUND CLOSURE

To determine whether exogenous galectin-7 would stimulate reepithelialization of corneal wounds in organ culture, corneas with alkali burn wounds were incubated in serum-free media in the presence or absence of recombinant galectin-7. After a 20- to 24-hour healing period, the remaining wound areas were quantified. Exogenous galectin-7 stimulated the rate of corneal epithelial wound closure (Figure 4). In corresponding experiments, recombinant galectin-1 did not stimulate the corneal epithelial wound closure rate. The stimulatory effect of galectin-7 on corneal epithelial wound closure was specifically inhibited by a competing sugar, β-lactose, but not by an irrelevant disaccharide, sucrose (Figure 4).

COMMENT

In the present study, evidence is provided indicating that galectin-7 is a potential mediator of corneal epithelial cell migration and reepithelialization of corneal wounds. Ex-
pression of galectin-7 was markedly up-regulated in mouse corneas after injury, and recombinant galectin-7 stimulated reepithelialization of corneal wounds. Immunohistochemical localization studies revealed that the increased expression of galectin-7 in healing corneas is not localized to the leading edge of the migrating epithelium; instead, after injury, there is increased expression of galectin-7 throughout the epithelium. In normal and healing corneas, galectin-7 was located in high density at sites of corneal epithelial cell-matrix adhesion, an ideal location for influencing cell-matrix interactions and cell migration. Healing corneas also contained elevated levels of galectin-7 mRNA transcripts compared with normal, uninjured corneas. These data are consistent with the results of a recent study comparing the gene expression patterns of normal and healing corneas using complementary DNA microarrays, which indicated that healing corneas contain 5.2-fold more gene transcripts for galectin-7 than normal corneas. Although galectin-7 protein and mRNA were up-regulated after corneal injury, the extent of up-regulation of the protein was higher than that for the galectin-7 mRNA. Although measured mRNA levels are implicitly extrapolated to indicate the levels of the corresponding protein in the cell, mRNA and protein levels often are not correlated for a variety of reasons, including variation in (1) posttranscriptional mechanisms controlling the protein translational rate, (2) half-lives of specific proteins and mRNAs, and (3) codon bias values.

In a recent study, Gygi et al compared the mRNA transcript and protein expression levels of many genes and concluded that mRNA transcript levels are poor predictors of protein expression. The authors found that a variety of genes with comparable mRNA levels exhibited a 20-fold difference in protein expression. Likewise, mRNA levels of some proteins expressed in comparable levels varied 30-fold.

A major finding of the present study is that exogenous recombinant galectin-7 stimulates reepithelialization of corneal wounds. The stimulatory effect of galectin-7 on the rate of corneal epithelial wound closure was partially abrogated by a competing disaccharide, β-lactose, but not by an irrelevant disaccharide, sucrose. This suggests that the carbohydrate recognition domain of the lectin is directly involved in the beneficial effect of the exogenous lectin on wound closure. As described earlier, exogenous galectin-3 also stimulates reepithelialization of corneal wounds in a lactose-inhibitable manner.

In contrast, another galactoside-binding protein, galectin-1, had no effect on the rate of reepithelialization of corneal wounds. Because each galectin has unique, fine specificity for more complex oligosaccharides, different galectins may mediate distinct biological functions. Currently, we are characterizing corneal epithelial cell surface receptors of galectin-3 and galectin-7 to identify specific cell surface glycoprotein molecules that play a role in galectin-mediated reepithelialization of corneal wounds.

Regarding the mechanism by which galectin-7 affects reepithelialization of corneal wounds, there are several possibilities. The potential of the lectin to interact with ECM glycoproteins and cell surface glycoproteins and glycolipids may permit the lectin to act as a bridge linking cells to ECM. Although the glycoconjugates of the corneal epithelial cell surface and ECM that serve as binding sites for galectin-7 have not been characterized, galectin-7, similar to galectin-1 and galectin-3, may bind to isoforms of well-known ECM molecules, such as laminin, fibronectin, and vitronectin, which contain polylactosamine chains. Another ECM glycoprotein that is expected to bind with high affinity to galectin-7 is lumican. Lumican is an unsulfated form of keratan sulfate proteoglycan, and it contains polylactosamine chains. In a recent study, Saika et al showed that although lumican glycoprotein is not expressed in normal mouse corneal epithelium, it is transiently expressed in the epithelial basement membrane of healing corneas and that the corneal epithelial wound closure rate is significantly reduced in lumican-deficient mice. Galectin-7 may also bind

![Figure 3. Semiquantitative reverse transcriptase–polymerase chain reaction showing that healing corneas express an increased amount of galectin-7 transcripts compared with normal corneas.](image)

![Figure 4. Exogenous galectin-7 (Gal-7) stimulates reepithelialization of corneal wounds. Corneas with 2-mm alkali burn wounds were allowed to heal in organ culture in serum-free media in the presence and absence of recombinant galectin-1 (Gal-1) or Gal-7 (20 µg/mL) and saccharides (0.1M) for 20 to 24 hours. At the end of the healing period, wound areas were quantified. Gal-7, but not Gal-1, stimulated corneal epithelial wound closure. β-Lactose (Lac), but not sucrose (Suc), inhibited the stimulatory effect of Gal-7 on the wound closure rate. Arrows indicate outlines of remaining wound areas from one of the experiments.](image)
with high affinity to the cell surface glycolipids of healing corneal epithelium. In this respect, it was previously demonstrated that migrating corneal epithelia express markedly elevated levels of neolactoglycosphingolipids containing polylactosamine chains compared with normal nonmigrating corneal epithelia and that antibodies to the polylactosamine chains inhibit corneal epithelial wound closure in vitro.

The findings presented in this article have broad implications for developing novel, galectin-based therapeutic strategies for treatment of not only nonhealing corneal epithelial defects but also possibly other epithelial tissues, such as skin, especially since it is known that mechanisms mediating reepithelialization of corneal and skin wounds are similar, at least in some respects. At present, treatment of nonhealing corneal defects is a major clinical problem. Also, the severity and intractability of nonhealing skin wounds have caused intense suffering in patients and have fueled a decades-long, dedicated search for clues to amelioration or cure by researchers and clinicians alike. Many growth factors, including epidermal growth factor, transforming growth factor α, fibroblast growth factor, keratinocyte growth factor, and hepatocyte growth factor (growth factors known to simulate mechanisms mediating reepithelialization of wounds) have been tested for usefulness in corneal and cutaneous epithelial wound healing with disappointing results. Moreover, epithelia of corneas treated with growth factors such as epidermal growth factor become hyperplastic. Perhaps the clinical potential of galectin-7 will prove more attractive than the growth factors.

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