

Colocalization of Galectin-3 With CD147 Is Associated With Increased Gelatinolytic Activity in Ulcerating Human Corneas

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Submitted: October 20, 2017

Accepted: December 3, 2017

Citation: Cruzat A, Gonzalez-Andrades M, Mauris J, et al. Colocalization of galectin-3 with CD147 is associated with increased gelatinolytic activity in ulcerating human corneas. *Invest Ophthalmol Vis Sci.* 2018;59:223–230. <https://doi.org/10.1167/iivs.17-23196>

PURPOSE. Galectin-3 is a carbohydrate-binding protein known to promote expression of matrix metalloproteinases, a hallmark of ulceration, through interaction with the extracellular matrix metalloproteinase inducer CD147. The aim of this study was to investigate the distribution of galectin-3 in corneas of patients with ulcerative keratitis and to determine its relationship to CD147 and the presence of gelatinolytic activity.

METHODS. This was an observational case series involving donor tissue from 13 patients with active corneal ulceration and 6 control corneas. Fixed-frozen sections of the corneas were processed to localize galectin-3 and CD147 by immunofluorescence microscopy. Gelatinolytic activity was detected by in situ zymography.

RESULTS. Tissue from patients with active corneal ulceration showed a greater galectin-3 immunoreactivity in basal epithelia and stroma compared with controls. Immunofluorescence grading scores revealed increased colocalization of galectin-3 and CD147 in corneal ulcers at the epithelial–stromal junction and within fibroblasts. Quantitative analysis using the Manders' colocalization coefficient demonstrated significant overlap in corneas from patients with ulcerative keratitis ($M1 = 0.29$; $M2 = 0.22$) as opposed to control corneas ($M1 = 0.01$, $P < 0.01$; $M2 = 0.02$, $P < 0.05$). In these experiments, there was a significant positive correlation between the degree of galectin-3 and CD147 colocalization and the presence of gelatinolytic activity.

CONCLUSIONS. Our results indicate that concomitant stimulation and colocalization of galectin-3 with CD147 are associated with increased gelatinolytic activity in the actively ulcerating human cornea and suggest a mechanism by which galectin-3 may contribute to the degradation of extracellular matrix proteins during ulceration.

Keywords: CD147, corneal ulceration, epithelial–stromal junction, galectin-3, matrix metalloproteinase

Estimates of the incidence of ulcerative keratitis remain high, with 71,000 new annual cases in the United States alone.¹ The disease can be initiated by infectious or noninfectious conditions that result in the disruption of the epithelial cell layer and the progressive loss of stroma, which can lead to corneal perforation and loss of vision. Infectious ulcerative keratitis is caused by various microbial pathogens that adhere to and penetrate the corneal epithelium, thereby gaining access to the corneal stroma. The etiology of noninfectious ulcerative keratitis is more diverse and less understood, often representing a diagnostic and therapeutic challenge.² Recognizing the causes that initiate and sustain corneal ulceration is critical for optimal clinical management. Although many pharmacologic approaches can be effective, in some cases, the ulcer worsens despite treatment, leading to the use of more aggressive surgical strategies.²

Restoration of an intact corneal epithelial cell layer is essential to reestablish corneal homeostasis. Conversely persistence of epithelial defects and rupture of the epithelial basement membrane are known to precede degradation of extracellular matrix components during corneal ulceration.^{3–6} These events result in the release into the stroma of a myriad of compounds, such as platelet-derived growth factor, which concentrates on the basement membrane, plus numerous epithelial cytokines involved in the modulation of the wound healing response.^{7,8} Failure to regenerate the basement membrane following injury has been associated with pathologic wound healing, corneal scarring and/or ulceration.⁶

The existence of extracellular matrix-degrading activity during the ulcerative process, originating primarily from the actions of specific members of the matrix metalloproteinase (MMP) family, is well known.^{9,10} The increased expression of

these proteolytic enzymes appears confined to the stroma underlying the healing epithelium, suggesting that epithelial-stromal interactions play a role in their induction.¹¹ Subsequent investigations have demonstrated that the extracellular matrix metalloproteinase inducer CD147 (or EMMPRIN) elicits MMP production in the epithelial-stromal interface and the ulcerating anterior stroma.¹² Consequently, it has been hypothesized that sustained activation of CD147 in chronic situations of delayed healing leads to excessive matrix degradation. However, a mechanism by which CD147 is persistently activated in these situations has not yet been verified.

Both oligomerization and glycosylation of CD147 are critical to the stimulation of matrix metalloproteinase production.¹³ It is now also clear that the actions promoted by CD147 can be modulated by galectin-3, a multivalent β -galactoside-binding lectin known to promote formation of plasma membrane lattices with unique functions.^{14,15} In corneal epithelial cells, galectin-3 interacts with and induces clustering of CD147, resulting in the expression of MMP9 and the initiation of cell-cell disassembly.¹⁶ Interestingly, epithelial-derived MMP9 is increased at the edges of corneal ulceration and participates in the degradation of basement membrane components.⁶ In this study, we investigated the distribution of galectin-3 in corneas of patients with ulcerative keratitis and its relationship to CD147 and the presence of extracellular matrix-degrading activity.

MATERIALS AND METHODS

Study Population

Corneas from patients with active corneal ulceration or perforation who required transplantation were obtained as discarded tissue from the Cornea Service of the Massachusetts Eye and Ear Infirmary. Corneal lesions were imaged by anterior segment optical coherence tomography using an RTVue OCT system (Optovue, Fremont, CA, USA). Corneal tissue for the nonulcerating control group comprised either eye bank donor corneas or keratoplasty tissue from nonulcerating corneas. Institutional Review Board approval (Ref# 13-013H) was obtained from the Massachusetts Eye and Ear Human Studies Committee.

Immunofluorescence

Corneal specimens were frozen in optimal cutting temperature compound for sectioning using a cryostat. Tissue sections (8 μ m thick) on glass slides were fixed in methanol at -20°C for 10 minutes, rehydrated with PBS, and then incubated with 3% BSA in PBS for 10 minutes. Slides were incubated overnight with mouse anti-human CD147 antibody (1:100; clone HIM6; BioLegend, San Diego, CA, USA) and rat anti-human galectin-3 antibody (1:100; clone M3/38; ATCC, Rockville, MD, USA) in 1% BSA in PBS. Samples were treated with the secondary antibodies Alexa Fluor 488 rabbit anti-mouse IgG (1:500) and Alexa Fluor 594 rabbit anti-rat IgG (1:500) for 1 hour at room temperature. Incubation with primary antibodies was routinely omitted in control experiments. Slides were finally washed, mounted in VectaShield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA), and observed and photographed on a Zeiss Axio Observer Z1 inverted fluorescent microscope (Carl Zeiss Microimaging GmbH, Jena, Germany). Histologic assessment was performed by light microscopy using hematoxylin and eosin.

In Situ Zymography

The in situ gelatinolytic activity was measured using the EnzChek Gelatinase Assay kit (Molecular Probes, Carlsbad, CA,

USA) following the manufacturer's instructions. Briefly, corneal tissue sections (8 μ m thick) in optimal cutting temperature compound were incubated with 20 μ g/mL DQ-gelatin-fluorescein isothiocyanate overnight at 37°C , washed with PBS, and mounted using coverslips and VectaShield mounting medium containing DAPI. Sections were then examined on a Zeiss Axio Observer Z1 inverted fluorescent microscope and photographed. As a negative control, frozen cryostat sections were incubated with reaction buffer supplemented with a general metalloproteinase inhibitor (1,10-phenanthroline).

Grading Scheme

Grading of immunofluorescence and in situ zymography photographs for the experimental and control groups was carried out independently by two masked observers guided by standard photographic references. Images were assessed for the presence and colocalization of galectin-3 and CD147, and for the presence of gelatinolytic activity, both at the basal epithelium and within the stroma. The staining intensity was scored in digital images as follows: no expression or very weak expression, -/+; moderate expression, ++; and strong expression, +++.

This scoring system was also used to evaluate whether the areas of colocalization within the tissue were none or minimal (-/+), partial (++) , or prevalent (+++). The photographic references included tissue with varying degrees of protein expression or colocalization. When the masked observers did not agree on their assessment, the individual scores were annotated in the tables using a slash punctuation mark. The observers were asked to specify whether colocalization was observed in the epithelial-stromal junction.

Image Analysis Software

The quantitative analysis of immunofluorescence images was performed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The colocalization of galectin-3 and CD147 was determined by measuring the Manders' coefficients M1 and M2.¹⁷ Values of these coefficients range from 0 to 1 and express the fraction of intensity in a channel that is located in pixels where there is above threshold intensity in the other color channel. For that purpose, the plugin Coloc 2 was applied to the red and green channels of the images. Subsequently, a comparison between the experimental and control groups was performed. A line-intensity scan analysis was likewise applied to determine the colocalization of galectin-3 and CD147 within the epithelial-stromal junction and stromal fibroblasts. Here, pixel intensity values for the red and green channels were determined within six linear regions of interest (20 μ m long). The intensity values were normalized to the maximum peak for each independent intensity curve.¹⁸

Statistical Analyses

Statistical analysis was carried out with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) for Macintosh.

RESULTS

Demographic Characteristics

The 13 keratoplasty specimens with active corneal ulceration comprised five patients with failed keratoplasties (Boston KPro), four failed corneal grafts, one rheumatoid arthritis, one Stevens-Johnson syndrome, one keratoconus, and one idiopathic, with a mean age of 54.7 ± 15.5 years (range, 28-75 years). The typical clinical appearance of corneal ulceration, as

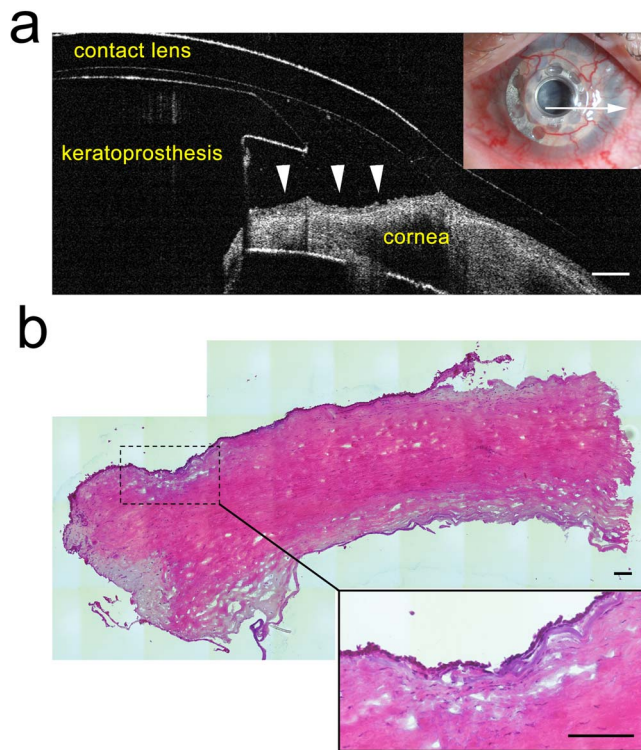


FIGURE 1. Cornea of a 38-year-old man with keratolysis after keratoprosthesis implantation. (a) Anterior segment optical coherence tomography reveals stromal thinning (*arrowheads*) in the area adjacent to the keratoprosthesis. *Scale bar* denotes 250 μm . (b) Hematoxylin and eosin staining demonstrates epithelial erosion with stromal dissolution and underlying inflammation. *Scale bars* denote 100 μm .

shown in a patient with a failed keratoprosthesis, was epithelial breakdown and stromal lysis (Fig. 1). No case evidenced active microbial infection. The six corneas comprising the control group included three keratoplasty specimens of central corneal scar and three normal eye bank donors. The mean age of the control group was 57.3 ± 15.1 years (range, 38–76 years). Demographic and clinical characteristics of the study population are provided in Table 1.

Localization of Galectin-3 and CD147 in Normal and Ulcerated Corneas

Galectin-3 in normal corneas is localized primarily on apical cells of the stratified epithelium, where it associates with transmembrane mucins to provide barrier function, and to a lesser extent, to basal and suprabasal epithelial cells.¹⁹ CD147 expression, on the other hand, has been reported across the entire normal corneal epithelium, with basal epithelial cells having stronger expression than superficial cells in central cornea.¹² Our observations of control corneas demonstrated galectin-3 and CD147 staining throughout the entire stratified epithelium, predominantly within cell membranes (Fig. 2a). Consistent with previous results, the strongest staining for galectin-3 was observed within apical membranes of apical epithelial cells.

Evaluation of active ulceration tissue revealed increased staining of galectin-3 and CD147 in the basal and suprabasal cell layers of the corneal epithelium. Interestingly, increased staining of these two proteins was also observed within stromal fibroblasts of ulcerating tissue samples (Fig. 2b). Visual scoring of the tissue samples showed limited colocalization of galectin-3 and CD147 in control corneas, in contrast to

TABLE 1. Demographic and Clinical Characteristics of the Study Population

No.	Age, y	Sex	Keratolysis	Etiology
Controls				
1	71	M	No	None (eye bank cornea)
2	63	F	No	None (eye bank cornea)
3	76	F	No	None (eye bank cornea)
4	45	M	No	Scar (trauma)
5	51	F	No	Scar (infection, vascularized)
6	38	M	No	Scar (infection, vascularized)
Cases				
7	65	F	Yes	Keratoprosthesis
8	47	F	Yes	Keratoprosthesis
9	73	F	Yes	Keratoprosthesis
10	75	M	Yes	Keratoprosthesis
11	38	M	Yes	Keratoprosthesis
12	63	M	Yes	Failed graft
13	55	F	Yes	Failed graft
14	63	M	Yes	Failed graft
15	51	F	Yes	Failed graft
16	72	F	Yes	Rheumatoid arthritis
17	48	M	Yes	Idiopathic
18	33	M	Yes	Stevens-Johnson syndrome
19	28	M	Yes	Keratoconus

ulcerating corneas, which were commonly characterized by the strong overlap of the two proteins in both the basal epithelium and stroma (Table 2). In these analyses, keratolysis was remarkably associated with the presence of galectin-3 and CD147 within the epithelial-stromal junction.

Quantitative colocalization analysis of digital immunofluorescence images further demonstrated increased overlap of galectin-3 and CD147 staining in ulcerating corneas (Fig. 3a; Supplementary Table S1). Here, full images in each of the ulcerating and control corneas were evaluated using the Manders' colocalization coefficients. The average M1 coefficient in ulcerating corneas was 0.29, indicating that approximately 29% of the galectin-3 staining in the entire image colocalized with the imaged CD147; the average M2 coefficient (i.e., the portion of CD147 that colocalized with the imaged galectin-3) was 0.22. On the other hand, the M1 and M2 coefficients in control corneas were 0.01 ($P < 0.01$) and 0.02 ($P < 0.05$), respectively. Line-intensity scan analysis evidenced that, similar to the visual scoring data, the colocalization of galectin-3 and CD147 occurred within the epithelial-stromal junction and stromal cells (Fig. 3b).

Gelatinolytic Activity and Galectin-3/CD147 Distribution in Cornea

The presence of gelatinolytic activity was evaluated in six control corneas and six ulcerating corneas by in situ zymography. In these experiments, we found that ulceration promotes the activation of gelatinases in corneal epithelium and stroma (Fig. 4a). Visual scoring of the tissue sections revealed that most control corneas had none or weak gelatinolytic activity, whereas most ulcerating corneas were characterized by moderate to strong gelatinolytic activity, particularly in the basal epithelium (Table 3). In addition, we found that the presence of galectin-3 and CD147 colocalization correlated with an increased gelatinolytic activity in the tissue sections. As shown in Figure 4b, there was a significant positive relationship between the degree of colocalization of these two proteins and the levels of gelatinolytic activity in the corneas analyzed (slope of regression, 0.61; $R^2 = 0.37$; $P < 0.01$).

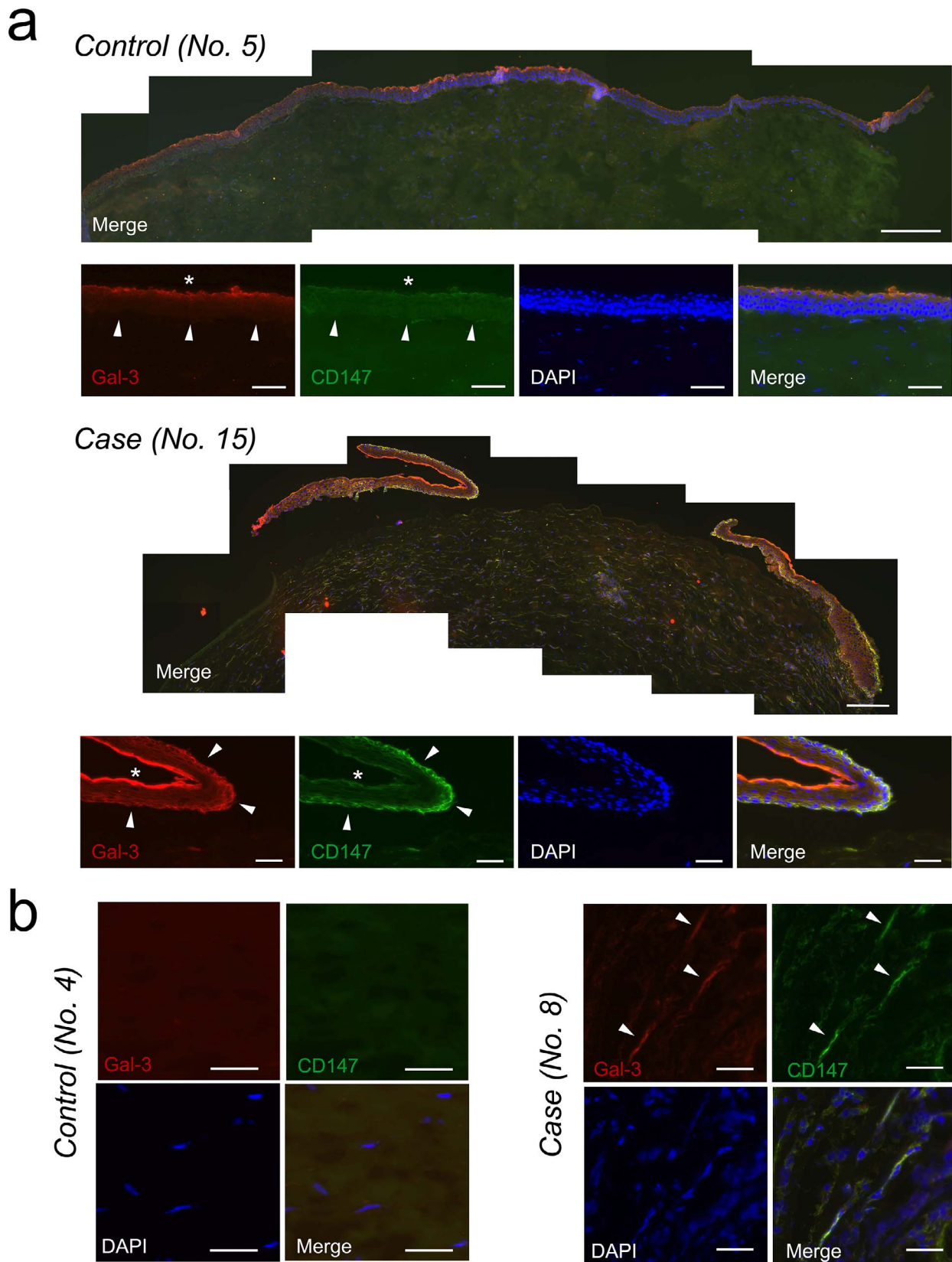


FIGURE 2. Immunofluorescence labeling of galectin-3 and CD147 in corneas of control subjects and patients with keratolysis. **(a, top)** Montage showing galectin-3 staining (red) predominantly in the apical surface epithelia of a control cornea. Weak membranous staining of CD147 (green) can be observed throughout the corneal epithelium. At higher magnification, it is possible to observe lack of galectin-3 and CD147 colocalization at the epithelial-stromal junction (arrowheads). **(a, bottom)** Cornea of a patient with keratolysis showing greater galectin-3 and CD147 immunoreactivity compared with control. At higher magnification, there is colocalization of galectin-3 and CD147 on cell membranes in the basal epithelium (arrowheads). Asterisks indicate the superficial layer. Scale bars denote 200 (montage) and 50 μ m (lower panels). **(b)** Weak galectin-3 and CD147 staining was detected within the stroma of control corneas. On the other hand, keratolysis was characterized by increased galectin-3 and CD147 immunoreactivity and colocalization within the stromal fibroblasts (arrowheads). Scale bars denote 50 μ m.

TABLE 2. Localization of Galectin-3 and CD147 in Corneal Tissue From Control Subjects and Patients With Keratolysis

No.	Basal Epithelium			ESJ	Stroma		
	Gal-3	CD147	Colocalization		Gal-3	CD147	Colocalization
Controls							
1	+	-	-	No	-/+	-	-
2	+	+	+	No	+	+	+
3	+	+	-	No	-	-	-
4	n.e.	n.e.	n.e.	n.e.	+	-	-
5	+	++	+	No	-	-/+	-
6	+	+ / ++	+ / ++	No	+	+ / ++	+
Cases							
7	++	+++	++ / +++	Yes	+	++	+ / ++
8	n.e.	n.e.	n.e.	n.e.	+++	+++	+++
9	+++	+++	+++	Yes	++	+	+
10	n.e.	n.e.	n.e.	n.e.	++	++	++
11	++	++	++	No/yes	++	++	++
12	+ / + / +++	+++	+++	Yes	++	+ / + / +++	+ / + / +++
13	++	+++	+ / + / +++	Yes	+ / ++	++	+ / ++
14	+++	+++	+++	Yes	+	+ / ++	+
15	+++	+++	+++	Yes	+ / + / +++	+ / + / +++	+ / + / +++
16	++	+++	+ / + / +++	Yes	+ / + / +++	+ / + / +++	+ / + / +++
17	+ / + / +++	+++	+++	Yes	+ / + / +++	+ / + / +++	+ / + / +++
18	+ / ++	+	+	Yes	+ / + / +++	+	+
19	+ / ++	+ / ++	+ / ++	Yes	+ / ++	+ / + / +++	+ / ++

ESJ, epithelial-stromal junction; n.e., no epithelium present.

DISCUSSION

Under physiologic wound healing conditions, increased gelatinolytic activity occurs in basal keratinocytes at the front of migrating epithelium, functioning to promote degradation of components of the epithelial basement membrane and to favor corneal reepithelialization.⁹ However, there are situations in which the reepithelialization process is delayed, and the

epithelial defects become chronic, leading to keratolysis. It has been proposed that, under these conditions, uncontrolled gelatinolytic activity contributes to the nonhealing process and the subsequent formation of stromal ulcers.^{4,9,20} Elucidating the molecular mechanisms leading to the abnormal expression of gelatinases remains complex and incompletely understood. Here we demonstrate that the presence of gelatinolytic activity in ulcerating human corneas positively correlates with the

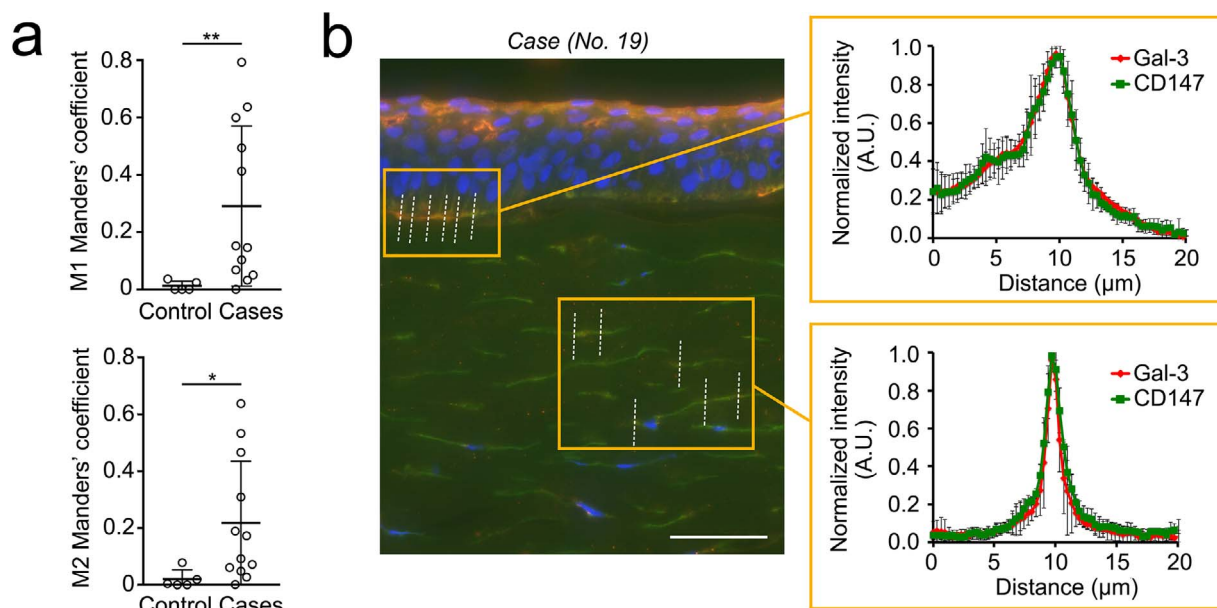


FIGURE 3. Quantitative colocalization analysis of galectin-3 and CD147. (a) The Manders' coefficients M1 and M2 revealed a significant increase in colocalization of galectin-3 and CD147 in corneas of patients with keratolysis compared with controls. Statistical significance between experimental and control groups was evaluated with the Mann-Whitney *U* test for nonparametric data. **P* < 0.05, ***P* < 0.01. (b) Line-intensity scan analyses for galectin-3 (red) and CD147 (green) were performed in two different areas corresponding to the epithelial-stromal junction and stromal cells, using the average of six regions of interest (dotted white lines). Measurement of the intensity values demonstrated colocalization in those regions. Scale bar denotes 50 μ m.

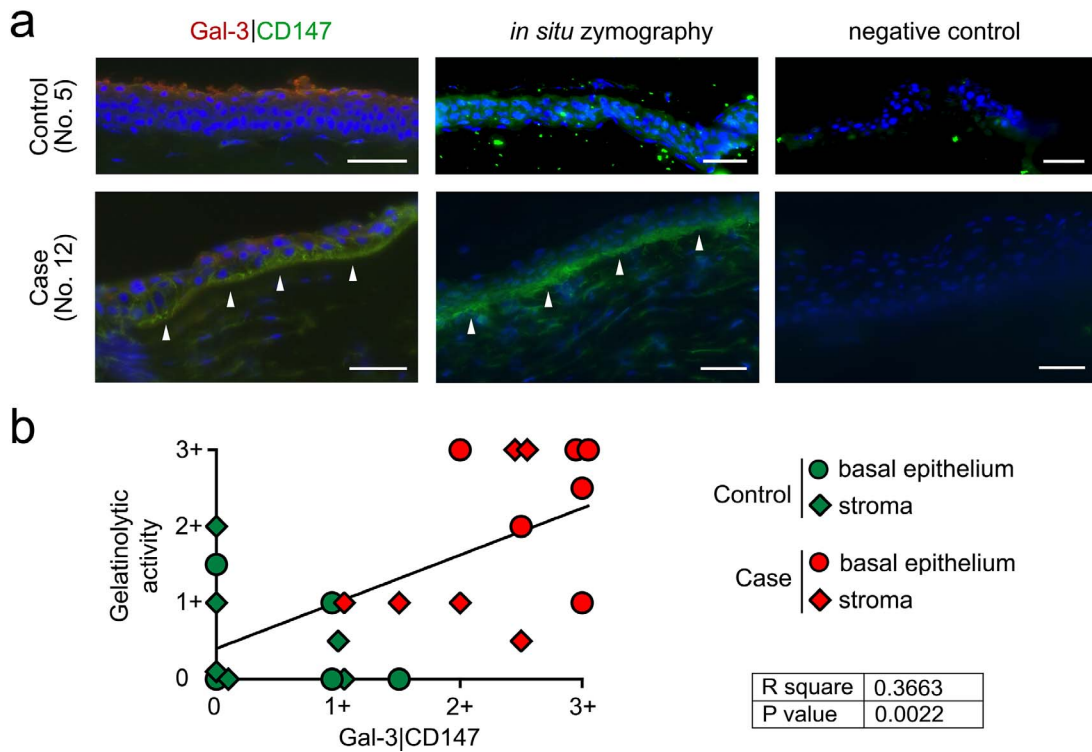


FIGURE 4. Gelatinolytic activity and galectin-3/CD147 distribution in cornea. **(a)** Increased gelatinolytic activity (*arrowheads*) was observed by *in situ* zymography in samples showing positive galectin-3 and CD147 colocalization. *Scale bars* denote 50 μ m. **(b)** Scatterplot demonstrating a significant positive correlation between gelatinolytic activity and the degree of colocalization based on visual scoring data. The *P* value is based on linear regression analysis. The straight line represents the regression line.

induction of galectin-3, a carbohydrate-binding protein known to promote expression of gelatinolytic activity through interaction with the extracellular matrix metalloproteinase inducer CD147.

CD147 is a widely distributed cell surface glycoprotein highly expressed on the surface of keratinocytes during wound healing, as well as of malignant tumor cells.^{6,21} Gabison et al. first reported its presence in ulcerated corneas in 2005 and demonstrated a role for CD147 in the induction of matrix metalloproteinases as a result of direct corneal epithelial-stromal interactions.¹² In these experiments, a remarkable increase in CD147 was observed in ulcerated areas, predominantly in the wounded stroma at the epithelio-stromal

boundary. MMP2 also increased in the anterior stroma and colocalized with CD147. Consistent with these observations, we found increased staining of CD147 and increased gelatinolytic activity at the epithelio-stromal boundary and within stromal fibroblasts in actively ulcerating tissue samples. Further, we observed increased expression of CD147 in basal epithelial cells that correlated with the presence of gelatinolytic activity in ulcerating corneas, suggesting a role for CD147 in modulating expression of gelatinases not only in stromal cells but also corneal epithelium.

It is the highly glycosylated species of CD147 that are responsible for the induction of matrix metalloproteinases,²² with β 1,6-branched poly lactosamine residues constituting

TABLE 3. Gelatinolytic Activity in Corneal Tissue From Control Subjects and Patients With Keratolysis

No.	Basal Epithelium		Stroma	
	Colocalization Gal-3/CD147	Gelatinolytic Activity	Colocalization Gal-3/CD147	Gelatinolytic Activity
Controls				
1	-	+ / ++	-	++
2	+	-	+	-
3	-	-	-	+
4	n.e.	n.e.	-	-
5	+	+	-	-
6	+ / ++	-	+	- / +
Cases				
11	++	+++	++	+
12	+++	+++	++ / +++	+++
13	++ / +++	++	+ / ++	+
14	+++	+	+	-
15	+++	+++	++ / +++	- / +
17	+++	++ / +++	++ / +++	+++

major components in the highly glycosylated CD147.¹³ These glycan residues are unique in that they mediate cell motility and reepithelialization via modulation of carbohydrate-dependent interactions between galectin-3 and many cell surface and extracellular molecules, such as fibronectin, laminin, and integrins.^{23–25} Recent experiments in vivo, using galectin-3-null mice, and in vitro using human corneal epithelial cells, have highlighted the requirement of galectin-3 for the induction of MMPs. Importantly, galectin-3 in these studies has been found to induce MMP expression in a manner dependent on the interaction with and clustering of CD147.¹⁶ Examination of tissue samples from patients with active corneal ulceration in the current study revealed a striking increase in expression of galectin-3 within the basal epithelium and stromal cells. Moreover, we found that galectin-3 colocalized with CD147 in areas showing increased gelatinolytic activity, supporting a mechanism by which sustained interaction between galectin-3 and CD147 contributes to the degradation of extracellular matrix proteins during corneal ulceration. It should be noted, however, that these experiments were performed in a heterogeneous group of pathologies and that inclusion of a larger cohort of patients may yield additional insights into the role of galectin-3 and CD147 within specific subtypes of ulcerative keratitis.

Progress in understanding the role of proteases in basement membrane and stromal dissolution has led to the use of inhibitors of matrix metalloproteinases in an attempt to reduce the severity of corneal ulceration.^{26,27} These include the tetracycline family of molecules, which have relatively weak inhibitory constants, but also potent synthetic inhibitors such as ilomastat, which has been shown to reduce deep ulcerations and perforations in a prospective clinical study of 300 patients with acute bacterial keratitis.² A major concern when using these inhibitors for the clinical management of ulceration is the assessment of potential risks.²⁸ Broad-spectrum inhibitors may be harmful to use as they can prevent the action of proteases and other nonrelated proteins associated with physiologic healing.²⁹ Furthermore, a complication with some protease inhibitors has been that the minimum effective concentration can be toxic or cause adverse reactions.³⁰ On the basis of this report, we envision that targeting the specific actions of and/or interactions between galectin-3 and CD147 may provide new therapeutic opportunities for preventing and treating corneal ulceration.

Acknowledgments

The authors thank Marium Aftab Ahmed for technical support and Marie-Claude Robert for her assistance in collecting tissue samples. Supported by the National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA (Grants R01EY024031 and P30EY003790) and the Boston-KPro research fund.

Disclosure: **A. Cruzat**, None; **M. Gonzalez-Andrades**, None; **J. Mauris**, None; **D.B. AbuSamra**, None; **P. Chidambaram**, None; **K.R. Kenyon**, None; **J. Chodosh**, None; **C.H. Dohlman**, None; **P. Argüeso**, None

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