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Hemangiogenesis and Lymphangiogenesis in Corneal Pathology

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Abstract

Purpose—We characterized the presence of hemangiogenesis (HA) and lymphangiogenesis (LA) in human corneal specimens exhibiting 13 underlying pathologies.

Methods—Human corneal specimens were obtained from consenting subjects (n = 2 or n = 3 for each pathology; total sample size, n = 35). The pathological specimens were stained with hematoxylin and eosin (H&E) to determine the presence or absence of corneal neovascularization (NV) and superficial or deep stromal distribution of NV. Immunohistochemical staining was then performed to differentiate HA (positive for CD31) from LA (positive for lymphatic vessel endothelial hyaluronan receptor-1 [LYVE-1]).

Results—The double-negative ($CD31^-$/$LYVE\text{-}1^-$) immunostaining, indicating the absence of NV, was exhibited by 21 specimens (60%). $CD31^+$/$LYVE\text{-}1^-$, indicating the presence of HA and absence of LA, was exhibited by 12 specimens (34%). The double-positive ($CD31^+$/$LYVE\text{-}1^+$) phenotype, indicating both HA and LA, was exhibited by 2 specimens (6%). Notably, the $CD31^-$/$LYVE\text{-}1^+$ phenotype, indicating the presence of LA and absence of HA, was not detected among the specimens. Deep stromal NV was exhibited in a 4:3 ratio to superficial stromal NV. The double-negative immunostaining was more prevalent in noninflammatory pathologies, particularly in comparison with combined neovascular phenotypes (ie, $CD31^+$ or $LYVE\text{-}1^+$). Among the neovascular phenotypes, HA was 7 times more common than LA. Specimens exhibiting LA presented only with the double-positive phenotype.

Conclusions—HA is the predominant component of NV in corneal pathologies. LA accompanies HA; however, isolated LA (from lymphatics in the conjunctiva) does not occur in these corneal pathologies. Our results suggest the potential therapeutic utility of targeting antineovascular therapies specifically for corneal HA and/or LA pathology.

Keywords

corneal neovascularization; hemangiogenesis; lymphangiogenesis; LYVE-1; CD31

Corneal avascularity, which reflects a balance between competing proangiogenic and antiangiogenic mediators, has been characterized as "angiogenic privilege."[1–5] Corneal neovascularization (NV), also referred to as angiogenesis or hemangiogenesis (HA), is the formation of ectopic corneal vasculature from preexisting vasculature.[6,7] Corneal NV is thus antagonistic to angiogenic privilege and mediates a number of visual morbidities,[8] such as

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blindness,[3] general visual impairment, and abnormal wound healing.[1,5,9] Corneal NV is promoted by a wide variety of proangiogenic factors—vascular endothelial growth factor, basic fibroblast growth factor, stromal derived factor-1, CXC chemokines, interleukin-6, platelet-derived growth factor, hepatocyte growth factor, and metalloproteinases (MMP2, MMP9, and MMP14).[5,6,10–13] In addition, numerous pathological insults are known to promote corneal NV, including various infectious, inflammatory, and chemical traumas.[14–16] Antiangiogenic factors include angiostatin (produced by the proteolytic cleavage of plasminogen) and endostatin (a type XVIII collagen proteolytic product).[5]

The proposed mechanisms of neovascular visual impairment consist of lipid keratopathy, corneal scarring, and edema.[17–20] In addition, preoperative corneal NV is associated with increased incidence of graft failure secondary to penetrating keratoplasty,[21,22] the most common form of allotransplantation.[23] Several conditions may cause graft failure, such as increased intraocular pressure, infection excluding endophthalmitis, and surface problems that are not immune mediated. However, graft rejection usually indicates the specific immunologic response of the host to the donor corneal tissue. A corneal graft with immunologic response may or may not cause corneal graft failure. Similarly, Regenfuss et al[3] report that the presence of lymphatic vasculature suggests a high-risk status of a recipient bed, and Cursiefen et al[1] report that HA concomitant with lymphangiogenesis (LA) poses an appreciable threat for graft rejection.

Corneal HA is the neovascular proliferation of blood vessels, whereas LA is the proliferation of lymphatic endothelial cells.[24,25] The characterization of LA has historically lagged behind that of HA because of a comparative dearth of known lymphatic vessel protein markers.[5,26] Recently, however, a number of LA-specific protein markers (vascular endothelial growth factor receptor-3 [VEGFR-3], podoplanin, prox1, and lymphatic vessel endothelial hyaluronan receptor-1 [LYVE-1]) have been identified.[27–29]

In the present study, we determined the presence of HA and LA in 13 underlying ocular pathologies by immunoassay with anti-LYVE-1 and anti-CD31 antibodies. Our findings demonstrated that these markers were spatially dispersed in stereotypical neovascular patterns.[30–32] We also reported that (1) a neovascular phenotype was detected in 40% of pathological specimens, (2) HA was detected 7 times more frequently than LA (LA did not occur in the absence of HA, whereas 86% of specimens with HA did not exhibit LA), and (3) the ratio of deep stromal NV to superficial stromal NV was 4:3. These findings suggest potential therapeutic utility of clinical interventions targeted specifically to HA and/or LA pathology.

MATERIALS AND METHODS

Specimen Selection

This was a descriptive observational study characterizing the presence of NV in corneal specimen exhibiting 13 underlying ocular pathologies. A total of 35 corneal buttons were selected from the archives of the Ophthalmic Pathology Laboratory at the University of Illinois at Chicago. Two or 3 examples of each of the following diagnoses were retrieved: *Acanthamoeba* keratitis, bullous keratopathy, chronic keratitis, corneal scarring, Fuchs dystrophy, fungal keratitis, graft failure, graft rejection, herpes simplex virus (HSV) keratitis, inflammatory pannus, keratoconus, pterygium, and ulcerative keratitis with perforation. Subjects provided informed consent for removal of corneal specimens in accordance with the guidelines for experimental investigations required by the Institutional Review Board at the University of Illinois at Chicago (Chicago, IL).

Immunohistochemistry

Formalin-fixed paraffin-embedded blocks were cut into 4-μm consecutive sections. Consecutive sections were individually stained with a hematoxylin and eosin (H&E) stain, an immunohistochemical stain using antibodies against CD31, and an immunohistochemical stain using antibodies against LYVE-1. H&E–stained sections were photographed under an Axioscope II bright field microscope (Carl Zeiss Micro-Imaging, Thornwood, NY) and examined for corneal NV.

For deparaffinization and antigen retrieval, the corneal sections were boiled at 95 to 100°C in 10 mM sodium citrate solution (pH 6.0) for 30 minutes. The sections were then incubated for 16 hours at 4°C with primary antibodies against the vascular endothelial cell marker CD31 (clone JC70A; Dako, Carpinteria, CA) and the lymphatic endothelial cell marker LYVE-1 (Abcam, Cambridge, MA). Sections were then incubated for 1 hour with the appropriate secondary antibody (fluorescein isothiocyanate-conjugated [FITC]-donkey anti-mouse IgG or Cy5 donkey anti-rabbit IgG; Jackson Immunologicals, West Grove, PA). The nucleus was stained with propidium iodide. Confocal microscopic analysis was performed on a Leica SP2 confocal system (Leica Microsystems, Bannockburn, IL) using sequential scanning to minimize bleed-through. Finally, the Cy5 chromophore was activated by 633-nm laser, and the signal was shown as a blue pseudocolor. Omission of primary antibodies in the immunofluorescence stains was used as the control.

Cases were characterized as either HA-only ($CD31^+/LYVE\text{-}1^-$) or LA-only ($CD31^-/LYVE\text{-}1^+$), double positive ($CD31^+/LYVE\text{-}1^+$) showing both HA and LA, and double negative ($CD31^-/LYVE\text{-}1^-$) showing neither HA nor LA. NV was confirmed via immunohistochemical visualization of vascular lumens peripherally bounded by either $CD31^+$ or $LYVE\text{-}1^+$ cells. Immunohistochemical and H&E assessments were used to differentiate superficial stromal NV from deep stromal NV. Propidium iodide counterstaining was used to discriminate nonspecific staining from neovascular immunohistochemical staining. A principal methodological complexity of our investigations, which has been well documented in the literature,[14,33] is the fact that lymphatic vasculature may seem disorganized and edematous, sometimes showing luminal collapse; thus occasionally making unambiguous immunohistochemical classification difficult.

RESULTS

Table 1 summarizes the results of immunohistochemical and H&E staining. Figure 1 presents representative corneal NV from 9 pathological buttons. NV (presence of either HA or LA) was seen in 14 cases (40%). Twelve cases (34%) exhibited an HA-only phenotype ($CD31^+/LYVE\text{-}1^-$; Fig. 2), and none showed LA-only phenotype ($CD31^-/LYVE\text{-}1^+$). The 12 HA-only cases represented the following diagnoses: bullous keratopathy, chronic keratitis, fungal keratitis, graft failure, graft rejection, HSV keratitis, inflammatory pannus, pterygium, and ulcerative keratitis with perforation. Two cases (6%), both with pterygium, exhibited the double-positive ($CD31^+/LYVE\text{-}1^+$) phenotype (Fig. 3). The double-negative ($CD31^-/LYVE\text{-}1^-$) immunostaining was the most prevalent, observed in 21 cases (60%; Fig. 4).

To characterize distribution of neovascular vessels as either superficial stromal or deep stromal, we subjected pathological corneal specimens to H&E staining and assessed for stereotypical presentation of neovascular structures. We then immunostained specimens presenting positively for NV for the presence of CD31 or LYVE-1, as described previously, and noted either superficial or deep stromal distribution. Deep stromal NV was exhibited in 8 of 14 corneal specimens (57% of NV) and was associated with $CD31^+/LYVE\text{-}1^-$ and

$CD31^+/LYVE\text{-}1^+$ phenotypes; bullous keratopathy, chronic keratitis, fungal keratitis, graft failure, graft rejection, HSV keratitis, and inflammatory pannus demonstrated NV (Figs. 1–4). Superficial stromal NV was exhibited in 6 of 14 corneal specimens (43% of NV) and was associated with the $CD31^+/LYVE\text{-}1^-$ phenotype; inflammatory pannus, pterygium, and ulcerative keratitis with perforation demonstrated superficial stromal NV (Figs. 1–4).

DISCUSSION

The association of corneal HA and LA is not completely understood. Some models of NV suggest that HA and LA, which may be induced by similar pathophysiological processes,[8,34] develop in parallel[8,34] but regress along different time courses.[1] An alternative framework focuses on the spatial distribution of HA and LA in vascularized corneas; this approach characterizes, for example, distinct patterns of polarization of HA and LA in murine corneal models.[14] Consistent with previous studies,[35–37] we detected HA 7 times more frequently than LA. However, because our study explicitly differentiated corneal HA and LA, it is complementary to those studies that only grossly characterize NV for epidemiological purposes.[2,38]

Our results also demonstrated a notable asymmetry in the frequency of HA and LA across our corneal buttons. Only 14% of $CD31^+$ specimens were also $LYVE\text{-}1^+$, whereas 100% of $LYVE\text{-}1^+$ specimens were also $CD31^+$. This datum highlights the question of whether LA is necessarily associated with HA in corneal NV—a historically fertile area of research inquiry.[8]

Additionally, we found that the double-negative immunostaining was more prevalent than HA-only neovascular phenotypes ($CD31^+$ and $LYVE\text{-}1^-$) and was particularly common in noninflammatory pathologies (eg, Fuchs dystrophy, keratoconus). This finding is consistent with published models of corneal NV that associate NV with inflammation.[14] In contrast, the double-positive phenotype was associated only with pterygium in the present study. Considering that pterygium is a lesion of the perilimbal conjunctiva that extends onto the cornea—characterized by elastotic degeneration of collagen and fibrovascular proliferation—and given the presence of lymphatics in the normal conjunctiva, it is, therefore, not surprising that pterygium would show the $CD31^+/LYVE\text{-}1^+$ phenotype.

Our final subject of investigation was the discrimination of superficial stromal NV from deep stromal NV. Here, we observed a 4:3 ratio of deep stromal distribution to superficial stroma distribution. In accordance with published models of NV,[39–42] we observed that specimens with bullous keratopathy, chronic keratitis, fungal keratitis, graft failure, graft rejection, HSV keratitis, and inflammatory pannus exhibited deep stromal NV, whereas specimens with inflammatory pannus, pterygium, and ulcerative keratitis with perforation exhibited superficial stromal NV. By permitting segmentation of corneal HA and LA distribution via CD31 and LYVE-1 immunohistochemistry, our results complement those corneal NV studies.

In sum, in the present study, the double-negative immunostaining was more prevalent among the pathological corneal specimens than were the neovascular phenotypes. HA-positive specimens predominated, and LA was strongly associated with the presence of HA (although the reverse was not observed). Deep stromal vascular distribution was more common than superficial stromal vascular distribution.

Our results suggest the potential therapeutic utility of targeting antineovascular therapies specifically to corneal HA and/or LA pathology. Further study is needed to elucidate patterns of HA and LA in other corneal diseases.

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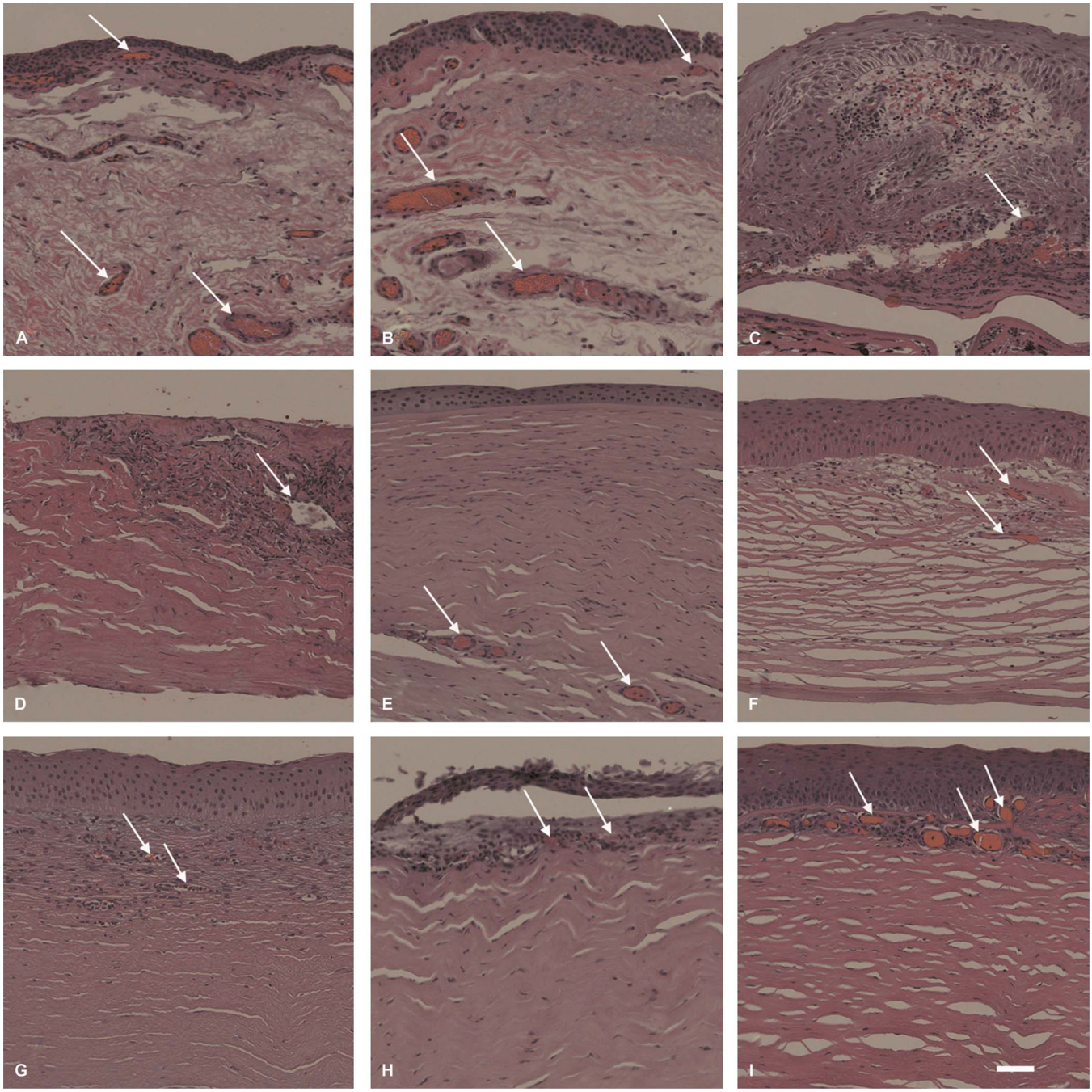

FIGURE 1.

Characterization of corneas exhibiting NV in various underlying ocular pathologies. H&E–stained specimens were assessed for histopathological characteristics of corneal vasculature. Arrows indicate neovascular formations. Pterygium presented with superficial stromal NV in subject 33 (A) and subject 32 (B). C, Ulcerative keratitis (subject 35) was also associated with superficial stromal NV. Specimens with (D) fungal keratitis (subject 16), (E) HSV keratitis (subject 24), (F) graft rejection (subject 21), and (G) chronic keratitis (subject 8) presented with deep stromal NV. Specimens (H and I) with inflammatory pannus in these images show NV distributed to superficial stroma (subjects 26 and 27); scale bar, 47.62 μm.

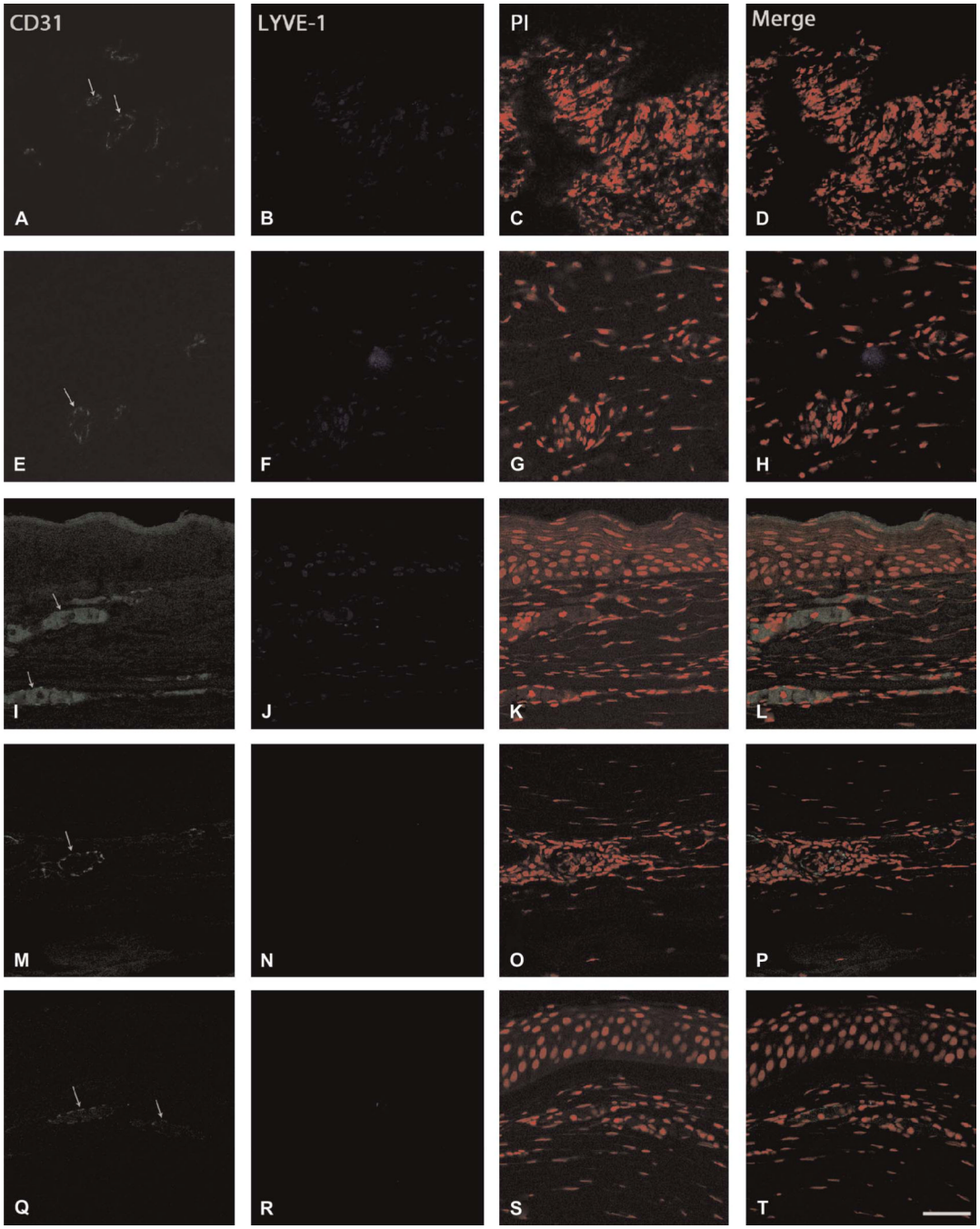

FIGURE 2.

Immunohistochemical visualization of the $CD31^{+}$/$LYVE\text{-}1^{-}$ phenotype. Specimens exhibiting vascular structures were immunostained for CD31 (green), LYVE-1 (blue), and propidium iodide (PI, red) to differentiate between HA and LA. Arrows indicate neovascular formations. Representative specimens showing (A–D) fungal keratitis (subject 16), (E–H) HSV keratitis (subject 24), (I–L) inflammatory pannus (subject 27), (M–P) inflammatory pannus (subject 25), and (Q–T) chronic keratitis (subject 8); scale bar, 47.62 μm.

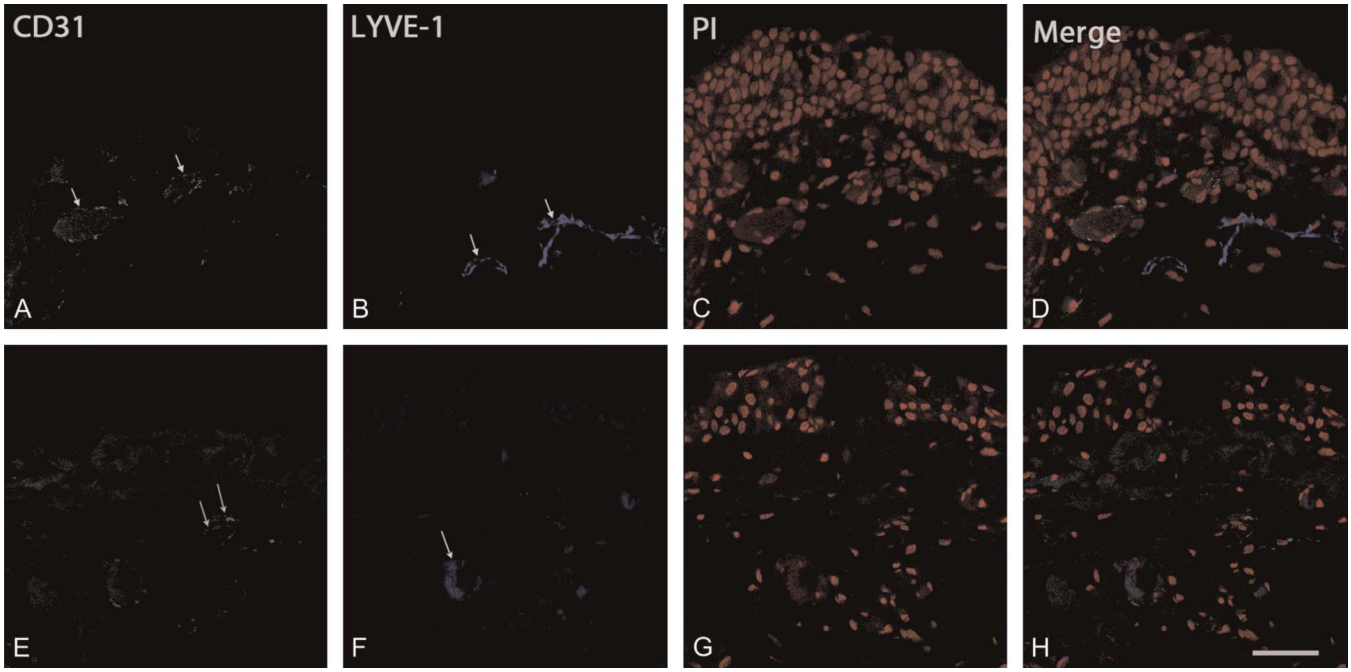

FIGURE 3.

Immunohistochemical visualization of the $CD31^+/LYVE\text{-}1^+$ phenotype. The specimens with pterygium exhibiting vascular structures were immunostained for CD31 (green), LYVE-1 (blue), and propidium iodide (PI, red). Arrows indicate neovascular formations. Immunostaining shows specimens positive for both CD31 and LYVE-1. Representative specimens showing pterygium (A–D) in subject 33 and (E–H) subject 32; scale bar, 47.62 μm.

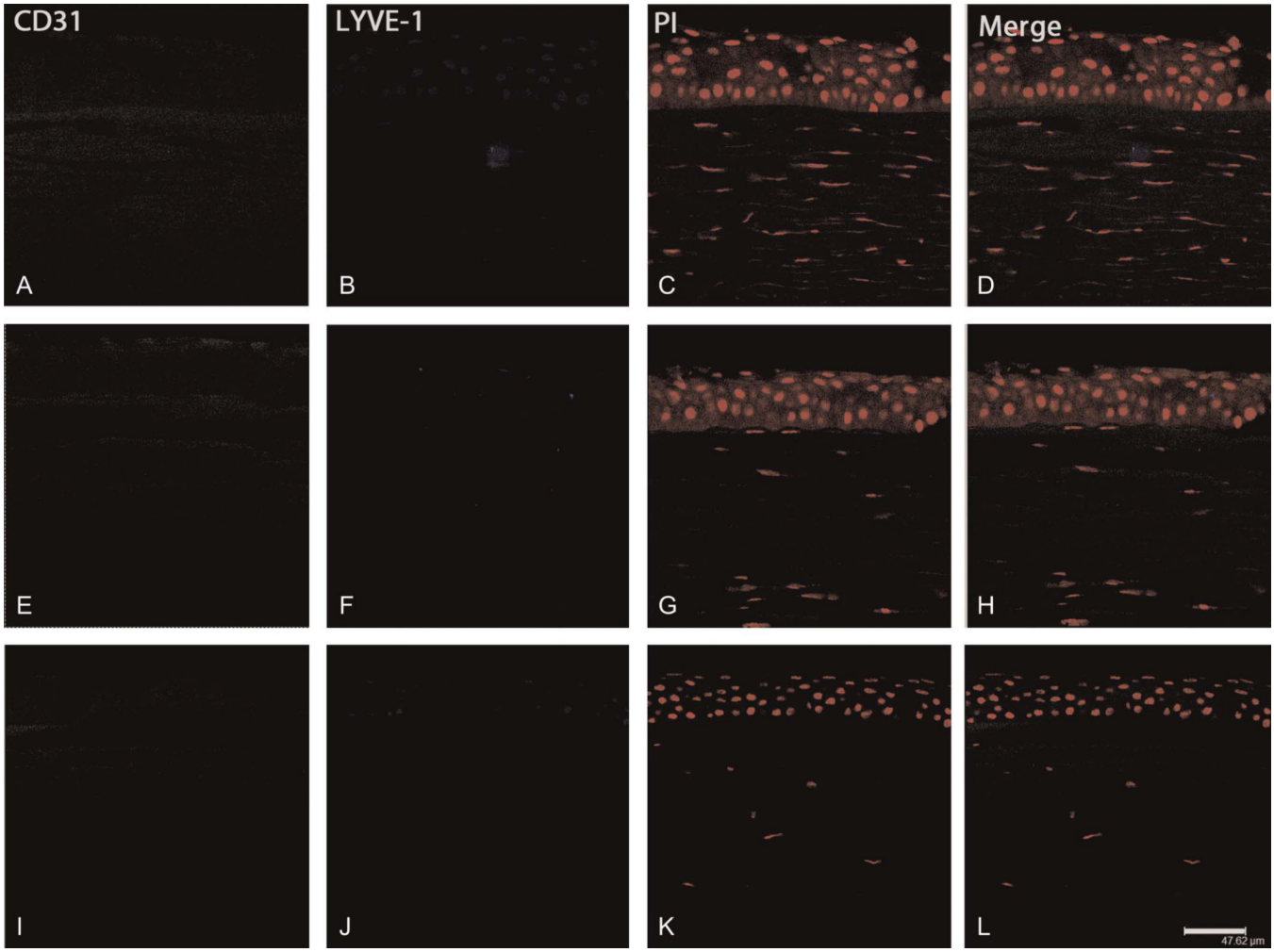

FIGURE 4.

Immunohistochemical visualization of the CD31⁻/LYVE-1⁻ phenotype. Specimens exhibiting no vascular structures were immunostained for CD31 (green), LYVE-1 (blue), and propidium iodide (PI, red). Representative specimens showing keratoconus (A–D) in subject 30, Fuchs dystrophy (E–H) in subject 14, and corneal scarring (I–L) in subject 11; scale bar, 47.62 µm.

TABLE 1

Results of Immunohistochemical and H&E Staining

Pathology	Subject	HA CD31	LA LYVE-1	Neovascular Distribution (Superficial Stroma or Deep Stroma)
Acanthamoeba keratitis	1	−	−	N/A
	2	−	−	N/A
	3	−	−	N/A
Bullous keratopathy	4	+	−	Deep stroma
	5	−	−	N/A
	6	−	−	N/A
Chronic keratitis	7	−	−	N/A
	8	+	−	Deep stroma
	9	−	−	N/A
Corneal scarring	10	−	−	N/A
	11	−	−	N/A
Fuchs dystrophy	12	−	−	N/A
	13	−	−	N/A
	14	−	−	N/A
Fungal keratitis	15	−	−	N/A
	16	+	−	Deep stroma
Graft failure	17	−	−	N/A
	18	+	−	Deep stroma
Graft rejection	19	−	−	N/A
	20	+	−	Deep stroma
	21	+	−	Deep stroma
HSV keratitis	22	−	−	N/A
	23	−	−	N/A
	24	+	−	Deep stroma
Inflammatory pannus	25	+	−	Deep stroma
	26	+	−	Superficial stroma
	27	+	−	Superficial stroma
Keratoconus	28	−	−	N/A
	29	−	−	N/A
	30	−	−	N/A
Pterygium	31	+	−	Superficial stroma
	32	+	+	Superficial stroma
	33	+	+	Superficial stroma
Ulcerative keratitis	34	−	−	N/A
With perforation	35	+	−	Superficial stroma

N/A, not applicable.