An Immunohistochemical Study of Inflammatory Cell Changes and Matrix Remodeling With and Without Acute Hydrops in Keratoconus

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Purpose. To determine the inflammatory cell and matrix changes in advanced keratoconus, including acute hydrops, using immunohistochemical analysis.

Methods. The corneal tissue from eight subjects with keratoconus undergoing corneal transplantation (three keratoconic buttons, five buttons post acute hydrops—one of them with extensive neovascularization following hydrops) was compared with tissue from two normal corneoscleral rims ($n = 10$). The corneas were sectioned and analyzed with specific markers for macrophages, lymphocytes, dendritic cells, and scar associated matrix molecules laminin, fibronectin, tenascin-C, and type III collagen.

Results. Populations of cells using markers for macrophages, leucocytes and antigen presenting cells were found to be associated with the epithelium and stroma of keratoconic tissue. Populations of these cells appeared decreased in hydrops-associated keratoconus except for a large increase in leucocytes in the stroma and endothelium associated with neovascularization. Extracellular matrix deposition was found to be uniquely demonstrated in localized areas of the stroma, corresponding to the site of hydrops involvement.

Conclusions. Immunohistochemical analysis revealed a chronic, inflammatory process with recruitment of immunoinflammatory cells and deposition of scar tissue in keratoconus. The inflammatory markers were somewhat attenuated in hydrops-associated keratoconus corneas and thus inflammation was not considered to be a major factor in the development of acute corneal hydrops.

Keywords: keratoconus, hydrops cornea, immunohistochemistry, microscopy

Keratoconus is an ectatic disorder of the cornea, classically described as progressive, noninflammatory, and characterized by central corneal thinning, protrusion, and irregular myopic astigmatism.[1] In New Zealand, approximately half of the penetrating keratoplasties performed each year are for the restoration of sight impaired by this disease.[2]

Acute corneal hydrops is a poorly understood complication of keratoconus. The development of marked corneal edema due to a break in Descemet's membrane usually occurs in eyes with advanced thinning and ectasia, with an incidence of 2.4 to 3.0% of eyes with keratoconus, typically causing a sudden further deterioration in vision.[3]

While acute hydrops in keratoconus usually resolves over 2 to 4 months,[4,5] it is associated with epiphora, photophobia and pain, thereby rendering the affected, but otherwise healthy young individuals, to a significantly impaired level of visual function. Furthermore, the condition frequently leaves a vision-impairing scar, expediting the need for corneal transplantation to achieve visual rehabilitation. Penetrating keratoplasty post hydrops is associated with greater risk of failure due to increased likelihood of neovascularization[6] and reportedly higher risk of allograft rejection.[3]

There are limited pathophysiological investigations into acute hydrops in keratoconus. The authors have previously conducted a prospective study assessing the clinical course of acute hydrops and its microstructural changes using in vivo confocal microscopy in 10 patients with acute hydrops in keratoconus and demonstrated novel findings.[4] Hyperreflective round cells in the epithelium and stroma were exhibited in 4 of 10 corneas. Elongated branching cells with small cell bodies were noted in the anterior stroma in two cases at 6 and 12 weeks, respectively. Three months after presentation, both cases also exhibited unusual stromal cells with large speckled cell bodies and elongated branching cell processes. Both cases subsequently developed corneal neovascularization.

In this follow-up study, we aimed to identify the specific microstructural and cellular changes following acute hydrops in keratoconus with the assistance of immunohistochemistry in a subset of the original cohort compared to keratoconic tissue without hydrops and to normal corneal tissue.

Materials and Methods

Subjects

Nine of the ten subjects with previously reported[4] keratoconus-related acute hydrops subsequently underwent penetrating keratoplasty for visual rehabilitation. A subset of five central corneal buttons, obtained at the time of transplant surgery, was examined with the permission of the subjects. Clinically, one of

Table 1. Panel of Antibodies Used in This Study

Antibody	Description	Dilution	Source
Laminin	Rabbit anti-mouse polyclonal IgG	1:60	Sigma, L-9393
Langerin	Monoclonal mouse anti-human IgG2b	1:20	Leica Biosystems, NCL-Langerin
CD11c-Cy5	Mouse anti-human IgG1, κ	2:1	BD Pharmingen, 551077
CD11b-Alexa 488	Rat anti-mouse IgG2b	2:1	BD Pharmingen, 557672
CD45-LCA	Monoclonal mouse anti-human, IgG1, κ	1:4	Dako, M0701
Fibronectin	Mouse anti-human monoclonal IgG1	1:100	Sigma-Aldrich, F-0916
Tenascin-C	Mouse anti-human monoclonal IgG1	1:100	Abcam, AB82449
Collagen III	Mouse anti-human IgM	1:80	AbD-Serotec, 2150-0081
Biotin	Goat anti-biotin polyclonal IgM	1:60	Sigma-Aldrich, S-9265
Streptavidin	Streptavidin-Cy3	1:200	GE Healthcare, PA-43001
HLA-DR-FITC	Monoclonal mouse anti-human IgG_{2a} H+L, κ	1:5	BD Pharmingen, 347363
Cy3	Goat anti-rabbit	1:400	Jackson ImmunoResearch, 115-165-003
Alexa 546	Goat anti-mouse IgG	1:1000	Molecular Probes, A-11003

these five buttons exhibited extensive neovascularization. Three buttons exhibiting keratoconus but no hydrops were also obtained at the time of penetrating keratoplasty. Two normal, peripheral, corneoscleral rims obtained following removal of the central 8.00 mm for penetrating keratoplasty, were also subjected to the cellular and microstructural analyses. Informed consent was obtained from each subject and research ethics approval was obtained from the Northern X Regional Ethics committee prior to tissue use. All subjects were treated in accordance with the Declaration of Helsinki. All corneal buttons were between 7.5 to 8.5 mm in diameter. Corneas were stored and transported in New Zealand Eye Bank medium (2% FCS, 2 mM L-glutamine, and 13 Anti–Anti in Eagle MEM) or New Zealand Eye Bank transport medium (additional 5% dextran in New Zealand Eye Bank medium).

Section Preparation and Immunohistochemistry

Tissue was fixed in 2.5% paraformaldehyde for 1 hour and followed by three washes for 15 minutes each in PBS prepared from tablets (BR14; Oxoid Ltd., Hampshire, UK). After snap-freezing tissue in OCT mounting medium (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), tissue was sectioned in 20-μm steps. Sections collected on slides were washed in 3 × 15 minutes in 0.1 M Tris saline buffer, pH 7.4. Slides were treated with 2 mg/mL testicular hyaluronidase for 1 hour at 37°C, followed by methanol at −20°C for 20 minutes, and 20 mM glycine for 30 minutes at room temperature. After applying 2% goat serum + 0.1% Triton X-100 for 30 minutes as treatment, slides were incubated with 1° antibody in 0.1% goat serum overnight. Slides were then incubated with 2° antibody for 2 hours at room temperature in the dark. Slides were subsequently labeled with DAPI for 10 minutes before sealing slides with coverslips. The details of the panel of antibodies used are presented in Table 1. Positive cells were calculated as the number of positive cells per tissue section.

Table 2. Details of the Corneal Buttons Analyzed in This Study

	Cornea	Age, y	Sex	Presence of NV Pre-PKP
Normal corneoscleral tissue	1	35	M	
	2	62	M	
Keratoconus	1	22	F	
	2	19	F	
	3	23	F	
Hydrops	1	17	F	N
	2	24	F	N
	3	22	F	N
	4	23	M	N
	5	18	M	Y

Image and Statistical Analysis

Montaged images of full-width, fluorescently labeled sections were collected using a fluorescence microscope with ×20 and ×40 lenses (Leica DR RA, Leica Microsystems, Heidelberg, Germany) via a digital camera (Nikon DS-5Mc; Nikon Corp., Tokyo, Japan) connected to a desktop computer (Dell Computer Corp., Austin, TX, USA) running a commercial operating system (Windows Vista; Microsoft Corp., Seattle, WA, USA) and imaging software (NIS-Elements BR; Nikon Corp.).

Tissue Information

Details of the human tissue used for this study are presented in Table 2. Specifically, five corneal buttons with keratoconus and acute hydrops (one with extensive neovascularization) and three corneal buttons with keratoconus but no history or clinical evidence of hydrops were collected during corneal transplant surgery. Two additional, normal corneoscleral rims were processed for analysis and comparison.

Immune Cell Detection

All corneal tissue was labeled with antibodies to:

1. CD11b: a marker for macrophages/monocytes.
2. CD45 (LCA) labels the cell membranes of almost all leucocytes. However, CD45 is expressed less on mature granulocytes than lymphocytes (http://www.dako.com/dist/ar45/p109660/prod_products.htm, Dako Corporation, Denmark) and as such it signifies primarily lymphocytic deposition in this setting.
3. Langerin detects a c-type lectin expressed by specific dendritic cell populations, including epithelial dendritic cells in the cornea.
4. HLA-DR is a cell receptor for human class II major histocompatibility complex (MHC) antigen, present on professional antigen-presenting cells (APCs), such as dendritic cells, B cells, Langerhans cells and macrophages.

Results

The distribution of the immune cell types in the layers of normal, keratoconic, and hydrops corneas is detailed in Table

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TABLE 3. Cell Counts of Positively Stained Cells Within the Corneal Layers of Each Tissue Sample

		CD11b			CD45-LCA			HLA-DR			Langerin		
		Epithelium	Stroma	Endothelium	Epithelium	Stroma	Endothelium	Epithelium	Stroma	Endothelium	Epithelium	Stroma	Endothelium
Normal corneoscleral tissue	1					2						2	
	2				1	3					1	8	
Keratoconus	1	1	5		6	25					3	3	
	2	2			39	161		2	36	14		5	
	3	5	2		7	26		6	112	2	2	1	
Hydrops	1	83			11	2		2			4		
	2												
	3				4							2	
	4							9			3		
	5				19	352	14						

3. Statistical analysis of the groups was not possible due to the small number of samples and the range of staining seen within each group. Thus quantitation of immune cell numbers is represented as a total cell count per section rather than a mean. Representative images of the labeling observed are shown in Figure 1.

All corneal tissue examined, including normal corneoscleral rims, exhibited sparse Langerin positive cells in the epithelium and stromal layers. No increase in these cells was observed in keratoconic or hydrops tissue.

The presence of leucocytes was noticeably higher within the epithelium of keratoconic tissues, with or without a history of acute hydrops, compared with normal corneoscleral tissue. Leucocytes were found in significant numbers within the stroma of all keratoconic buttons examined, but were not seen in hydrops-associated keratoconic tissue. Very high numbers of leucocytes were present in the stroma of the corneal button with both hydrops and neovascularization and notably leucocytes were identified in the endothelium of this tissue sample alone.

Antigen-presenting cells were only identified in the stroma of two-thirds of the keratoconic buttons and not in the hydrops-associated keratoconic buttons or normal tissue; while macrophages were found to be present in the epithelium and stroma of the keratoconic tissue samples but not in hydrops or normal corneoscleral tissue.

Matrix Deposition Associated With Scar Formation

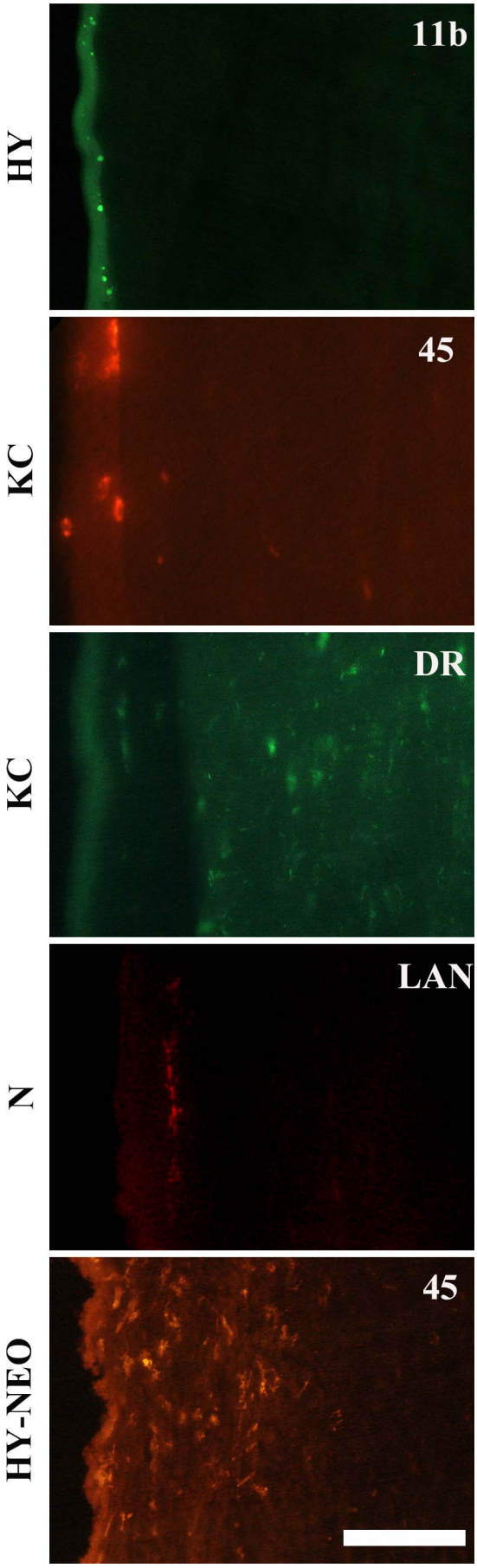
Extracellular matrix molecule production associated with scar formation was assessed using labeling to fibronectin, collagen III, tenascin-C and generic laminin.

In normal tissue, fibronectin, collagen III and laminin are all detected within the sclera and limbal regions (Figs. 2A–C). Positive staining for fibronectin is also visible in the peripheral corneoscleral stroma (Fig. 2A) and small amounts of collagen III are also visible (Fig. 2B). Examination of keratoconic tissue showed significant and extensive deposition of fibronectin (Fig. 2D) and collagen III (Fig. 2E) in the mid- and anterior stroma, while laminin showed some deposition in the mid/anterior stroma, but most deposition occurred in the epithelial basement membrane and Bowman's layer (Fig. 2F). In keratoconic tissue with associated hydrops, deposition of small amounts of fibronectin in Descemet's membrane was observed (Fig. 2G, arrowheads) and punctate staining of collagen III throughout the stroma with strongest labeling in the posterior stroma immediately adjacent to Descemet's membrane (Fig. 2H, arrowheads). Similarly, staining for laminin in hydrops associated keratoconic corneas was observed in the stroma immediately adjacent to Bowman's layer and Descemet's membrane (Fig. 2I, arrowheads).

No labeling was observed in any tissue sections labeled with the tenascin-C antibody (data not shown).

DISCUSSION

In undertaking this study, we aimed to investigate the immune cell and matrix changes associated with advanced keratoconus, with and without acute corneal hydrops, in direct comparison with normal corneoscleral tissue. To our knowledge, this is the first immunohistochemical investigation of corneal hydrops in keratoconus, with earlier published laboratory studies in the literature of hydrops consisting mainly of histological case reports.[7,8] More recently, in vivo confocal microscopy (IVCM) and other anterior segment imaging technologies have expanded our understanding of the pathophysiological process of acute hydrops in keratoconus.[9,10] However, without



immunohistochemical labeling, it is difficult to accurately interpret the ultrastructural changes beyond speculation.

A Role for Inflammation in Keratoconus?

Perhaps the most important overall observations are the extensive presence of inflammatory cells in keratoconic corneas. $CD11b^+$ monocytic cells, APCs including Langerhans cells, and leucocytes have been demonstrated throughout layers of keratoconic corneas. In recent years, a paradigm shift has taken place and the tenet that the human cornea is devoid of all bone marrow–derived cellular elements has been refuted. Independent research groups have identified the presence of $CD11c^+$ $CD11b^-$ Langerhans cells in the epithelium,[11,12] Leucocytic cells that are $CD45^+$ in the corneal stroma,[13,14] a separate, distinct population of myeloid monocytic ($CD11b^+$) $CD11c^+$ dendritic cells in the very anterior portions of the cornea stroma,[13] and a population of $CD14^+$ undifferentiated cells have been identified throughout the stroma.[11,13] Therefore, the discovery of the presence of some of these cells in keratoconic corneal tissue is perhaps not surprising.

However, this study demonstrated the presence of $CD11b^+$ monocytic cells, likely macrophages, in both the stroma and the basal epithelium of the keratoconic corneas. This cell type is now understood to be part of the resident myeloid cell population of the normal corneal stroma. Following insults to the cornea, macrophage infiltration of the corneal stroma following infiltration by neutrophils recruited from the limbal cell population is part of the inflammatory cascade, usually in response to a breach of the blood-aqueous barrier.[15,16] The presence of macrophages in the basal epithelium in the current study is likely to be representative of inflammatory recruitment of macrophages, as monocytic cells have not previously been found in the normal healthy corneal epithelium nor were they found in the normal corneoscleral tissue examined in this study. One hydrops-associated keratoconic button showed greater numbers of macrophage recruitment to the epithelium and this may represent a cornea with hydrops extending to the peripheral cornea and limbus.

A chronic inflammatory process in the setting of keratoconus is further supported by the presence of increased leucocytic deposition in the anterior to mid stroma, as demonstrated by positive labeling with CD45 (LCA) antibody.

Interestingly, large numbers of leucocytes were present in the stroma, epithelium and even the endothelium of the single cornea that sustained hydrops extending to the limbus with subsequent neovascularization.

We postulate that the presence of these cells is directly associated with the development of neovascularization as leucocytes that mediate these immuno-inflammatory responses are derived from the (limbal) intravascular compartment and as such, the greater the surface area the blood vessels have with the tissue, the more pronounced the effect of the inflammatory process.[18]

In contrast, the presence of langerin-positive dendritic cells in the corneal epithelium and anterior stroma likely does not necessarily signify an alteration in the cell population in keratoconic tissue, since resident dendritic cells have been demonstrated in the corneal basal epithelium previously.[12,19]

Figure 1. Macrophages (11b) are identified within the epithelium of a hydrops associated keratoconic cornea (HY). Leucocytes (45) and APCs (DR) are seen within the epithelium and stroma of keratoconic tissue (KC). Langerhans cells (LAN) are associated with the basal epithelium of normal cornea (N) and vast amounts of leucocytes (45) are seen within the stroma and epithelium of a neovascularized hydrops cornea (HY-NEO). *Scale bar*: 100 μm.

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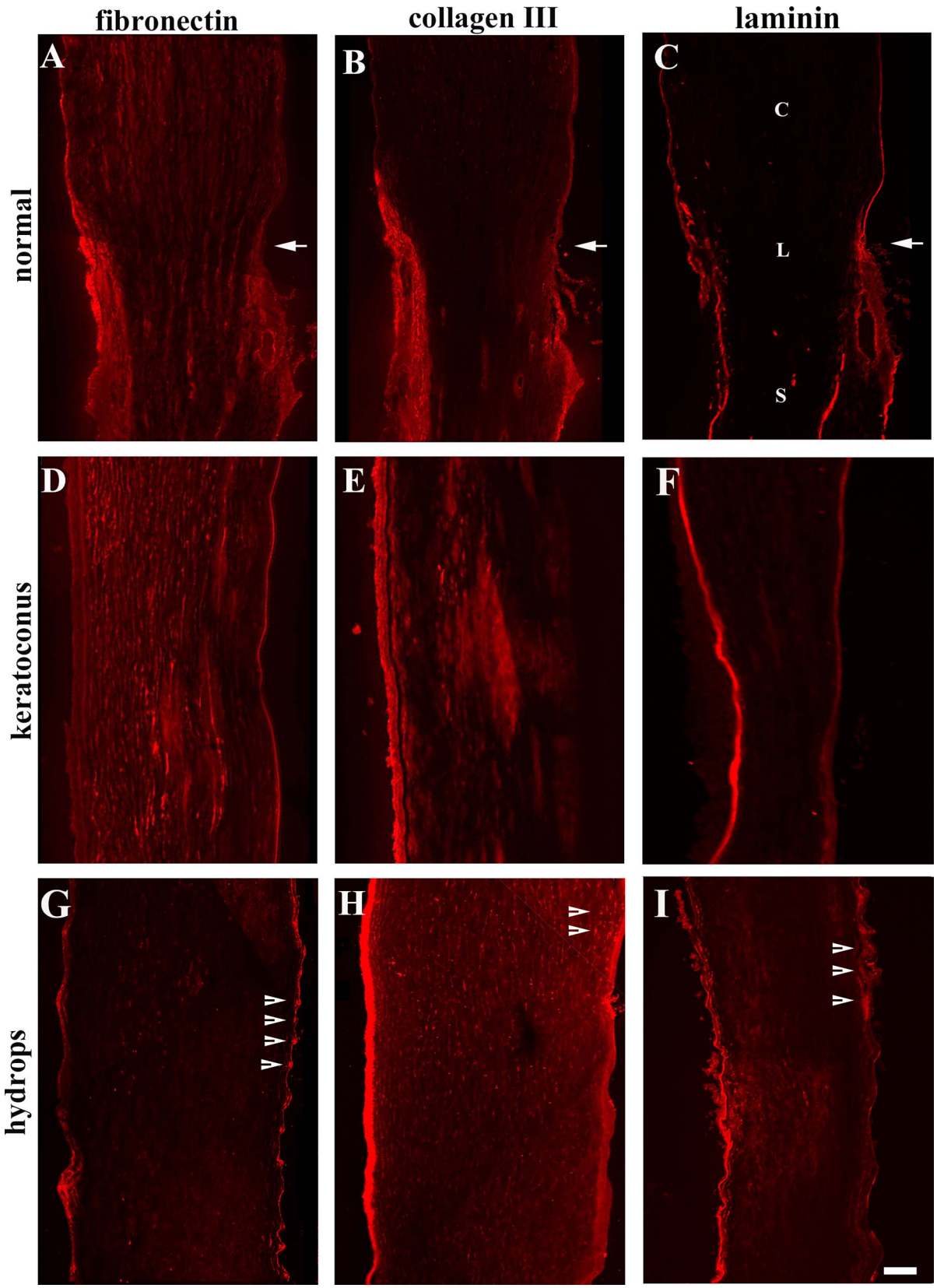

FIGURE 2. Representative cross sections of corneal tissue from normal corneoscleral buttons (**A–C**) keratoconic (**D–F**) and hydrops associated samples (**G–I**). All sections are oriented with the epithelium to the left and endothelium to the right. The normal tissue was obtained from a limbal rim and thus shows the transition from sclera (S) through the limbus (L) and into the peripheral cornea (C). *Scale bar*: 50 μm.

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Further support of this is that these cells are located paracentrally, and very sparsely. This is consistent with the findings by Mayer et al.[20] in a population of post herpes keratitis, post graft rejection, and keratoconic corneas post penetrating keratoplasty.

Interestingly, the immune cell populations that were elevated in keratoconic tissue compared with normal corneoscleral tissue mostly seemed to be down-regulated in the hydrops-affected corneas, which may indicate that inflammation is associated with advanced keratoconus but not with hydrops formation.

Matrix Remodeling

This study also examined the matrix molecule deposition and remodeling that occurred within the keratoconic process and whether distinct differences could be identified following hydrops.

Analysis of fibronectin, collagen III and generic laminin were used as indicators of tissue remodeling indicative of scar formation and all three molecules exhibited distinct profiles within the corneas with keratoconus but no hydrops, with fibronectin being deposited within the anterior and central two-thirds of the stroma and collagen III appearing in less organized areas within the middle third of the stroma. Laminin staining was diffusely deposited around the epithelial basement membrane and Bowman's layer.

These patterns were distinctly different from those observed in corneas with keratoconus and hydrops where the staining pattern of all three molecules became punctate with deposition primarily seen in Descemet's membrane and the immediately adjacent stroma. It is likely that the deposition of these matrix molecules is in direct response to the matrix damage caused by the edema that classically defines hydrops and that the deposition occurs at the site of swelling and water influx sustained by the breaches in Descemet's membrane. Future coordination of clinical imaging and laboratory studies may even be able to colocalize the site of hydrops initiation and matrix remodeling.

Interestingly, laminin deposition following trauma may be slightly increased in keratoconic corneas compared with nonkeratoconic corneas.[22] In the present study, laminin deposition has been demonstrated in very localized areas of the stroma, more prominently in the anterior-to-mid-stroma, representative of a fibrotic process. Increased laminin deposition in the epithelial basement membrane, gaps in Bowman's layer and anterior stroma has previously been described in scarred keratoconic corneas (a history of hydrops not indicated).[25] Sparse laminin deposition has also been documented in association with subepithelial fibrosis in bullous keratopathy,[26] a process not too dissimilar to acute hydrops.

Unfortunately, we were unable to identify the unusual stromal cells (with large speckled cell bodies and elongated branching cell processes) previously reported by our team[4] on IVCM analysis of two corneas with hydrops that developed neovascularization (one of which was analyzed in this study). While these cells were postulated to be specific APCs, we have not been able to isolate them ex vivo. This is despite the fact that other investigators have found higher dendritic cell density ex vivo with immunohistochemistry than with IVCM.[20] The possible reasons for this are: first, that the cells were no longer present at the site identified by the in vivo investigation, either due to their disappearance following hydrops resolution or they were sensitive to the tissue processing regimen; or second, that the appropriate antibody markers were not utilized to identify the cells.

In conclusion, with the assistance of immunohistochemistry, this study has confirmed the presence of a chronic inflammatory process in advanced keratoconus. However, the inflammatory process was not enhanced, possibly even attenuated in hydrops-associated keratoconus specimens, indicating that inflammation may not be involved in hydrops development or had resolved by the time of corneal transplantation. Specific matrix deposition profiles are seen in keratoconic tissue with and without associated hydrops that may correlate well with the scarring and loss of vision in these associated conditions, given that the majority of eyes post hydrops require corneal transplantation for visual rehabilitation.

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