Retinal Microvessel Extracellular Matrix: An Immunofluorescent Study

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The vasculature of the retina functions within a sheath of extracellular matrix (ECM). Unfortunately, little is known about the biochemical composition of this matrix. Abnormalities in the ECM of the retinal microvasculature are important in diabetic retinopathy as well as vasculopathies associated with connective tissue disorders. The ECM of unfixed frozen human retinal blood vessels was examined by indirect immunofluorescence using antibodies raised against collagen types I, II, III, IV, and V as well as the structural glycoproteins laminin and fibronectin. Antibodies against collagen types I and IV as well as laminin and fibronectin stained a broad spectrum of retinal vessels, from large thick-walled vessels down to microvessels less than 10 μm in diameter. In contrast, antibodies against types III and V collagen were seen to stain primarily the walls of the larger vessels. Antibodies against type II collagen did not react with retinal vessels. Preincubation with the appropriate antigen or preimmune serum eliminated staining of the vessels by the antisera. Invest Ophthalmol Vis Sci 27:194-203, 1986

In a normally functioning vessel, the endothelial cells are surrounded by a sheath of extracellular matrix. The structure of this extracellular matrix is likely to play an important role in both health and disease. Abnormalities of the retinal vascular extracellular matrix play an important role in diabetic retinopathy.1 In addition, retinal vascular abnormalities have been observed in cases of systemic connective tissue diseases such as lupus erythematosus, dermatomyositis, and scleroderma.2-4

Extracellular matrices differ qualitatively from region to region and within various organs. They are generally characterized by a collagenous network to which cells are attached by means of proteoglycans and large glycoprotein attachment factors such as laminin and fibronectin.5 Basement membranes constitute a particular subgroup of extracellular matrices and typically contain types IV and V collagen as well as laminin.5 The ability of the vascular endothelium to synthesize components of its own microenvironment has been reported elsewhere.7-14 In this study we describe the in vivo characteristics of the retinal vascular microenvironment.

Materials and Methods

Tissue Preparation

Retinas used in this study were dissected from ten human eyes of donors 18–76 yr of age obtained from the Medical Eye Bank of Maryland within 24 hr of death. Following dissection, the central portions of the retinas were cut into fragments (10 mm × 5 mm). These fragments were gradually frozen in OCT compound (Tissue-Tek II, Miles Laboratories, Inc.; Naperville, IL) without prior fixation, and the blocks were stored in airtight containers at −70°C until they were sectioned. The retinas were cut on an IEC Minotome cryostat at −26 to −22°C, and the 8–12 μm sections were placed on albumen-coated slides.

Immunohistochemistry

Following sectioning, the slides were stained either with a modified Wright–Giemsa stain (Harleco; Gibbstown, NJ) or with one of an array of polyclonal connective tissue antisera. The antisera used in this study, with the exception of antiserum against type III collagen, were the generous gift of Drs. Kleinman, Chandrasekhar, and Grotendorst of National Institute of Dental Research, National Institutes of Health. The type III collagen antiserum was the gift of Dr. L. Paglia, Pfizer Hospital Products; Groton, CT. Following incubation in a 10% solution of preimmune serum in phosphate buffered saline (PBS) to decrease nonspecific binding of the secondary antibody, the sections were incubated in 1:100 dilutions in PBS of primary antiserum against laminin, fibronectin, or collagen types I, II, IV

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or V in PBS for 1 hr at 25°C followed by incubation at 4°C overnight. Antiserum against type III collagen was used at a 1:40 dilution in PBS under similar conditions. The sections were extensively rinsed in PBS and then incubated in rhodamine-conjugated secondary antibodies (Cappel Labs; Melvern, PA) at a 1:40 dilution in PBS for 1 hr at room temperature. The sections were again extensively rinsed in PBS, then excess liquid was removed and coverslips were mounted in Gelvatol (Monsanto; Springfield, MA). The slides were then stored at 4°C in the dark until microscopic examination and were found to be stable to multiple examinations.

In order to determine the level of nonspecific immunofluorescent staining of the tissue, control sections of the same tissue were incubated in preimmune serum overnight and treated subsequently with rhodamine-conjugated secondary antibodies in parallel with the experimental slides. To examine specific binding of the primary antiserum to the sections, the various antisera were each preincubated for 3 hr at 25°C with an excess amount of the antigen against which it was raised or with potential contaminating collagen types. Following this blocking, the antisera so treated were used to stain sections of the same retina in parallel with experimental slides. In the case of antiserum against type III collagen, an equivalent dilution of preimmune serum was used as the control for nonspecific staining by the primary antiserum because type III collagen was not available for antiserum blocking.

**Examination of Tissue**

Sections were examined under epifluorescent illumination using a Zeiss microscope (Carl Zeiss, Inc.; Oberkochen, West Germany) equipped with an Olympus OM-2 camera (Olympus Products Corp.; Woodbury, NY). In order to compare levels of fluorescence seen in experimental and control slides, exposure times of experimental tissues were measured, and comparable times were used when photographing both types of control conditions.

**Results**

Low and high-powered fields of stained tissue were examined, and fluorescent patterns were compared.
Type I Collagen

Following incubation of tissue in antiserum against human dermal type I collagen, strong staining could be seen in the walls of retinal vessels of all sizes, from thick-walled arterioles down to vessels about 8 \( \mu \)m in diameter (Fig. 1, 2). At the level of the capillary-size vessels, cell profiles are frequently seen on the outside wall which may represent pericytes (Figs. 2A–B). Preincubation of antiserum with laminin, fibronectin, and type IV collagen had no effect on the staining pattern (data not shown). In contrast, when sections were incubated either with the secondary antibody alone (Fig. 3A) or with antiserum preincubated with type I collagen (Fig. 3B), only low levels of background fluorescence were observed in both vessels and surrounding tissue.

Fibronectin and Laminin

When antiserum against human serum fibronectin was used, staining of vessel walls was most prominent in larger vessels (Figs. 4A–B), although staining of smaller vessels was occasionally seen (Fig. 4C). Preincubation of antiserum with laminin or collagen types I and IV had no effect on the subsequent staining pattern (data not shown). Preincubation with fibronectin decreased the level of fluorescence to background (Figs. 3A–B).

After staining with antiserum against murine EHS sarcoma laminin a much broader spectrum of retinal vessels were stained, from large thick-walled vessels down to those approximately 8 \( \mu \)m in diameter (Figs. 5A–B). In capillary-size vessels presumptive pericytes were frequently outlined (Fig. 5B). Preincubation of the antiserum with laminin dropped the level of staining to that of control slides (Fig. 6). Preincubation of the antiserum with types I and IV collagen, however, had no effect on the staining pattern of retinal vasculature (data not shown).

Types IV and V Collagen

Incubation with antiserum against EHS sarcoma type IV collagen resulted in dramatic fluorescent staining of a wide spectrum of retinal vessels similar to that seen with antisera against type I collagen and laminin (Fig. 7A–C). When tissue was incubated with antiserum against bovine placental type V collagen, the staining of the large vessels was much more pronounced and consistent than that of the small vessels (Fig. 8). Preincubation of these antisera with their appropriate antigen resulted in a decrease of fluorescent intensity down to control levels, while preincubation with laminin or
Figs. 3A–B. Frozen sections of unfixed human retina at high magnification showing the levels of nonspecific immunofluorescence seen in this study. In A, the section was incubated with the rhodamine-conjugated secondary antibody alone (×350). In B, the section was incubated with antiserum which had been previously incubated with type I collagen as described in Materials and Methods (×800). In both A and B, vessel profiles can be seen (asterisks), but in both cases the level of fluorescence is not significantly increased over the background.

collagen type I had no such effect on subsequent staining (data not shown).

Type II Collagen

Following incubation of tissue with antiserum against rat chondrosarcoma type II collagen, no fluorescent staining of the retinal vessels was seen (Fig. 9A) above the control background levels (Fig. 9B).

Type III Collagen

When antiserum against bovine type III collagen was used, strong staining was seen only in the region of the larger retinal vessel walls (Fig. 10). In comparison to the reaction seen with antisera against collagen types I and IV as well as laminin, the staining of small vessels seen with anti-type III collagen was not consistently increased over background levels, although an occasional vessel was clearly stained. Preincubation of the antiserum with types I and IV collagen as well as laminin did not influence this staining pattern.

Discussion

Vessels in the retina, as is the case elsewhere in the body, exist within a sheath of basement membrane and
extracellular matrix. In this study we probe the retinal vascular microenvironment of the human eye by indirect immunofluorescence. Using antisera, a variety of extracellular matrix components were examined, including the interstitial collagens type I, II, and III, the basement membrane collagens type IV and V, and the glycoprotein attachment factors laminin and fibronectin.

In a recent study, microvessel preparations from rabbit cerebrum have been shown to contain type I collagen. The isolation of type I collagen from bovine retinal microvessel preparations has also been reported. More recently, Madri et al have described the cytoplasmic localization of both types I and III procollagen in cultured capillary endothelial cells from the rat epididymal fat pad. We were unable to observe a clear and consistent reaction of retinal capillaries with antisera against type III collagen. Type III collagen may in fact be present in the capillary extracellular matrix at levels too low to be effectively monitored by our antisera. In the study reported here, capillary-size microvessels are observed to be enclosed within an extracellular matrix containing types I and IV collagen as well as laminin. The association of type I collagen with microvessels reported in these studies is somewhat surprising, since interstitial collagen is not usually found in capillary extracellular matrix. This staining pattern did not vary significantly within the group of ten retinas used in this study, and microvessels at all levels within the retina showed the same staining profile.

We also found types I, III, IV, and V collagen as well as laminin and some fibronectin associated with large retinal vessels in all tissue samples examined. The presence of such extracellular matrix components as laminin and collagen types IV and V suggests that the
larger retinal vessels have a microenvironment comparable to vessels elsewhere in the body. Although the presence of type I collagen in the wall of these larger vessels is not typical of basement membranes, this collagen has been described in the media of larger vessels, a region of the vessel wall composed primarily of smooth muscle.

The in vitro synthesis of extracellular matrix components by endothelial cells from larger vessels has also been described. Cultured human umbilical vein endothelial cells have been reported to synthesize collagen types III and IV but not the other collagen types. Various studies have suggested that when cultured vascular endothelial cells change in phenotype from contact-inhibited "epithelioid" cells to elongated overgrown "sprout" cells, the type of extracellular matrix components synthesized by these cells also changes. McAuslan et al reported the synthesis of type I collagen by cloned bovine aortic endothelial cells having a "sprout" phenotype whereas "epithelioid" lines synthesized type III collagen instead. Cotta-Pereira et al described the synthesis of types I, II and IV collagen by monolayer cultures of "epithelioid" bovine aortic endothelial cells whereas "sprouting" cells synthesized primarily type I collagen. Expression of the epithelioid phenotype and synthesis of types III, IV, and V collagen was reported to be maintained in the presence of fibroblast growth factor, while in the absence of this polypeptide growth factor the "sprout" phenotype appeared concomitant with the synthesis of type I collagen. The synthesis of type I collagen by vascular endothelial cells therefore appears to be possible, at
least in vitro. Such phenotypic and biosynthetic modulations in endothelial cells may possibly reflect in-vivo adaptations of these cells to local environmental differences within a complex vascular bed.

Our results suggest that the extracellular matrices of even the smallest retinal microvessels in vivo contain type I collagen as well as laminin and type IV collagen. In view of these results, reports of synthesis of an enzyme capable of type I collagen degradation by bovine capillary endothelial cells\(^{16}\) and enzymes capable of the degradation of types IV and V collagen by fetal bovine aortic endothelial cells\(^{17}\) are intriguing. Syn-

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**Fig. 6.** A section of unfixed human retina incubated with the same antiserum against laminin, used in Figure 5, which had been previously incubated with laminin as described in Materials and Methods. In this tissue examined at high magnification, profiles of vessels can be seen (arrowheads), but these profiles are not significantly brighter than the level of background fluorescence (×800).

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**Fig. 1A–C.** Sections of unfixed human retina incubated with antiserum against type IV collagen. In low magnification (×150) shows staining of both large and small vessels (×150). At high magnification, B and C show that the antiserum (arrowheads) stains microvessels of 8–10 μm in diameter (×800). A profile in C typical of a pericyte (asterisk) can be seen in the wall of one of these microvessels (×550).
thesis of these collagenases may be necessary early in the process of neovascularization to allow endothelial cells from microvessels to migrate through the basement membrane into the interstitium. In addition to endothelial cells, both pericytes and smooth muscle cells are possible sources of the microvessel extracellular matrix examined here. Synthesis of type III collagen by pericytes in vitro has been reported, and cultured arterial smooth muscle cells synthesize both type I and III collagen. Therefore, some contribution to the ex-

Figs. 9A-B. Sections of unfixed human retina incubated with antiserum against type II collagen. The vessel profiles (asterisks) are not significantly brighter than the surrounding tissue. In A, the tissue was incubated with the antiserum whereas in B the tissue was incubated with the same antiserum which had been previously incubated with antigen as described in Materials and Methods. The low level of nonspecific fluorescence is typical of the control levels seen elsewhere in this study (×800).
Figs. 10A-B. Sections of unfixed human retina incubated with polyclonal antiserum against type III collagen. The larger retinal vessels (arrowheads) are significantly brighter than the surrounding tissue (×350).

tracellular matrix components we have described by both smooth muscle cells and pericytes cannot be ignored.

Following extensive trypsin digestion of human retinas, vascular integrity at all levels is maintained. Trypsin digestion of human cortex results in a similar degree of vascular integrity. This technique has yielded a great deal of information on both the normal and pathological retinal microvasculature. Microvasculature elsewhere in the body, however, does not appear to be trypsin-resistant. The presence of type I collagen in the extracellular matrix at all levels of the retinal vasculature may explain why capillary-size retinal microvessels maintain their integrity after trypsin digestion. The extensive alpha-helical structure of type I collagen results in an increased stability to digestion by trypsin.

Historically, the extracellular matrix was thought to serve as an inert scaffold providing structural support for tissues. More recently, a wealth of data has accumulated to show that the extracellular matrix plays a far more important role in regulating the behavior of cells that contact it. Alterations in the extracellular matrix of the retinal vasculature may play an important role in many retinal disorders. It is, therefore, important to understand the structure and composition of the retinal vascular extracellular matrix in both health and disease. The current report provides information regarding the collagen and glycoprotein environment of the normal human retinal vasculature.

Key words: retinal capillary, immunofluorescence, extracellular matrix

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