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Collagen Distribution in the Human Vitreoretinal Interface

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Purpose. To evaluate the presence of collagen types I to VII, IX, XI, and XVIII at the posterior pole, the equator and the pre-equatorial area in human donor eyes, since collagens are important macromolecules that contribute to vitreoretinal adhesion at the vitreoretinal interface.

Methods. Freshly isolated human retinectomy samples from the equator were used for reverse transcription–polymerase chain reaction to detect mRNA of the above-mentioned collagens. In addition, human donor eyes and equatorial retinectomy samples were embedded in paraffin, stained with antibodies against the collagens and evaluated by light microscopy (LM).

Results. Retinectomy samples expressed mRNA of all tested collagen types. By LM, vitreous cortex was positive for collagen types II, V, IX, and XI. In all three regions within the donor eyes and in the retinectomy samples, the internal limiting membrane (ILM) showed types IV, VI, and XVIII; the retinal vasculature was positive for types I to VI and XVIII in most specimens; and the retinal layers showed condensed spots of type VII. In addition, type VII increased in density and in distribution over the retinal layers toward the posterior pole.

Conclusions. Staining patterns of collagen types I to V, IX, XI, and XVIII confirmed previous observations. Important new findings include the presence of type VI in the ILM and type VII in several layers of the retina. Both collagens can anchor matrix components, and type VI could be involved in vitreoretinal attachment. Furthermore, the presence of collagen mRNA in human retinectomy samples may be an indication of postnatal collagen production by retinal cells. (*Invest Ophthalmol Vis Sci.* 2008;49:4089–4095) DOI:10.1167/iovs.07-1456

The vitreous body (or vitreous) of the human eye is the transparent extracellular matrix (ECM) located between the lens and the retina. It is the largest structure of the eye and consists of 98% to 99% water and of just 0.1% macromolecules, such as glycosaminoglycans (such as hyaluronan),[1] proteoglycans,[2,3] glycoproteins[4] (such as opticin[5,6]), collagens,[7–15] and noncollagenous structural proteins[4,5,16,17] (e.g., fibrillin[5]). The most important macromolecules are the collagens, which form a network of heterotypic fibrils (types II, V/XI, and IX) and presumably maintain the gel structure.[5,7,18,19] Collagens present in the vitreous are types II,[7,8] V and XI,[9–11] VI,[11,20] and IX.[8,10–15]

The vitreous cortex is situated against the internal limiting membrane (ILM) of the retina. Strong vitreoretinal adhesions have been described at the vitreous base,[21] at the equator,[22] over retinal blood vessels,[23] at the optic disc,[24,25] and at the macula.[24] Furthermore, morphologic studies have revealed a regional variability in thickness of the ILM, consisting of an increase in thickness from the vitreous base toward the macular area, with a thinning over the fovea, optic disc, and retinal blood vessels.[23,26–28] Finally, attachment plaques (i.e., hemidesmosomes) are present in the equator and absent from the posterior pole, with the exception of the fovea.[26] In the vitreous base area, which is known for its very strong vitreoretinal attachments, direct insertions of vitreous fibrils into Müller cells and/or into crypts between adjacent Müller cells have been found.[26,29,30] Immunohistochemical studies on ILM composition have shown the presence of the noncollagenous components laminin, fibronectin, proteoglycans, and several glycoconjugates[31,32] as well as collagen types I, IV, and XVIII (Jerdan JA, et al. *IOVS* 1986;27:ARVO Abstract 230).[33,34]

In the adult human retina, the collagens that have been described (starting from the photoreceptor layer to the ILM) are types I to VI[26,35–40] and XVIII.[34] In retinas of nonpathologic donor eyes, isolated deposits of type II collagen have been found in the pre-equatorial and equatorial areas.[26] In several studies, type II was also present in retinal blood vessels,[36,38,40] although this finding was ambiguous.[37] It is also unclear whether there is preference for an anterior location, as suggested in a histopathologic pilot study on inherited rhegmatogenous retinal detachment.[40] Collagen types I, III, IV, V, VI, and XVIII have been described as components of retinal vasculature.[34–39]

The present study focused on the presence and distribution of collagen types I to VII, IX, XI, and XVIII in the vitreoretinal interface at the pre-equatorial area, the equator, and the posterior pole, by studying human donor eyes and human retinectomy samples. The knowledge about the distribution of collagens can be useful in understanding the (patho)physiology of a spontaneous, mechanical, or enzymatically induced posterior vitreous detachment (PVD).

Materials and Methods

Reverse Transcription–Polymerase Chain Reaction

Four fresh retinectomy samples (70, 74, 86, and 87 years) were acquired from patients with exudative macular degeneration during a surgical procedure in which a full-thickness healthy equatorial autologous retinal pigment epithelium (RPE) and choroid graft is transplanted to the macular area and in which the retina of the graft is not used in the procedure.[41,42] Informed consent was obtained before surgery

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with the approval of the medical ethics committee in accordance with the standards laid down in the 1964 Declaration of Helsinki. The retinectomy samples were taken from the equatorial retina at the 12 o'clock position and were immediately put into lysis buffer (Qiagen, Venlo, The Netherlands). Total RNA was extracted from the samples (RNeasy Mini Kit; Qiagen), according to the manufacturer's instructions. To eliminate DNA contamination, RNA samples were treated with DNA-free DNase (Ambion, Austin, TX). RNA concentration and purity were determined on a spectrophotometer (Nanodrop; Isogen, Maarssen, The Netherlands) by calculating the ratio of optical density at wavelengths of 260 and 280 nm. RNA (2 μg) was reverse transcribed into cDNA by using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocol (total reaction, 20 μL).

For the PCR reaction, 1 μL cDNA was added to 23 μL master mix, consisting of 2.5 μL 10× PCR buffer, 2.5 μL 2 mM dNTP mix, 1.5 μL 25 mM $MgCl_2$, 0.25 μL (5 U/μL) *Taq* DNA polymerase (MBI Fermentas), and 16.25 μL ultrapure water (Milli-Q; Millipore, Billerica, MA). Finally, a total of 1 μL of the two specific flanking primers (50 μM) was added (Table 1). The mixtures were initially denatured at 94°C for 5 minutes. The PCR consisted of 35 cycles in the following conditions: denaturation at 94°C for 0.5 minute, annealing at 55°C (for collagen types I, II, III, V, and IX) and 58°C (for types IV, VI, VII, XI, and XVIII) for 1 minute, and an extension period at 72°C for 1 minute. These cycles were followed by a final extension period of 10 minutes at 72°C. PCR products were analyzed by agarose gel electrophoresis (1%) with 500 ng/mL ethidium bromide. Human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin were used as internal positive control. No amplification was obtained from the water control (data not shown).

Paraffin Embedding Procedure

Three human eyes (three donors; aged 44, 55, and 74 years) with no known ophthalmic disorder were obtained from the Cornea Bank (Amsterdam, The Netherlands). After removal of small parts of the globe, the eyes were fixed by immersion within 36 hours postmortem in 2% paraformaldehyde (PF; Polysciences Inc., Warrington, UK) in phosphate-buffered saline (PBS) overnight at 4°C. To achieve good penetration of the fixatives, the washing, dehydration, and infiltration steps were facilitated by gently rotating the specimens. The eyes were washed in PBS and dehydrated by graded ethanols (50%–100%). Finally, the specimens were embedded in paraffin. In addition, three retinectomy samples of three patients (aged 74, 75, and 75 years) were put into 2% PF immediately after surgical removal and embedded in paraffin according to the same procedure.

Immunohistochemistry

The pre-equatorial area, the equator, and the posterior pole were selected from the donor eyes (Fig. 1). Both the selected areas and the equatorial retinectomy samples were cut in sections of 5-μm thickness and studied by light microscopy (LM). The slides were deparaffinized by the addition of xylene followed by short hydration steps with ethanols (100%–50%). After they were washed with demiwater, 1% type XXIV protease (Sigma-Aldrich, St. Louis, MO) was added for 30 minutes. Sections were washed with PBS, and endogenous peroxidases were blocked. Then, sections were exposed to PBS with 2% bovine serum albumin (BSA; Sanquin, Amsterdam, The Netherlands) and 5% serum of the producer of the secondary antibody at room temperature. The primary antibody was diluted 1:50 in PBS with 1% BSA and added for 1 hour. The primary antibodies included (1) rabbit polyclonal antibodies against human collagen types I, III, V (Abcam, Cambridge, UK) and XI (the kind gift of Julia Thom Oxford, Boise State University, Boise, ID) and against endostatin, the product of the C-terminal of type XVIII (Abcam); (2) biotinylated rabbit polyclonal antibody against human type VI (Abcam); (3) goat polyclonal antibodies against human types II and IV (Southern Biotechnology Associates [SBA], Birmingham, AL); and (4) mouse monoclonal antibodies against human types VII (Abcam) and IX (US Biological, Swampscott, MA). After the sections were washed, the peroxidized secondary antibody diluted to 1:100 in PBS, 1% BSA, and 2% human serum was added for 1 hour at room temperature. Secondary antibodies included goat- and swine-anti-rabbit peroxidases (GARPO and SARPO; Dako, Glostrup, Denmark), a rabbit-anti-goat peroxidase (RAGPO; Dako), and a rabbit-anti-mouse peroxidase (RAMPO; Dako). For biotinylated anti-type VI collagen antibody, a streptavidin peroxidase (SAPO; Dako) was used. After the sections were washed with PBS, they were stained with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) and hematoxylin.

Negative controls specimens underwent the entire procedure, except for the substitution of the primary antibody. Three human corneas were used as positive control specimens for collagen types IV and VI (not shown). As an extra control for collagen type VII, a different rabbit polyclonal antibody against type VII (Calbiochem, Darmstadt, Germany) was used to confirm the data of the mouse monoclonal antibody against type VII.

Morphologic Analysis

The morphologic data were semiquantitatively analyzed as follows: within each donor eye ($n = 3$) the pre-equatorial area, the equator, and the posterior pole were identified. One author (RJW) took pictures from the collagen labeling in the three areas in the donor eyes and from the retinectomy samples. All pictures were randomly and digitally presented at the same magnification to two independent masked observers (TLP and LIL). Collagen labeling intensity was defined on a scale of 0 to 2 (0, negative; 1, weakly positive; and 2, strongly positive).

Results

Reverse Transcription–Polymerase Chain Reaction

The retinectomy samples expressed mRNA of all tested collagen types (Fig. 2). Amplimers were seen at the expected positions: *COL1A1* at 254 bp, *COL2A1* at 419 bp, *COL3A1* at 369 bp, *COL4A2* at 648 bp, *COL5A1* at 454 bp, *COL6A1* at 342

Table 1. Primers Used in the RT-PCR Analyses

Collagen	Forward Primer: 5′→3′	Reverse Primer: 5′→3′	Size (bp)
COL1A1	TCG GCG AGA GCA TGA CCG ATG GAT	GAC GCT GTA GGT GAA GCG GCT GTT	254
COL2A1	GTG GAA GAG TGG AGA CTA CTG	TGT ACG TGA ACC TGC TAT TG	419
COL3A1	ACC GAT GAG ATT ATG ACT TCA CT	CTG CAC ATC AAG GAC ATC TTC AG	369
COL4A2	ATC GGC TAC CTC CTG GTG AA	GCT GAT GTG TGT GCG GAT GA	648
COL5A1	GAC TAC GCG GAC GGC ATG GAA	CCT GCC AGG CCA CTG ACT GGT A	454
COL6A1	GGA GCT CAA GGA AGC CAT CAA G	TCC TCC AGC AGC TCT GCA TAG T	342
COL7A1	CCG AGG ACG AGA TGG TGA AGT TG	CTG GCT CCA GGT CCT GTG TCT AC	261
COL9A1	GCC TCT GGT GAA GAA GGT GAA	TGC TGA TCT GTC GGT GCT CTA	245
COL11A1	CAG CAG GCT CGG ATT GCT CTG A	GGC CAT CTA CAC CTG CCA TAC C	460
COL18A1	TCT ACG TGG ACT GTG AGG AGT T	CTG CTC CTC GAC TTC TCC ACT T	380

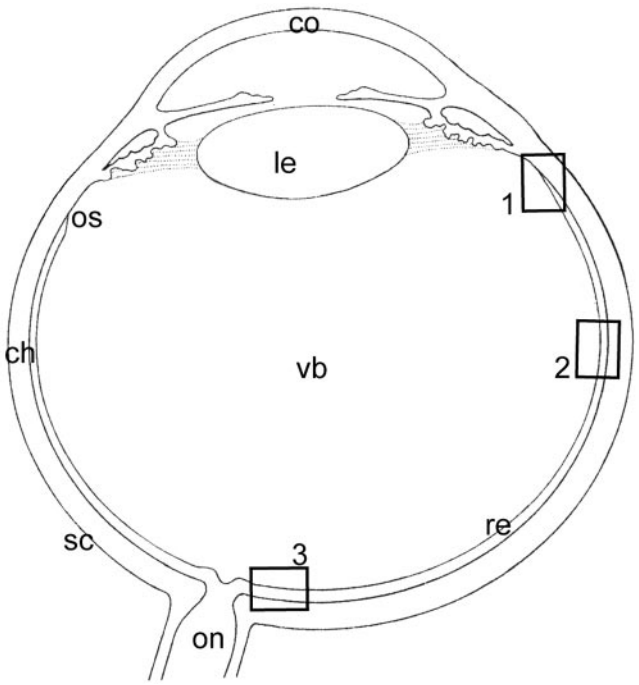

FIGURE 1. Schematic overview of an eye. Boxes 1, 2, and 3 indicate the pre-equatorial area, the equator, and the posterior pole, respectively. Co, cornea; le, lens; sc, sclera; on, optic nerve; ch, choroid; re, retina; os, ora serrata; vb, vitreous body.

bp, *COL7A1* at 261 bp, *COL9A1* at 245 bp, *COL11A1* at 460 bp, and *COL18A1* at 380 bp.

Light Microscopy

General Observations. Cross-sections and longitudinal sections through donor eyes revealed that in each eye at least part of the retina with the pigment epithelium had detached from the choroid and sclera, probably as a result of the embedding procedure. Sections through the retinectomy samples showed no adherent vitreous because of the preceding vitrectomy. In addition, the retinectomy samples contained no fragments of the RPE layer. The judgments of the masked observers were very similar in positive or negative scores, but showed differences in the intensity of positivity (weak or strong). Since quantitative results on immunohistochemical pictures appeared less reliable, we used only positive or negative scores in our results. In the cases in which the pre-equatorial area, the equator, and the posterior pole stained positive or negative, the intervening areas (not shown) were similarly stained.

Vitreoretinal Interface. The vitreoretinal interface is the area of contact between the vitreous body and the retina. The vitreous cortex when present was clearly positive for type II collagen (Fig. 3B) and variably for types V, IX, and XI (Figs. 3E, 3G, 3H). The vitreous cortex showed no staining with the antibody against type VI (Fig. 3F). The ILM was clearly positive for types IV, VI, and XVIII in all three regions (Figs. 3D, 3F, 3I). The staining patterns of types IV and VI at the ILM were the same. In the case of type II, the ILM was not discernible as a separate entity from the vitreous cortex (Figs. 3B, 3D). It was observable only at places with a local vitreous detachment. The retinectomy samples confirmed the presence of collagen types IV and VI and the absence of type II in the ILM (not shown). Human corneas stained specifically for types IV and VI (data not shown).[43]

Retinal Blood Vessels. In almost all retinal layers and in all three regions, small and large blood vessels were present. In the donor eyes, blood vessels were strongly positive for collagen types IV and VI and positive for types I, II, III, and XVIII and for type V in two eyes (55 and 74 years; Figs. 3A–F, 3I). However, type IX collagen was found only in the pre-equatorial area, and the equator in two eyes (74 and 55 years, respectively; Fig. 3G). The results of the retinectomy samples were very similar, except for type IX, which was not detected.

Retina from the Photoreceptor Layer to the ILM. In all three donor eyes, both antibodies against type VII collagen (Fig. 4) showed multiple, positive, and circular spots occasionally in the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), and inner nuclear layer (INL). Besides the circular spots, dotted spots positive for type VII were found more often in the vicinity of a nucleus. An obvious finding was the increase in the amount as well as the spread over the retinal layers of type VII–positive spots toward the posterior pole (Figs. 4A–C). In the 69-year-old donor eye, type XI collagen was faintly present in the GCL in the posterior pole and the equator (Fig. 3H).

In the retinectomy samples, type VII collagen was found in a spot-like configuration variably in the NFL, GCL, IPL, INL, outer plexiform layer (OPL), and outer nuclear layer (ONL).

DISCUSSION

By immunohistochemical staining and LM evaluation, we detected the collagens of interest. Our interest was primarily in collagens with a potential role in vitreoretinal adhesion. These can be subdivided into those collagens present both in the vitreous cortex and the retina (such as collagen types II, V, VI, and XVIII) and collagens which in other tissues are known to mediate anchoring of one tissue structure to another (e.g., type VII). New findings in this study are that type VI is located in the ILM (previously only described in retinal blood vessels)[38,44] and that type VII is widely distributed in several retina layers with increasing density from the pre-equatorial area toward the posterior pole. Furthermore, type II was present in human retinal vasculature and was probably absent from the ILM. In addition, previous published observations on collagen distribution in the vitreous and retina were confirmed.

In retinectomy samples, mRNA of α1-chains from types I to III, V to VII, IX, XI, and XVIII collagen and mRNA of α2-chain from type IV collagen were found. The presence of collagen mRNA in this equatorial part of the retina is an indication that these collagens can be synthesized by cells present in the sample (e.g., Müller glial and endothelial cells). Besides the ciliary body, which is often indicated as a possible source of ILM and vitreous collagens,[45–48] equatorial retinal cells may be able to synthesize vitreous and ILM collagens. A comment should be made that only collagen types II, III, VII, and XVIII consist of three identical procollagens (α1), and thus other

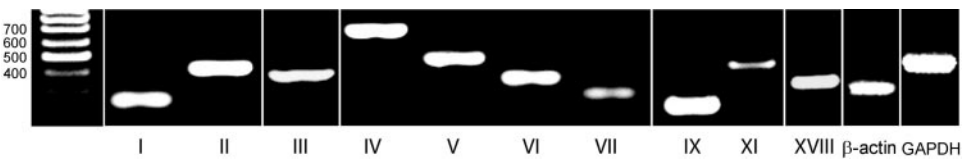

FIGURE 2. RT-PCR on an equatorial retinectomy specimen (74-year-old patient). *Left* to *right:* bands indicating the positions of collagen types I, II, III, IV, V, VI, VII, IX, XI, and XVIII. Human β-actin and GAPDH were positive. *Left*: 100-bp DNA ladder.

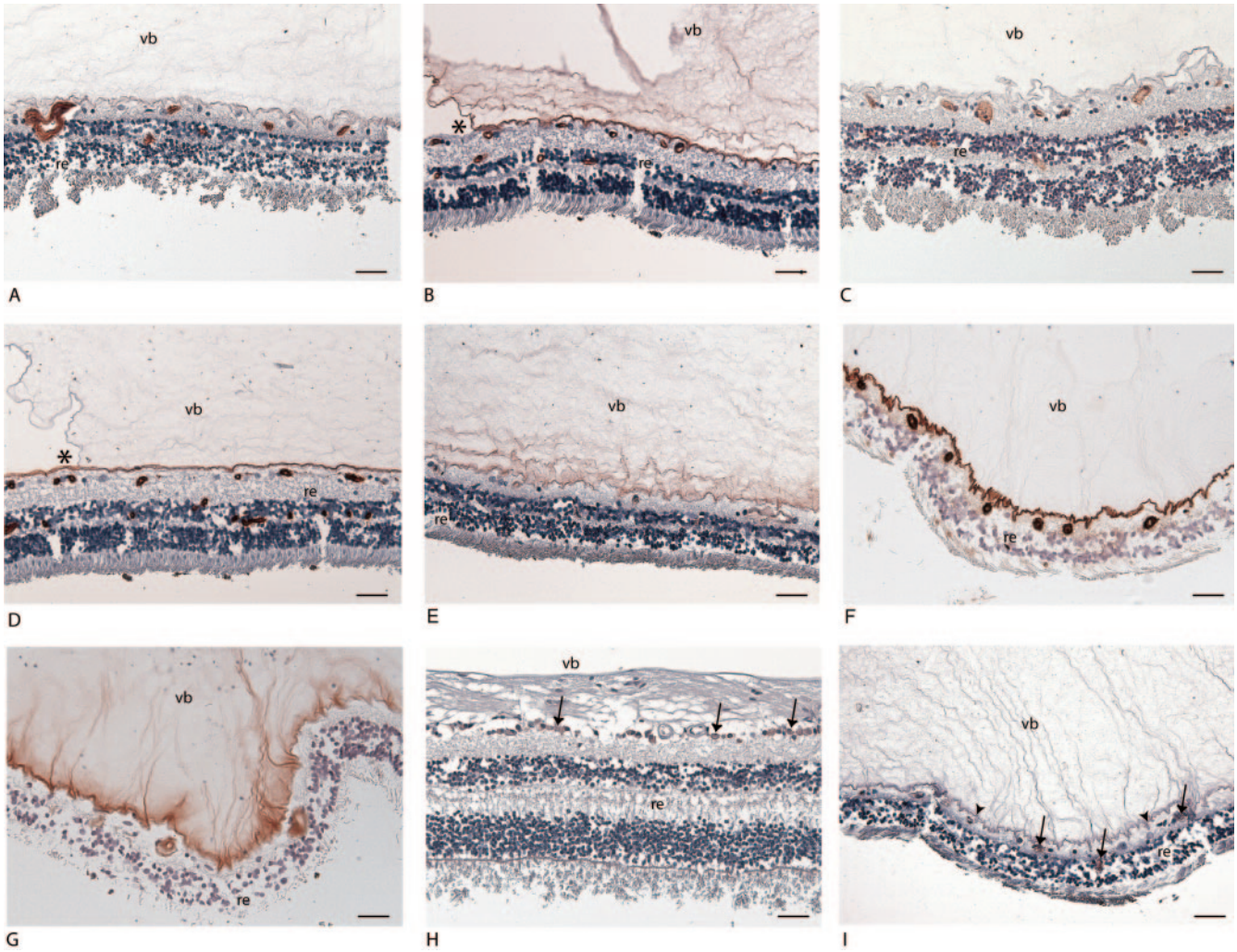

FIGURE 3. Immunohistochemical analyses of the collagens at the equator of human donor eyes evaluated by LM. (**A**) Type I (55 years) was present in the smaller and larger retinal blood vessels. (**B**) Type II (74 years) was found in the vitreous cortex and in retinal blood vessels. The ILM showed no type II at the place where the vitreous body was detached from the retina (✻). (**C**) Type III (55 years) was visible in the retinal blood vessels. (**D**) Type IV (74 years) was present in the ILM and retinal blood vessels. At the site of the local vitreous detachment (✻), the ILM remained positive for type IV and the vitreous cortex did not stain. (**E**) Type V (55 years) was found in the retinal blood vessels and vitreous cortex. (**F**) Type VI (55 years) was clearly present in retinal blood vessels and in the ILM, whereas the vitreous cortex shows no staining. (**G**) In this section, type IX was present in retinal vasculature and in the vitreous cortex (55 years). (**H**) Type XI (*arrows*) was faintly stained in the GCL in the posterior pole (69 years). (**I**) Type XVIII was present as a faint staining in the ILM (*arrowheads*) and retinal blood vessels (*arrows*; 55 years). Vb, vitreous body; re, retina. Bars, 50 μm.

chains may be essential for types I, IV, V, VI, IX, and XI to build up a functional triple helical molecule.[35]

As previously described, the vitreous cortex was positive for collagen types II, V/XI, and IX.[5] We did not find type VI collagen in the vitreous cortex, although this collagen has been reported to be present in the vitreous.[20] The light microscopic absence of type VI in the vitreous cortex and the variable presence of type V, IX, and XI may be explained by the small amount present or by a masked epitope. At the vitreoretinal interface, it was impossible to distinguish between the vitreous cortex and the ILM, sometimes making it difficult to determine whether labeling was at the vitreous cortex, the ILM, or both. The difficulty in discriminating both structures is a known problem described in previous studies.[33,49] Based on specimens with a local vitreous detachment and on the retinectomy samples after vitrectomy, we concluded that the ILM contained collagen types IV, VI, and XVIII, but not type II.

Type VI collagen is essentially a glycoprotein that belongs to the non–fibril-forming collagens and forms a (beaded) filamentous network in most ECMs.[50,51] It has a predominant role in linking cells and matrix macromolecules.[52,53] It has shown specific interactions with (1) hyaluronan in calf skin, which is interesting since vitreous is also rich in hyaluronan,[54,55]; (2) the striated collagen fibers of the inner and outer layers of Bruch's[39]; (3) collagen types I, III, and V within the scleral and the corneal stromal collagen network[56–58]; (4) type IV in Bowman's layer[57,58]; and (5) pericytes at the choroidal side of the choriocapillaris.[59] In human iris and ciliary body, type VI was found in the direct vicinity of the basement membranes, but not in the vicinity of the epithelial basement membranes of the ciliary and iris muscle cells.[60] From the present study, based on the widespread presence of type VI throughout the ILM, we conclude that the entire vitreous is probably surrounded by this type of collagen, which thus could mediate an overall anchoring between the ILM and vitreous cortex.

Type XVIII collagen (of which endostatin, a potent angiogenesis inhibitor, is a proteolytically derived fragment) was found in the ILM, as previously described.[34,61] Based on mouse

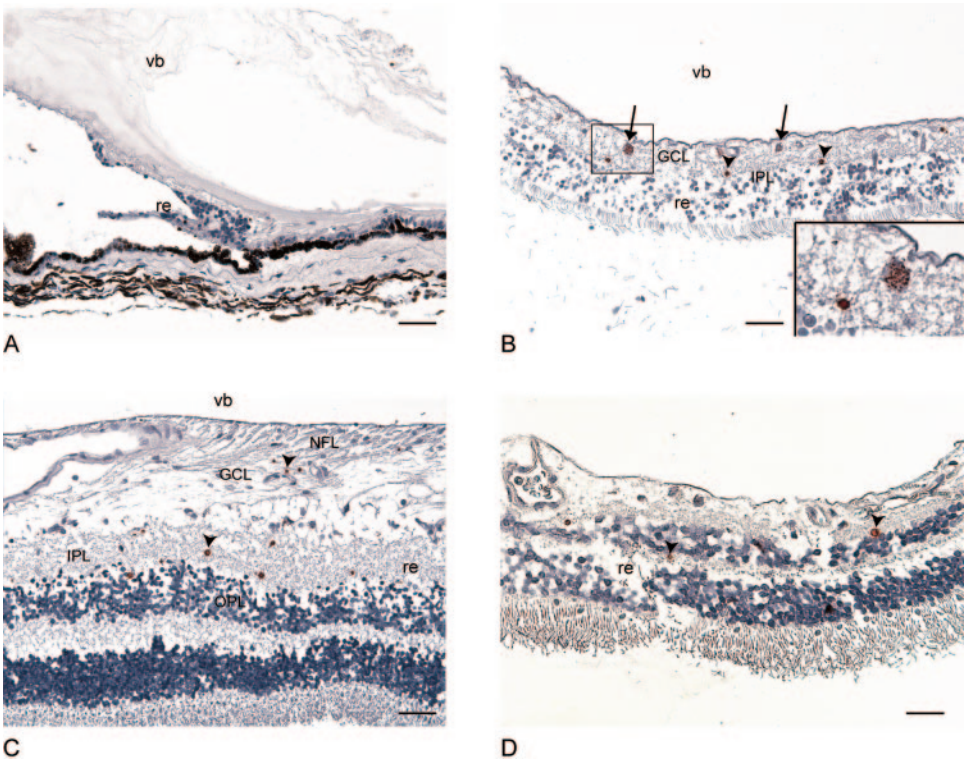

FIGURE 4. The distribution of type VII collagen in the 74-year-old donor eye (**A–C**) and in the equatorial retinectomy sample (**D**) (results of the mouse monoclonal antibody are shown). In the pre-equatorial area (**A**), type VII collagen was not found. In the equator (**B**), it was visible as small positive spots in the GCL and IPL, whereas, in the posterior pole (**C**), it was present in nerve fiber layer, the GCL, the IPL, and the OPL. *Arrows*: dotted aspect; *arrowheads*: circular spots; *inset*: circular spots (*left*) and dotted spots (*right*) positive for type VII collagen. In the equatorial retinectomy sample (**D**), type VII collagen was also found in the OPL and ONL. *Arrows*: spots with the dotted aspect; *arrowheads*: circular spots. Vb, vitreous body; re, retina. Bars, 50 μm.

studies, its function may be twofold: (1) part of an anchoring complex between the vitreous fibrillar collagens and the ILM, and (2) responsibility for the disappearance of vitreous hyaloid vasculature in the embryonic period.[62] Thus, its presence could theoretically be associated with a protection against PVD or against vascular neovascularization. It would therefore be interesting to study type XVIII collagen/endostatin on ageing and in diseases characterized by retinal neovascularization.

Retinal blood vessels contained collagen types I to VI and XVIII , whereas types V and IX were variably present, which is largely in agreement with previous studies on human retinal vasculature. Previous immunohistochemical studies did not uniformly confirm the presence of types II and IX.[34,35,37–39] In our study, the presence of type IX was very variable and needs further investigation. With regard to type II, one study[37] questioned its presence, which could be explained by the used antiserum, and another found only type II in the peripheral vasculature.[40] However, Western Blot analysis on bovine retinal blood vessels confirmed the presence of types I to V, of which types II and IV were prominently present.[36]

The presence of type II collagen is interesting in the light of strong interconnections between vitreous and retinal vasculature[23] and the possible source of this collagen. As a consequence of the strong connection, vitreous hemorrhage can occur during a posterior vitreous detachment.[63] The presence of mRNA *COL2A1* in freshly isolated human retina could indicate the retina as a possible production place of type II. The producing cell of type II has still to be established, but Müller cells are good candidates, since they are attached to the retinal vasculature and ILM[64] and their end feet are closely related to sublaminar intraretinal type II collagen.[33]

Surprisingly, we found a condensed appearance of type VII collagen, an anchoring fibril, in the retina. The staining pattern, consisting of dotted spots and larger globular structures in multiple retina layers, differed clearly from the superficial linear staining pattern previously found by LM in other tissues (e.g., cornea).[65] The presence of type VII in the retina is a new finding and it is as yet unknown whether it is located intra- or extracellularly. As far as is known from other tissues (e.g., skin and cornea),[66] functional type VII collagen is an extracellular matrix component; it is the primary structural element of anchoring fibrils, and it forms anchoring plaques (connection areas between several anchoring fibrils) together with type IV.[67] Because its staining pattern is at variance with patterns found in other tissues, where it connects ectodermal and mesodermal tissue components, its retinal function is not immediately clear and has to be determined in future studies.

Collagen types VI, VII, and XVIII are all able to anchor matrix components to each other. Their presence in the retina and their functions suggest an involvement in the (posterior) vitreoretinal attachment and thus also in the mechanism of (posterior) vitreoretinal detachment. At the moment, several types of enzymes (e.g., (micro)plasmin and collagenase) are used to pharmacologically induce liquefaction and PVD in humans and animals, both therapeutically and experimentally, but in most cases the mechanism of action remains unclear.[68–71] When we focus on collagens, we see that subtypes of collagenases should be able to degrade specific collagens,[71] nattokinase can hydrolyze vitreous collagen fibers,[72] thrombin and plasmin can cleave type V collagen,[73] and exogenous plasmin can activate matrix metalloproteinase-2,[74] which is capable of degrading types IV and VII.[75] Care should be taken when enzymes are used to induce liquefaction and PVD. Little is known about their mechanism of action on the vitreoretinal interface, and it is questionable whether the action of these enzymes stops at the ILM.

The presently described distribution patterns of different collagen types in the human vitreoretinal interface emphasize the possible interactions between the vitreous cortex and retina. Future research should determine the exact roles of the various collagens in vitreoretinal adhesions and interface pathology, the process resulting in PVD, and the potential effects of enzymatic vitreolysis on the vitreoretinal interface and retina.

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