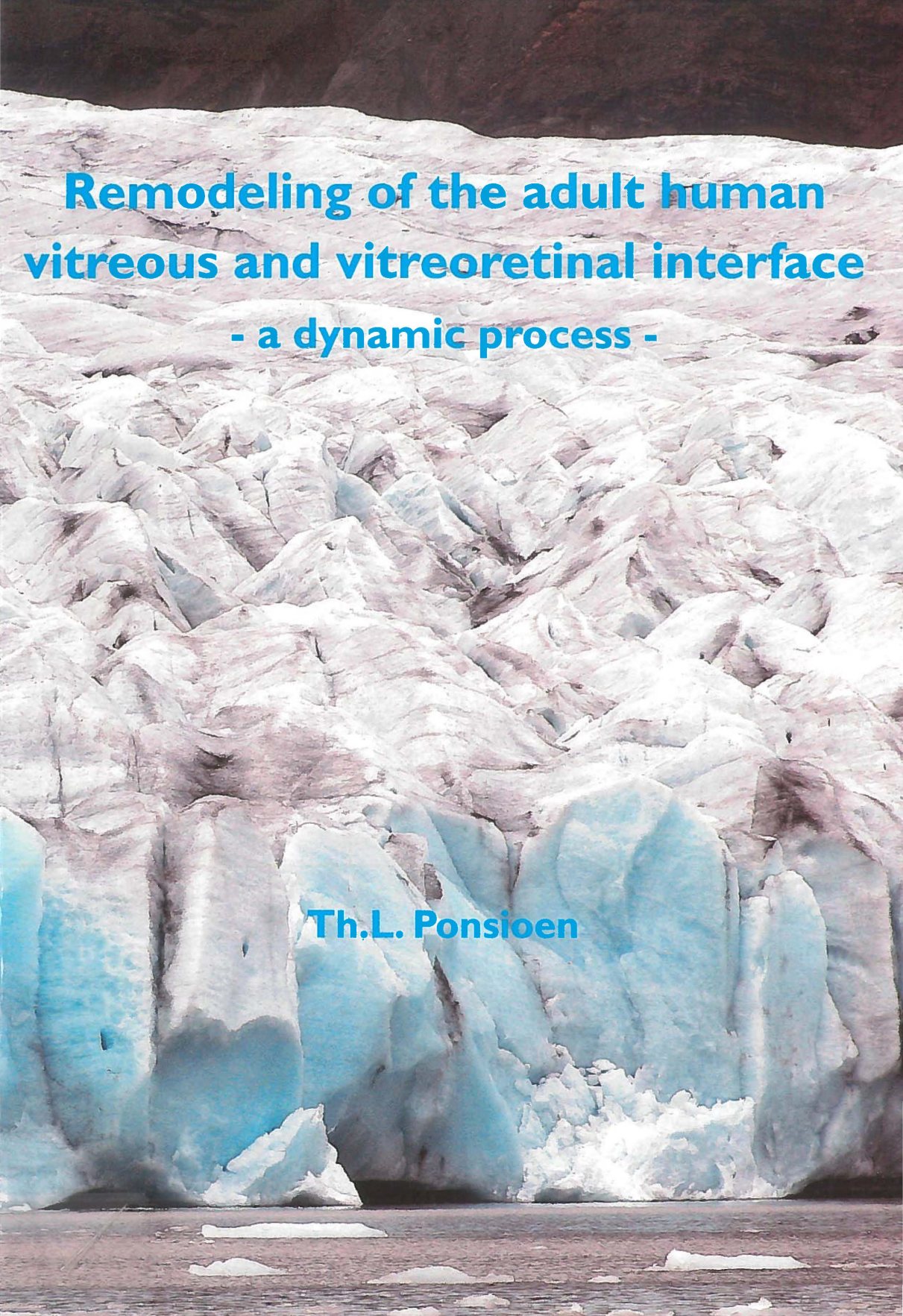
Remodeling of the adult human vitreous and vitreoretinal interface

Ponsioen, Theodorus Leonardus



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- a dynamic process -

Th.L. Ponsioen

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Stellingen

bij het proefschrift

Remodeling of the adult human vitreous and vitreoretinal interface

- a dynamic process -

1. Het verouderingsproces van het humane glasvocht is dynamisch en niet slechts een samenklontering van collageen. (dit proefschrift)

2. Door de Müller cel is de retina als producent van glasvochtcollageen weer in beeld. (dit proefschrift)

3. Het gebruik van enzymen voor het creëren van een achterste glasvochtmembraan-loslating (PVD) is een riskante aangelegenheid. (dit proefschrift)

4. Als het antigeen in het glasvocht gemaskeerd is, blijft de chemie tussen het antigeen en zijn specifieke antilichaam afwezig. (dit proefschrift)

5. In de immuno-transmissie elektronenmicroscopie is de zilververstersterking een gouden standaard. (dit proefschrift)

6. Het zien van Abraham of Sarah betekent voor het glasvocht-collageen het enzymatisch hoogtepunt. (dit proefschrift)

7. De definitieve boekvorm van het proefschrift draagt bij aan duurzame CO_2 opslag.

8. Als we wisten wat we deden, was er geen onderzoek.

9. You can't depend on your eyes when your imagination is out of focus.

10. Life is all about priorities, first things first – Marlies Ponsioen

11. If the whole materia medica could be sunk at the bottom of the sea it would be all the better for mankind and all the worse for the fishes.
Holmes (1891)

12. Live today, cause yesterday is gone and tomorrow may never come.
Dr Donald "Ducky" Mallard, NCIS

Dirk Ponsioen
Groningen, 19 mei 2008

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Πάντα ρεῖ και ουδεν μενεῖ
(Alles stroomt en niets blijft)

Herakleitos van Efese

Aan vader en moeder
Voor Rosette en onze Marius

Table of contents

General Introduction

The vitreous body (or vitreous) of the human eye is the clear gel, which is located behind the lens and is surrounded by and attached to the retina (Fig. 1). The vitreous is the largest structure of the eye, represents approximately 80% of its volume, and consists of 98-99% water, of 1% inorganic salts with organic lipids of low molecular weight and of just 0.1% macromolecules, such as glycosaminoglycans (GAGs),[1] proteoglycans (PGs),[2,3] glycoproteins[4] (GPs; 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 and the GAG, hyaluronan (HA).[18,19] Collagen types present in the vitreous are types II,[7,8] V/XI,[9-11] VI,[11,20] and IX.[8,10-15] A collagen network of heterotypic fibrils (types II, V/XI, and IX) presumably maintains the gel structure and HA fills the spaces between these collagen fibrils and stabilizes the gel.[5,7,21]

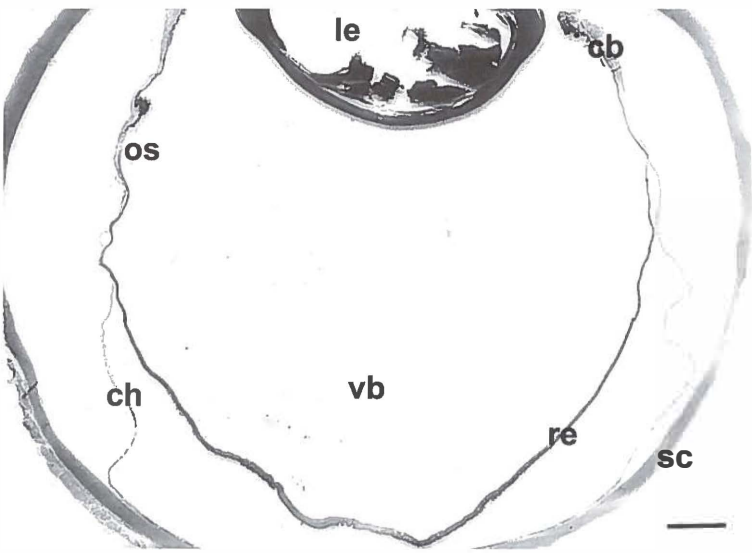


Figure 1 Macroscopic overview of a Technovit embedded human donor eye. Cb: ciliary body, le: lens, os: ora serrata, ch: choroid and retinal pigment epithelium, vb: vitreous body, sc: sclera, and re: retina. Bar = 2.5 mm.

The vitreous has a refractive index of 1.3349 and a specific gravity of 1.0053-1.0089 g/ml.[22] It has several (supposed) functions including involvement in growth of the embryonic eye and media transparency. The vitreous transmits 90% of light with a wavelength between 300 and 1400 nm. In addition, the vitreous plays a physiological role in lens transparency and metabolism, which can be divided in a depository for nutrients and metabolic wastes and a scavenger of free radicals.[23,24] Other functions include protection of eye structures during a mechanical trauma through its viscoelastic properties and it

can have a preventive effect in the case of a retinal detachment (the cortex can close a fresh hole). Finally, it could inhibit angiogenesis by the presence of low molecular weight growth inhibitors of endothelial cells and by its high concentration of ascorbic acid.[25,26] On the other hand, the vitreous can almost completely be removed by vitrectomy without marked damage to the eye (nuclear sclerotic cataract can be induced), and the gel does not reform to any significant degree.[5]

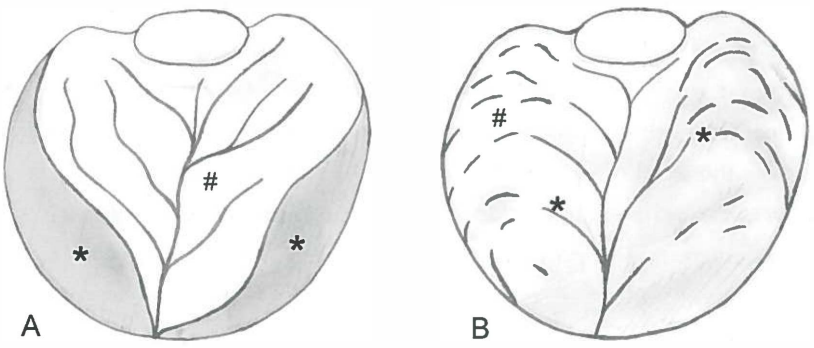
With aging, the vitreous is subjected to progressive morphological changes. In adult extracellular matrices (ECMs) other than the vitreous, morphological changes have been described as matrix remodeling.[27-30] Supported by some recent biochemical and morphological publications, which find evidence of collagen turnover (synthesis and breakdown) in the vitreous and in the vitreoretinal interface, the concept of matrix remodeling could also apply to the aging vitreous.[31-37]

Because the development of the eye is almost complete before birth except for the macula, we will start with a summary of the vitreoretinal embryology (1.1). Subsequently, some anatomical features of the vitreous will be highlighted (1.2). This is followed by observations about the postnatal vitreous from the newborn through the elderly (1.3). Finally, our research topics are introduced (1.4).

1.1 The human embryonic vitreous

In the earliest stages of the human embryo (4-5 mm stage or third to fourth week), evidence for vitreous formation has been found when the neural ectoderm separates from the surface ectoderm in the optic vesicle. Fibrils or proteoplasmic protrusions, which are continuous with the retina and lens, fill the narrow space between the lens vesicle and the inner retinal layer: the primordial vitreous. In the lentiretinal space of the optic cup, the primary vitreous develops along with the hyaloid vasculature in the 10-13 mm stage (fifth week). It consists of ectodermal components, which are the fibrils produced by the inner layer of the future sensory retina and the posterior side of the lens vesicle. At this stage, it is a matter of debate whether the primary vitreous also consists of mesenchymal components, which enter posteriorly with the hyaloid blood vessels and anteriorly through the space between the anterior rim of the optic cup and the lens vesicle.[18,19,38-41] The cells of the inner layer of the cup differentiate into an inner and outer neuroblastic layer, which both will develop into retinal neurons and glia whereas the outer layer will also form rods and cones.[42-44]

Together with the closure of the optic fissure around the 14 mm stage (end of the sixth week), the period of the secondary vitreous (avascular vitreous) begins.[18,19,39,41] For a long time, it was generally accepted that the secondary vitreous and primary vitreous are spatially separated structures (Fig. 2A). The former appears on the inside of the sensory retina pushing the primary vitreous and the hyaloid system forward and inward, except at the periphery and optic disc.[18,19,39,41] In contrast to the theory of strict spatial separation between primary and secondary vitreous, there is also evidence that retracting hyaloid blood vessels may act as a scaffold along which fibers of the definite vitreous organize themselves (Fig. 2B).[45-49] Both in human and rabbit vitreous, the course of vitreous fibrils and lamellae can be retranslated into the course of the hyaloid blood vessels of the primary vitreous.[45-48] This latter theory implies a gradual remodeling of the primary into the secondary vitreous. The secondary vitreous is in essence an ECM that consists primarily of type II collagen.[19,39,50] From the retina, processes of Müller cells start to unite and form the internal limiting lamina (ILL), which begins in the posterior pole. The ILL is not a surface of separation yet and Müller cells seem continuous with the vitreous fibrils.[18,19,41] The foot-plates of the Müller cells are supposed to be concerned in the synthesis of vitreous fibrils during the growth of the eye. Mesenchymal cells accompanying the hyaloid blood vessel, which grows in the vitreous as an arteriole giving off a complicated system of branches (vasa hyaloidea propria and tunica vasculosa lentis), may also contribute to the formation of vitreous.[41] Around 40-48 mm (ninth week) the maximum size of the hyaloid system is reached, the atrophy of the posterior vasa hyaloidea propria has begun and the primary vitreous has ceased to grow. This is followed by degeneration of the tunica vasculosa lentis.[18,19,41] The hyaloid artery persists much longer and increases with the growth of the whole eye, because it is attached to the lens and to the center of the optic disc. At the place where primary vitreous (adhering to the blood vessel and consisting of a looser meshwork) and secondary vitreous meet, a transition can be seen: the wall of Cloquet's canal.[39,41] Between 70-100 mm (fourth month), the tertiary vitreous or zonular system is produced at the level of the developing ciliary body by surface ectoderm and fibroblasts.[19,41] Around 240 mm (seventh month) the blood flow in the hyaloid artery ceases, followed by glycogen and lipid deposition in the endothelial cells, and, finally, cells in the blood vessel wall become atrophic and are phagocytozed by mononuclear phagocytes.[18,19,39,51] At birth, all blood vessels have disappeared from the vitreous. The eye itself is then almost completely developed except for the macula, which reaches its adult configuration at 45 months.[39,52-55]



Figures 2A-B Schematic representation of two views on the development of the primary and secondary vitreous during human embryology in the tenth week or 50 mm stage. **A**. Primary (#) and secondary (*) vitreous are spatially separated structures.[18,19,39,41] **B**. Primary vitreous (#) is gradually replaced by secondary vitreous which forms around the retracting hyaloid blood vessels (*).[45-49]

1.2 Macroscopic and microscopic anatomy of the vitreous body

The ECM structure of the vitreous is preserved by interactions between its main macromolecules.[2,21,56-58] Concerning the anatomy of the vitreous body, there is no absolute distinctness in the literature, which might be explained by the high water content of the vitreous, by the use of different preservation methods (fixatives), and by the variable visualization techniques. For example, fresh vitreous has no structure when examined unstained with the light microscope.[18]

The vitreous body (Fig. 3) is almost spherical apart from the patellar fossa where the posterior part of the lens indents the vitreous anteriorly. At the edge of this fossa, a firm attachment of 1-2 mm in width and 8-9 mm in diameter is located between the vitreous and the lens, which is known as the ligamentum hyaloideocapsulare or Wieger's ligament.[59] Between the posterior lens capsule and the patellar fossa and within the attachment zone of the Wieger's ligament is a potential space: the space of Berger.[60]

Within the vitreous body, (i) the basal vitreous, (ii) the vitreous cortex, and (iii) the central vitreous are recognized. The vitreous base is a three dimensional annular zone, which extends 1.5-2 mm anteriorly to the ora serrata and 1-3 mm posteriorly to the ora serrata.[61] The basal vitreous is composed of dense bundles of collagen fibrils, which firmly adhere to the retina and to the non-pigmented ciliary epithelium. Upon aging, the

posterior edge of the vitreous base enlarges posteriorly.[37,62] The increase in width of the posterior vitreous base is greater in the nasal half than the temporal half of the eye.[37]

The cortex (100-300 μm) surrounds the central gel and adjoins the retina. It is composed of the anterior vitreous cortex, which runs forward from the anterior vitreous base to cover the central part of the posterior surface of the lens, and of the posterior vitreous cortex, which runs posteriorly from the posterior vitreous base and is adherent to the retinal surface. The posterior vitreous cortex is absent over the optic disc and thinned over the macula.[5,63] A prepapillary hole can sometimes be visualized clinically when the posterior vitreous is detached from the retina (Weiss' ring). Strong vitreoretinal adhesions have been described at the vitreous base,[64] at the macula,[65] at the optic disc,[65,66] and over retinal blood vessels.[67] From the cortex to the central vitreous, the concentration of collagen fibrils decreases: from a high density in the cortex via an area with intermediate density to a loose-meshed network in the center.[68,69]

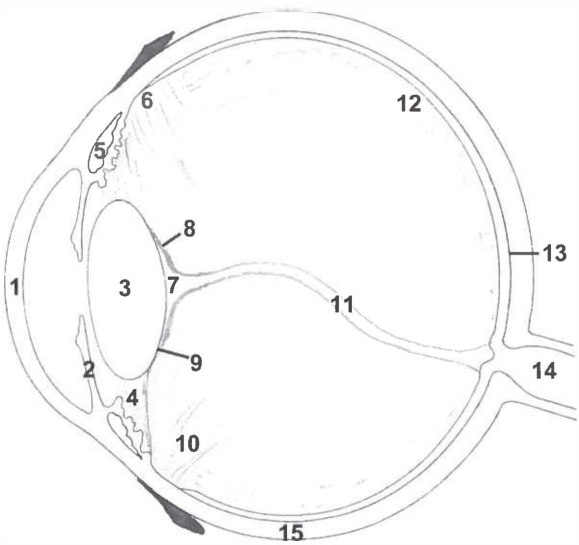
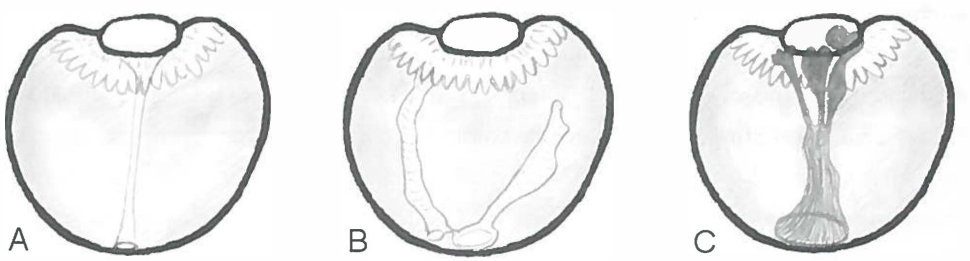


Figure 3 Anatomical considerations and lamellar organization of the vitreous. 1. cornea; 2. iris; 3. lens; 4. zonules; 5. ciliary body; 6. ora serrata; 7. space of Berger; 8. patellar fossa; 9. Wieger's ligament; 10. basal vitreous; 11. (supposed) Canal of Cloquet; 12. vitreous cortex; 13. macula; 14. optic nerve; 15. equatorial area.

The central vitreous is the largest part and could still contain the canal of Cloquet (Fig. 4A), which represents the remnant of the embryonic hyaloid vasculature (see also vitreoretinal development).[5,18] In opposite, some studies question the existence of the canal of Cloquet as described above. Several ink injection studies show a distinct morphology of transvitreal channels in the adult eye, i.e. central canal(s) which end behind the ciliary

body (Fig. 4B).[70-72] The embryonic and early postnatal Cloquet's canal disappears around the third postnatal month.[69]

Furthermore, spaces and condensations have been described in the vitreous body. Spaces are found in the vitreous cortex in front of the optic disc, the macula, and blood vessels. However, the latter finding remains unclear.[64,68,73] Within the vitreous body, several authors have observed sacs,[74] tracts,[68,75] and cisterns, which are spaces at different levels in a circular arrangement.[64] The presence of these structures as components of vitreous anatomy has not generally been accepted.



Figures 4A-C Three different concepts of "Cloquet's Canal". **A**. The papillo-lenticular canal through the central vitreous represents a remnant of the embryonic hyaloid blood vessel.[5,18] **B**. Two cilio-papillo-macular channels run through the intermediate vitreous and extend towards the retrociliary area.[72] **C**. A relatively large central canal begins in the posterior pole and ends in the perilenticular or retrociliary area.[71]

Most tissue condensations found in the vitreous cortex follow a course parallel to the retina, while some follow a course perpendicular to the retina.[69] In the area with an intermediate density, condensations come from the vitreous base. They are changeably described as tracts,[68,75] septa,[64,70] lamellae,[69,76,77] membranes,[68,69,78] and fiber bundles.[79,80] Their origin, organization pattern and contents are still discussed in literature. Via slit lamp microscopy, condensations were thought to be collagenous in origin.[80] By transmission electron microscope (TEM), vitreous collagen fibrils could aggregate into bundles.[80] In a more recent study, however, structures in the vitreous were not just fiber bundles but more complex lamellae.[72]

Cells in the vitreous

The adult vitreous body contains only a small number of cells predominantly located in the vitreous cortex. Hyalocytes represent approximately 90% of the cells and fibroblasts the other 10%. In the region of the vitreous base, the highest cell density of hyalocytes is found

followed by the posterior pole, with the lowest density at the equator.[81,82] There is also a preferential location of these cells along retinal blood vessels.[82] Hyalocytes are 10-15 µm in diameter with a lobulated nucleus and depending on their activity and location, these cells can be round, oval, spindle-shaped, or flattened.[83,84] These cells contain a well-developed Golgi complex, a moderate amount of mitochondria, basophilic Periodic Acid Schiff (PAS)-positive granules (0.2-2.0 µm), lysosomes, and smooth and rough endoplasmatic reticulum phagosomes.[18,83] In hyalocyte granules, several substances are found: GAG, GP, nucleic acid, acid phosphatase, beta-glucuronidase, and alkaline phosphatase.[85,86]

In the posterior vitreous cortex, laminocytes were found.[87] These cells were predominantly present at the side of the ILL and absent from the rest of the vitreous. They could be involved in maintaining the adhesion between the posterior vitreous cortex and the ILL.

Fibroblasts are localized in the cortex at the ciliary processes, vitreous base, and adjacent to the optic disc.[88,89] They differ form hyalocytes by the presence of longer extensions (with a maximum length of 260 µm) and the absence of PAS-positive granules.[24,63,82]

The tertiary vitreous

In embryology, the zonules are traditionally classified as the tertiary vitreous, because of their intimate association with the vitreous body. From an anatomical point of view, the posterior zonular fibers run (partly) through the anterior vitreous. However, the zonular system is in fact neither structurally nor biochemically part of the vitreous: the zonules are the suspensory system through which the ciliary muscle can alter the curvature of the lens.[90]

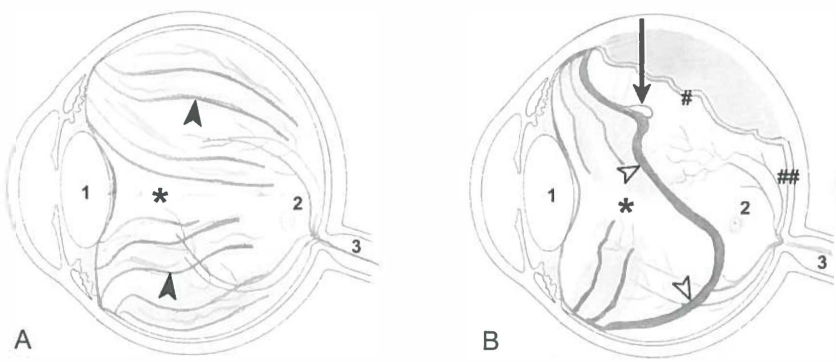
The vitreoretinal interface

The vitreoretinal interface is the area of contact between the vitreous body and the retina. Centrifugally, the interface consists of a vitreous cortex, retinal ILL, and Müller cell endfeet. Müller cells are radially oriented macroglia that traverse the retina from its inner (vitreal) border to the outer limiting membrane. They have many local functions: they stabilize the retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space.[91,92] It has been suggested that they are responsible for the production of vitreous and ILL collagens during growth and in adulthood, but this remains a matter of debate.[7,41,43,93-100]

1.3 Aging of the postnatal vitreous

At birth, the human vitreous has an ultrasonic length of 10.48 mm and 10.22 mm for males and females, respectively. Through the years, this difference is preserved. After fast growth in the first three postnatal years (around 3.5 mm), growth slows down and the vitreous is almost full-grown at the age of 13 with measurements of 16.09 mm for males and 15.59 mm for females.[101,102]

With aging, two morphological changes in the vitreous have been described: a progressive increase in the volume of liquefied spaces (synchisis)[103-105] and an increase in optically dense areas (syneresis).[68,69,106-108] Synchisis generally starts in the central part and is characterized by the replacement of vitreous gel by liquefied vitreous, which is typically free of collagen fibrils and surrounded by optically dense structures or condensations (Fig. 5A).[57,108] As these morphological alterations progress with age, they may locally interfere with the passage of light and cause symptoms referred to as 'mouches volantes' or floaters. Furthermore, posterior vitreoretinal attachments weaken,[73] while anterior vitreoretinal adhesions develop with aging.[37,62] Finally, these processes may lead to a posterior vitreous detachment (PVD), a separation between the vitreous cortex and the retina. In most eyes, PVD is not a serious condition, but it may induce pathology, such as retinal tears, retinal detachment (Fig. 5B), intravitreal hemorrhage, and cystoid macular edema.[109-111] PVD is particularly associated with senescence and the amount of liquefied spaces,[104,106] and less with myopia, which mainly causes an earlier onset,[109,112,113] female gender,[104] ischemia of Müller cells,[114] and several metabolic disturbances, e.g. diabetes mellitus.[114-116]



Figures 5A-B **A**. Vitreous aging with an increase in the volume of liquefied spaces (*) and an increase in optically dense structures (closed arrowheads). **B**. A PVD induces a retinal hole followed by a retinal detachment starting at the place where the vitreous cortex has a firm attachment with the retina (arrow). Liquefied and collapsed vitreous (*), vitreous cortex (open arrowhead), detached retina (#), attached retina (##). 1. lens; 2. macula; 3. optic nerve.

The (patho)physiological mechanisms underlying the morphological changes and PVD have not yet been clarified. Generally, synchisis is supposed to start with changes in the noncollagenous components of the matrix and to result in aggregation of collagen fibrils.[2,5,21,56,57,108] Following this theory, synchisis and syneresis are the structural manifestation of a destabilization of the vitreous matrix.[21,103,108] Based on more recent studies, we propose an alternative hypothesis, in which breakdown of vitreous matrix (synchisis)[117] would coincide with synthesis of vitreous collagen[31-36] leading to an increase in optically dense structures upon aging (syneresis). Since collagens are held responsible for the preservation of the gel structure,[5] these macromolecules are of primary interest. This hypothesis is also in line with adult ECMs other than the vitreous (e.g. gingiva and cartilage), in which collagens undergo turnover (synthesis and breakdown).[27-30]

In those ECMs, collagen breakdown follows mainly an intracellular route for remodeling under physiological conditions.[29] When large amounts of collagen have to be broken down in a relatively short interval (i.e. inflammation), extracellular breakdown is more important with a main role for matrix metalloproteinases (MMPs).[118,119] However, in the vitreous, physiological collagen breakdown could also follow the extracellular route, because cellular structures are absent near liquefied spaces, where collagen fragments are found.[117]

Fibril-forming collagens (e.g. type II) are synthesized intracellularly and in general procollagen molecules are secreted into the ECM, where collagen fibrils assemble and collagen cross-link formation occurs.[29,120,121] The formation of fibrils may not always apply to the vitreous, since procollagens were observed in adult human, rabbit and bovine vitreous.[31,32,34,35] In addition, mature bovine vitreous contained more immature collagen cross-links than articular cartilage, indicating a slower or incomplete maturation process and/or a more active synthesis of collagen in the former.[33]

1.4 Aim and outline of this thesis

Since the first moment the vitreous is formed, its structure is not static, but subject to changes. During embryonic development, the vitreous progresses from the primordial (avascular) via the primary (vascular) into the secondary (avascular) vitreous. From the age of four onwards, the first evidence for synchisis is described[103] and besides synchisis, syneresis occurs.[68,69,106-108] Both processes progress slowly with aging and 45% of the persons between 60-69 years old have at least 50% liquefaction.[105] The pathogenesis of

aging of the human vitreous is a process that still needs to be elucidated. The morphological changes, synchisis and syneresis, are traditionally seen as the structural manifestation of a destabilization of the vitreous matrix. The alternative proposed hypothesis, in which collagen turnover, i.e. synthesis and breakdown, plays a role, will be a central feature in this thesis. Our main goal is to find and add evidence to the dynamic view of collagen turnover. To achieve our goal we study:

(i) the human vitreous and its interaction with the vitreoretinal interface,

(ii) retinal Müller cells to determine their possible role in collagen turnover, and

(iii) the role of enzymatic collagen cross-links in the human vitreous with aging.

Chapter 2 is an LM and TEM study on the morphology of the vitreoretinal interface in the (pre-)equatorial area of the human eye. It discusses vitreoretinal attachments and provides additional information on newly synthesized intraretinal collagen (networks and packages).[37] In most eyes, Müller cells were closely related to the collagen packages. According to our morphological observations, these cells could be involved in the breakdown and synthesis of intraretinal collagen.

In **chapter 3**, the presence of types I-VII, IX, XI, and XVIII collagen in the vitreoretinal interface is studied in fresh retinectomy samples from the equator and in human donor eyes at the pre-equator area, equator area, and posterior pole. We use reverse transcriptase-polymerase chain reaction (RT-PCR) to detect mRNA of above-mentioned collagens and LM immunohistochemistry to localize the collagens. Important findings include the detection of type VI collagen in the ILM (internal limiting membrane, an LM description) and type VII collagen in superficial retinal layers.

In **chapter 4**, human retinal Müller cells are evaluated to elucidate their possible role in collagen breakdown. Cultured Müller cells (MIO-M1[122]) are exposed to fluorescent latex beads coated with types II (vitreous) and IV (ILL) collagen. To influence phagocytosis, cytochalasin B and anti-integrin subunits (α1, α2, and β1) are added to the cells. Phagocytosis is evaluated by TEM, flow cytometry, and confocal microscopy.

Chapter 5 discusses whether cultured Müller cells (MIO-M1) can be involved in the synthesis of types I-VII, IX, XI, and XVII collagen. Type XVII collagen is not related to the natural vicinity of the retinal Müller cell. We use RT-PCR, immunohistochemistry (LM) and Western Blotting to detect (a) the presence of collagen mRNA, (b) the cellular expression of collagens, and (c) the secretion of collagens, respectively.

Chapter 6 describes the mature enzymatic collagen cross-links, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), in the human vitreous with aging. The pyridinoline cross-links form the last enzymatic step in collagen maturation. They

provide physical and mechanical strength to the collagen network and thus contribute to its integrity.[123] Whole human vitreous bodies are analyzed by high performance liquid chromatography (HPLC). Our findings can contribute to the insight in the age-related processes synchisis and syneresis in the concept of collagen turnover.

Chapter 7 is a general discussion and a review of recent literature addressing remodeling of the human vitreous and vitreoretinal interface with aging.

Chapters 8 and 9 provide an English summary and a Dutch summary, respectively.

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2

Packages of vitreous collagen (type II) in the human retina: an indication of postnatal collagen turnover?

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Abstract

The purpose of this study was to evaluate the vitreoretinal border in the (pre-)equatorial area in nonpathologic human donor eyes, because the majority of retinal defects induced by posterior vitreous detachment (PVD) are located there. Nine eyes (24–80 years) were fixed and embedded in Technovit 8100. After evaluation by light microscope, areas of interest were selected for immunotransmission electron microscope. Anti-type II collagen antibody was used to stain vitreous fibrils and lamellae; anti-type IV collagen antibody was used to identify the internal limiting lamina (ILL); anti-vimentin and anti-CD-68 antibodies stained retinal Müller cells and macrophages, respectively. Observations included fusing of lamellae with the ILL, an intravitreal course of the ILL, and clear focal interruptions in the ILL. In addition, an obvious finding was the presence of intraretinal packages of type II collagen. Interestingly these collagen packages were closely related to Müller cells and, in several eyes, also to macrophages, cell debris and interruptions in the ILL. In our opinion, the collagen packages can reflect the net result of a process of interactive remodelling, in which both breakdown and synthesis of vitreous and ILL collagens take place. Connections between vitreous and intraretinal collagen networks can make the (pre-)equatorial area more vulnerable to tearing and retinal detachment in the case of liquefaction and PVD.

1. Introduction

The vitreoretinal interface is the area of contact between the vitreous body and the retina. Centrifugally, the interface consists of a vitreous cortex, retinal internal limiting lamina (ILL), and Müller cell endfeet. The clinical importance of this area becomes apparent from interface pathology induced by posterior vitreous detachment (PVD), such as retinal tears, retinal detachment, and intravitreal haemorrhage. PVD is mostly associated with senescence and the amount of liquefied spaces (Foos, 1972a; Foos and Wheeler, 1982), and less with myopia, which mainly causes an earlier onset (Linder, 1966; Jaffe, 1968; Akiba, 1993), female gender (Foos and Wheeler, 1982), ischaemia of Müller cells (Sebag, 1987a), and several metabolic disturbances, e.g. diabetes (Akiba et al., 1993; Deguine et al., 1998; Sebag, 1987a).

On ageing, the human vitreous undergoes two morphological changes in matrix structure. One is a progressive increase in liquefied spaces (synchysis) (Favre and Goldmann, 1956; O'Malley, 1976; Foos and Wheeler, 1982; Balazs and Denlinger, 1982); the other is an increase in optically dense structures (syneresis) (Szent Györgyi, 1917; Eisner, 1971; Foos, 1972a; Oksala, 1978; Sebag, 1987b). In general, liquefaction starts in the central vitreous (Sebag, 1987b). At the border of liquefied spaces, aggregation of proteoglycans and fragmentation of collagen fibrils have been described (Los et al., 2003). In the end, synchysis leads to PVD (Favre and Goldmann, 1956; Foos, 1972a,b; O'Malley, 1976; Foos and Wheeler, 1982).
The (patho)physiological mechanisms underlying these processes have not yet been clarified.

Whether a PVD will induce interface pathology depends on the nature of vitreoretinal adhesions and the thickness of the ILL. Strong adhesions have been described at the vitreous base (Teng and Chi, 1957), the equator (Worst, 1977), over retinal blood vessels (Foos, 1977), at the optic disc (Grignolo, 1952; Foos and Roth, 1973) and macula (Grignolo, 1952). Transmission electron microscopic (TEM) studies revealed a regional variability in thickness of the ILL, consisting of an increase in thickness from the vitreous base towards the macular area with a focal thinning over the fovea and optic disc (Foos, 1972b; Heegaard, 1997). Foos (1977), and Spencer and Foos (1970) further observed thinning of the ILL and Müller cell processes in areas overlying retinal blood vessels. Furthermore, attachment plaques (i.e. hemidesmosomes) were present in the equator and absent from the posterior pole with exception of the fovea (Foos, 1972b). In the vitreous base area, which is known for its extremely strong vitreoretinal attachments, several variations in the vitreoretinal border have been described. These include direct insertions of vitreous fibrils into Müller cells and/or into crypts between adjacent Müller cells (Foos, 1972b; Gloor and Daicker, 1975;

Malecaze et al., 1985). These and other variations have been interpreted as degenerative or interactive remodelling of the vitreoretinal juncture (Foos, 1972b; Malecaze et al., 1985). By scanning electron microscopy, Wang et al. (2003) observed sublaminar collagenous fibres expanding into collagen networks, which increase on ageing.

The present study focuses on the vitreoretinal border in the (pre-)equatorial area, because the majority of retinal defects induced by PVD are located there (Sebag, 1989). Donor eyes were embedded in Technovit 8100; morphology and immuno-TEM identified specific structures and cells in the vitreoretinal border. Previous immunohistochemical studies on ILL composition have shown the presence of the noncollagenous components laminin, fibronectin, proteoglycans, and several glycoconjugates (Kohno et al., 1987; Russell et al., 1991), as well as type I and type IV collagens (Jerdan et al., 1986). The latter authors were not able to demonstrate type II collagen in the ILL. Here, the location of the ILL and vitreous collagen is determined with an anti-type IV collagen antibody and an anti-type II collagen antibody, respectively. Studying lamellae inserting on the ILL in the equatorial area by TEM, we discovered packages of type II collagen in the retina. Therefore, we also focus on these collagen packages and related accessory findings.

2. Materials and methods

2.1. T8100 embedding procedure

Nine human eyes (nine donors) with ages varying between 24 and 80 years (mean age 62 years; Table 1) and with no known ophthalmic disorder were obtained from the Cornea Bank (Amsterdam, The Netherlands) after removal of the corneas for cornea transplantation. The eyes were studied by light microscope (LM) and by immunotransmission electron microscope (immuno-TEM). Eyes were fixed by immersion within 48 hr post mortem in 2% paraformaldehyde (PF, Polysciences Inc., Warrington, UK) diluted in 0.1 M phosphate buffer, pH 7.4 for 1 hr. After removal of small parts of the globe, the eyes were fixed for an additional 4 hr in PF. In order to avoid vitreous loss, washing, dehydration and infiltration steps were promoted by gently rotating the specimens. The eyes were washed in 6.8% sucrose in phosphate buffered saline (PBS) for 16 hr, briefly in doubly distilled water, dehydrated in acetones (30–100%) and embedded in Technovit 8100 (T8100, Heraeus Kulzer, Wehrheim, Germany). After infiltration with T8100A (without accelerator) at 4°C, the specimens were transferred to –20°C for infiltration with T8100ACB (with accelerator), and again to 4°C for polymerisation.

The embedded eyes were cut and sections of 3–4 μm thickness were stained with toluidin blue (TB) for evaluation by LM. Areas of interest were selected for morphological and immunohistochemical evaluation by TEM. Sections with a thickness of approximately 200 nm were mounted on formvar-coated nickel grids, subjected to the immuno-TEM procedures described below, counterstained with uranyl acetate in 25 cp methylcellulose, and evaluated in a Philips 201 TEM operated at 80 kV.

2.2. Immunohistochemical staining

The following primary antibodies were used: goat polyclonal antibodies against human type II collagen (vitreous; Southern Biotechnology Associates (SBA), Birmingham, USA) and type IV collagen (ILL; SBA) and mouse monoclonal antibodies against human vimentin (retinal Müller cells; DAKO, Glostrup, Denmark) and CD-68 (macrophages; DAKO).

The immuno-TEM procedure was as follows: thin sections were pretreated with 0.1% (for anti-type IV collagen antibody) and 0.01% (for the remaining antibodies) trypsin (Gibco, Paisley, Scotland) in Tris–HCl, pH 7.8 containing 0.1% $CaCl_2$, for 15 min at 37°C. The sections were then washed in PBS pH 7.4, incubated in 0.1 M citric acid pH 3 (for anti-type II collagen antibody containing 0.5 mg ml^{-1} pepsin (Boehringer Mannheim, Mannheim, Germany)) for 45 min at 37°C, washed in PBS, incubated in PBS with 0.15% glycine, 5% Bovine Serum Albumin (BSA; CLB, Amsterdam, The Netherlands), 2% rabbit serum (for both anticollagen antibodies) or 2% mouse serum (for antivimentin and anti-CD-68 antibodies) for 30 min at room temperature (RT) to block nonspecific binding of the primary antibody. Thereafter, sections were incubated with the primary antibody diluted in PBS (1:150) with 1% BSA-c (Aurion, Wageningen, The Netherlands) first for 2 hr at 37°C, and then overnight at RT. The next day, sections were washed in PBS, incubated in Rabbit anti-Goat IgG or Rabbit anti-Mouse IgG conjugated to 6 nm gold (Aurion, Wageningen, The Netherlands) diluted in PBS (1:150) for 60 min at RT, washed in PBS, fixed in 2% glutaraldehyde/PBS for 2 min and finally washed in doubly distilled water. This step was followed by incubation with silver enhancement solution (Aurion R-gent enhancer, Aurion, Wageningen, The Netherlands) for 5 min at RT. After washing in doubly distilled water, the sections were counterstained as described above. Control sections underwent the entire procedure, except for the substitution of the primary antibody.

Table 1 Overview of the donor eyes

Donor eye	Age	Section location	Distance col pack to ILL (μm)	Length of col pack (μm)	Lamellae perpendicular	Lamellae parallel	Interruption ILL	Müller cell	Macrophage
70 702	24	Equator	7-10	4-7	++	+	n.p.	n.p.	n.p.
70 734	29	Pre-equator	0-44	2-17	+	+	n.p.	n.p.	n.p.
81 778	56	Pre-equator	0-24	6-25	n.p.	n.p.	+	n.p.	+
81 791	67	Pre-equator	0-38	3-18	+	n.p.	+, **	+	+
85 117	70	Pre-equator	30-36	1-4	+	+	+	+	n.p.
76 587	74	Equator	0-2	3-24	++	+	+, **	+, displaced	+
81 784	77	(Pre-)equator	0-20	2-28	+	+	+, **	+, displaced	+
85 116	77	Pre-equator	0-28	5-25	+	+	+, **	+, displaced	+
74 656	80	Equator	0-20	6-11	++	+	+, **	+	n.p.

*Col, collagen (type II); pack, package; CC, many present; C, present; K, negative; n.p., not present in the (labelled) sections; **, type II collagen filling or passing through the interruption; displaced, clear displacement of Müller cell processes by collagen packages.*

3. Results

3.1. Light microscope

Cross-sections and longitudinal sections revealed that in every eye at least part of the retina with the pigment epithelium detached from the choroid and sclera, probably as a result of the embedding procedure (Fig. 1). The vitreous remained largely in the eyeballs, although two holes were cut in the sclera for impregnation with T8100. In all eyes, the vitreoretinal border was intact and thus no (complete) PVD was observed. Lamellae were visible as thick threads crossing through the vitreous partly inserting on the ILL, and together with (larger) superficial retinal blood vessels they proved to be good landmarks for TEM evaluation.

Furthermore, the vitreous base of each specimen had a clear and thick attachment at the ora serrata. After the novel finding of intraretinal packages of type II collagen by TEM (see below), many larger packages were seen under the ILL in LM sections (Fig. 2).

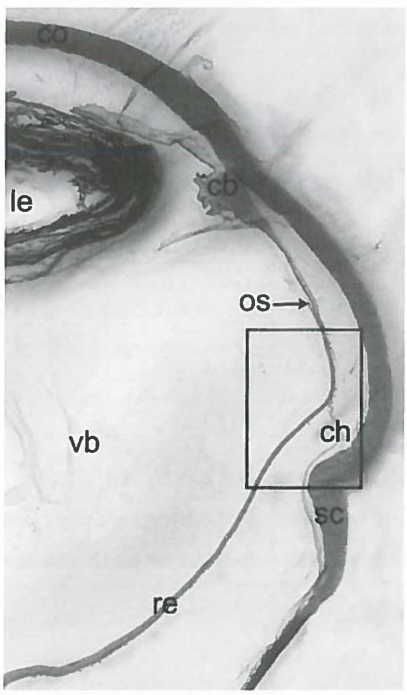

Figure 1 Macroscopic overview of eye 85 117. The box shows the evaluated equatorial and pre-equatorial areas. Co, cornea; cb, ciliary body; le, lens; os, ora serrata; ch, choroid and retinal pigment epithelium; vb, vitreous body; sc, sclera; and re, retina.

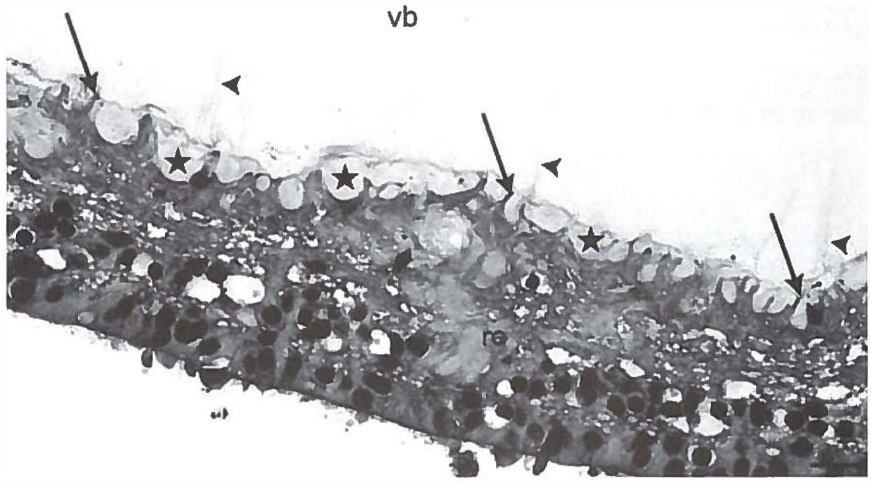

Figure 2 LM of eye 81 784: overview of the vitreoretinal border containing perpendicular lamellae (arrowheads) on the side of the vitreous body (vb). In the retina (re), many collagen packages (★; see Fig.3 for type II collagen staining) are located subjacent to the ILL. Note the displacement of the Müller cell processes (arrows). Bar=10 μm.

3.2. Transmission electron microscope

3.2.1. General observations

Details of the LM selections of the vitreoretinal border, which contained part of the vitreous and the retina, were evaluated by (immuno-)TEM and clarified part of the vitreoretinal morphology, because the same areas were compared after staining with different antibodies (Table 1).

Vitreous and collagen packages were positive for type II collagen in all eyes. By the use of pepsin in the pretreatment, the morphology was of a lesser quality (Fig. 3A).

The retinal basement membrane (ILL) was positive for type IV collagen (Fig. 3B). The ciliary basement membranes, retinal blood vessels and the lens capsule also contained type IV collagen (not shown).

In six cases, anti-vimentin antibody staining resulted in a clear distinction of Müller cells and their processes in the retina. The characteristic triangular endfeet adjacent to the ILL were hardly seen, instead of that we found, in half of the cases, clear displacements of Müller cell processes by collagen packages (see below; Fig. 3C).

Macrophages, demonstrated by an anti-CD-68 antibody labelling, were clearly observed in five eyes, and in those cases they appeared to be closely related to collagen packages in the vitreoretinal border (Figs. 3D and 4). In these eyes, other parts of the peripheral retina without light microscopic collagen packages did not contain any macrophages.

All control sections (i.e. without primary antibody) were negative (not shown).

3.2.2. Vitreoretinal interface

A striking observation in these human donor eyes was the variability in structure of the vitreoretinal interface in the (pre-)equatorial areas. Even though the expected organisation pattern, consisting of vitreous cortex–retinal ILL–retinal Müller cell surfaces, was most frequently seen, we also observed vitreous lamellae fusing with the ILL, direct vitreoretinal adhesions without ILL interpositioning, and focal interruptions in the ILL as described below (1–3):

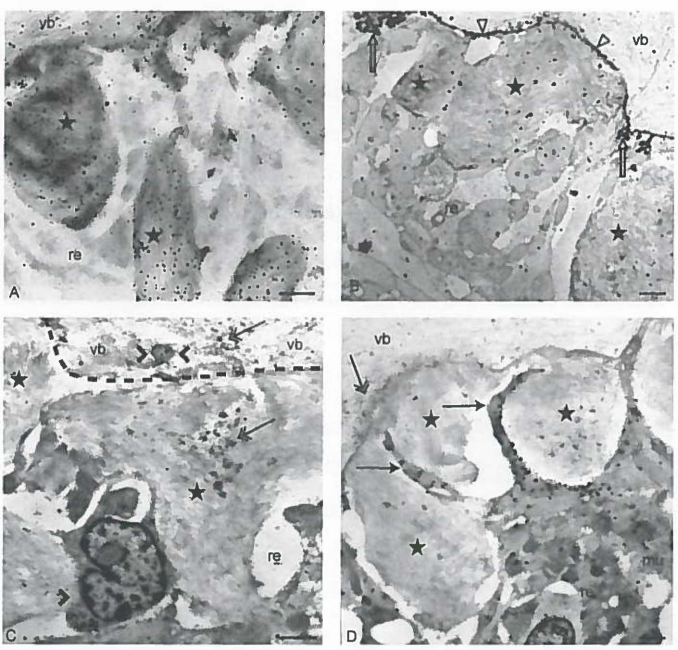

Figure 3 Immuno-TEM of eye 81 784 shows details of the vitreoretinal border. (A) Anti-type II collagen antibody indicates the vitreous body (vb) and typeII collagen positive packages (★) in the retina (re). (B) Anti-type IV collagen antibody clearly stains the ILL (open arrowheads) with a few thickened notches (open arrows). There is also a little nonspecific staining. (C) Anti-CD-68 antibody indicates a macrophage (>) located near collagen packages (★) in the retina (re) with probably cell debris (double arrow) and a CD-68 positive cell process (>) in the vitreous body (vb). The dotted line indicates the position of the ILL. (D) Anti-vimentin antibody shows the intracellular filaments of a Müller cell (mu) and the displaced processes (arrows) in between the collagen packages (★). Again, cell debris (double arrow) is probably present in the vitreous body (vb). Re, retina. Bars=2 μm.

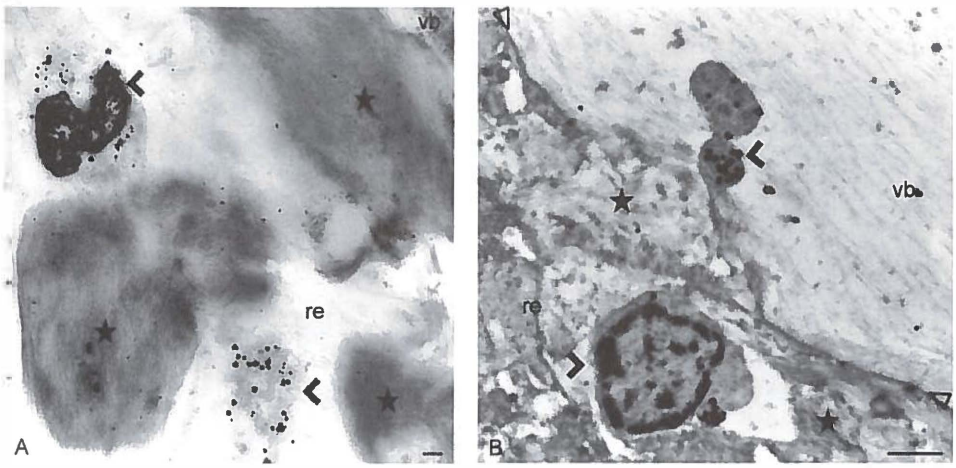

Figure 4 Immuno-TEM CD-68 staining shows: (A) (eye 81 778) intraretinal (re) macrophages (>) near the collagen packages (★) and (B) (eye 76 587) a macrophage (>) located under the ILL (open arrowheads) between packages of collagen and a positive cell process within the vitreous body (vb). Bars=1 μm.

1. In eight donor eyes (all except 81 778), vitreous lamellae perpendicular to the ILL were seen. In seven of these eyes (all except 81 791), there were also lamellae parallel to the retina (see Table 1). Vitreous lamellae running parallel to the retina were clearly fusing with the ILL in three eyes (70 702, 70 734, and 74 656). In these areas, the ILL and vitreous lamella ran separately over large areas, while sometimes an overlap between the two structures was noted. In 70 702, type II collagen fibres also penetrated through the ILL, and these were connected with a fine-meshed type II collagen network in the retina (Fig. 5A and B).

2. In four cases the ILL had an intravitreal course. This was observed in the pre-equatorial (70 734, 81 784, and 85 116) and equatorial (76 587) retina. In all instances, vitreous collagen was located between the retinal Müller cells and the elevated ILL, resulting in focal vitreoretinal adhesions.

3. Clear focal interruptions within the (elevated) ILL were observed in seven eyes (all except 70 702 and 70 734). In these observations, anti-type IV collagen labelling played a crucial role in detecting breaks in the ILL. In five eyes (74 656, 76 587, 81 784, 81 791, and 85 116), vitreous collagen filled or passed through the interruption (Fig. 6A and C). This was shown by comparing the same areas after anti-type II collagen antibody and anti-type IV collagen antibody staining in each eye (Fig. 6A and B).

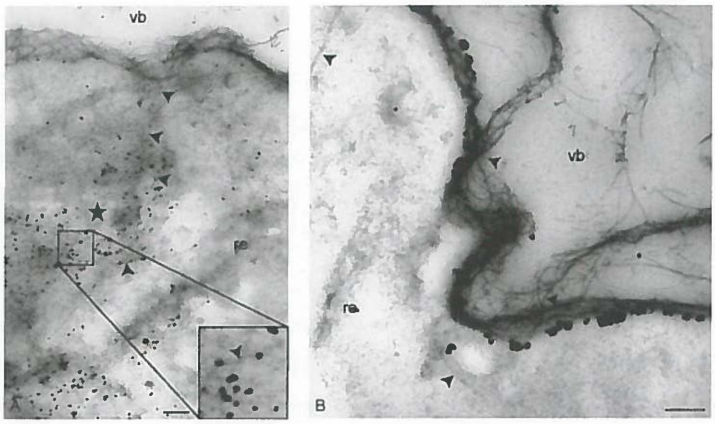

Figure 5 Immuno-TEM of eye 70 702: (A) Anti-type II collagen antibody demonstrates the presence of fine type II collagen fibres (arrowheads), which pass through the ILL and make contact with a fine-meshed intraretinal type II collagen network (★). The insertion shows details of the type II collagen fibres. (B) Anti-type IV collagen indicates the ILL, with which vitreous fibrils (arrowheads) fuse. Loose (vitreous) fibrils are located directly under the ILL (arrowheads). Re, retina and vb, vitreous body. Bars=500 μm.

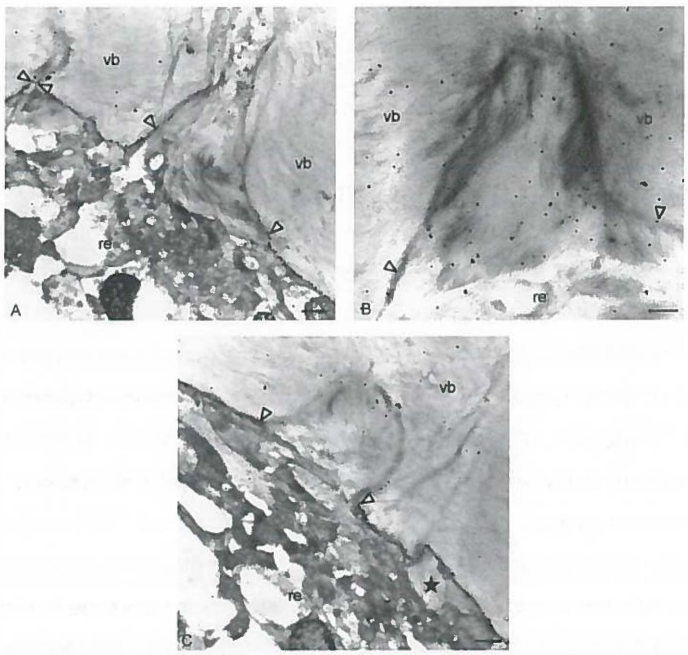

Figure 6 Immuno-TEM of eye 76 587: (A) Staining of the vitreoretinal border shows a clear interruption (between open arrowheads) in the type IV collagen positive ILL and, partly, an intravitreal course of the ILL. (B) Type II collagen staining of the same area shows that the vitreous collagen (type II positive) is partly enclosed by an aberrantly running ILL (open arrowheads). (C) In another area with an intraretinal package of collagen (★), anti-type IV collagen antibody staining indicates an interruption in the type IV collagen positive ILL (between open arrowheads). Re, retina and vb, vitreous body. Bars=2 μm.

3.2.3. Intraretinal packages of vitreous collagen

In all donor eyes, type II collagen packages were observed in the retina. The distance of these packages to the ILL ranged from 0 to 44 μm and their diameter varied from about 1 to 28 μm (Table 1). The collagen fibrils in different packages ran parallel and perpendicular to retina and vitreous. In addition, collagen fibrils in most packages appeared to be more densely packed than in the vitreous.

During the analysis of collagen packages, we repeatedly found, besides some vitreous lamellae inserting perpendicular on the retina, cells, cell processes, and electron-dense structures (most probably cell debris) in their surrounding. It appeared that the ILL over the collagen packages was largely intact, and focal interruptions were mainly observed in their neighbourhood. In five eyes (76 587, 81 778, 81 784, 81 791, and 85 116), the vitreous cortex and retina adjacent to packages contained macrophages (CD-68 positive cells), and in three (76 587, 81 784, and 85 116) displacements of Müller cell processes (vimentin positive) embracing intraretinal collagen were clearly visible. Because Müller cells were also observed in the vicinity of the collagen packages in three other eyes (74 656, 81 791, and 85 117), most collagen packages, both superficially, i.e. close to the ILL, and deeper in the retina, seemed to be surrounded by Müller cell processes.

4. Discussion

For the evaluation of the vitreoretinal border, our main focus was on adhesions between vitreous lamellae and the retinal surface and on the course of the ILL in the (pre-)equatorial area. Before vitreous fibrils, lamellae, ILL, and intraretinal packages of type II collagen are discussed in more detail (see below), it should be noticed that the postembedding labelling procedure on T8100 (Los et al., 2000) played an essential role in determining the exact course and location of the above-mentioned structures and the position of the retinal Müller cells and macrophages. Morphological research on LM and TEM level alone could not have convincingly demonstrated this. Postembedding labelling thus appeared to be a very useful tool to study the nature and distribution patterns of collagens in the vitreoretinal interface. Postembedding labelling studies commonly encounter the masking of epitopes, which need (enzymatic) pretreatment steps for the unmasking of epitopes. With various pretreatment steps, specific labelling patterns were obtained for each antigen/antibody. Pepsin pretreatment was desirable in anti-type II collagen antibody labelling, but it affected overall cell morphology. This step can be skipped in the other labelling procedures.

After specific labelling with anticollagen antibodies, we focussed firstly on vitreous lamellae, which can be considered as local condensations of vitreous fibrils. Due to their structure, lamellae are the most likely candidates to cause focal traction upon the retina leading to tear formation. Macroscopically, they present themselves as sheet-like structures; by LM, they are visible as groups of parallel-running collagenous threads, and by TEM they appear as concentrations of vitreous fibrils, in a mainly parallel organisation, with occasional smaller fibrils crossing the spaces between parallel fibrils (Los, 1997; Los et al., 2000). In eight of nine eyes, lamellae inserted clearly perpendicular to the retina. In one case, vitreous fibrils passed the ILL (70 702).

Secondly, the course of the ILL was followed by immunohistochemical staining with anti-type IV collagen antibody, which showed, besides variability in structure of the ILL, clear interruptions and an intravitreal course. We established clear fusing of vitreous lamellae with the ILL, which was accompanied in some places by vitreous fibrils passing through the ILL (Fig. 5). Furthermore, we observed and via immuno-TEM expanded on impressions described by Foos (1972b) in the (pre-)equatorial area, i.e. macrophages, cell debris, and interruptions in the ILL with collagen fibres trying to penetrate.

Finally, we found intraretinal collagen packages positive for type II collagen in the (pre-) equatorial area. Moreover, these packages were closely surrounded by Müller cells. Our results correspond and add information to the SEM findings of Wang et al. (2003), who observed collagen fibres, without cellular relations, under the ILL developing into dense collagen knots on ageing in the basal area. Their findings were interpreted as de novo synthesis of collagen. Although the sample size in our study was too small to detect age-related phenomena, the youngest eye (70 702) contained an intraretinal fine-meshed collagen network (Fig. 5), and the oldest eyes (e.g. 81 784) contained much denser collagen packages (Fig. 3A).

A few decades ago, Foos (1972b), Gloor and Daicker (1975),and Malecaze et al. (1985) described direct adhesions between vitreous fibres and Müller cells at the vitreous base and the ora serrata, so-called crypts. In the equatorial area, Malecaze et al. (1985) also observed widening of intracellular spaces partly filled with fibres adhering to Müller cells, which were covered by an ILL. These and other variations had been interpreted as degenerative or interactive remodelling of the vitreoretinal juncture (Foos, 1972b; Malecaze et al., 1985). The results of our study differ to some extent as to the location of the interruptions, the depth and size of the collagen packages, and their close relation to the Müller cells. Furthermore, these packages were observed in the vicinity of inserting lamellae, but a direct connection between collagen packages and vitreous lamellae was not found.

To explain the intraretinal packages, our findings are placed in a more current view of extracellular matrix (ECM) remodelling. Several studies have described remodelling in adult ECMs other than the vitreous (Svoboda et al., 1981; Everts et al., 1996; Antoniou et al., 1996; Blair et al., 2002). Collagens, which are also the main macromolecular components of the vitreous (type II) and vitreoretinal border (type IV), appear to undergo turnover, which means that there is some synthesis and breakdown. Collagen breakdown follows primarily an intracellular route in case of remodelling in physiological conditions (Everts et al., 1996). When large amounts of collagen have to be degraded in a relatively short interval (i.e. inflammation), extracellular breakdown is more important with a main role for matrix metalloproteinases (MMPs) (Everts and Beertsen, 1992; Nagase and Woessner, 1999). Collagen synthesis occurs intracellularly, and in general procollagen molecules of fibrillar collagen types (e.g. type II) are secreted into the extracellular space, where collagen fibrils are formed (Alberts et al., 1994; Everts et al., 1996). The formation of fibrils may not always apply to the vitreous, since Bishop et al. (1994) observed procollagens in mature bovine vitreous. In addition, Snowden et al. (1982) found more immature collagen cross-links in mature bovine vitreous than in articular cartilage, indicating a slower or incomplete maturation process and/or a more active synthesis of collagen in the former.

The observed type II collagen packages could thus be the net result of a process of interactive remodelling, in which both breakdown and synthesis of vitreous and ILL collagens take place. Phenomena suggestive of breakdown include the presence of macrophages, cell debris and focal interruptions in the ILL. A strong argument in favour of net synthesis of collagen is the observation by Wang et al. (2003) of intraretinal collagen networks expanding upon ageing. In addition they showed continuities between vitreous collagen fibres and sublaminar collagen networks. Because we used different techniques, we were only able to confirm this finding once (70 702). Since mechanical forces in general are well known for their ability to induce matrix remodelling (e.g. in bone and cartilage), we propose that vitreous base and equator remodelling, resulting in the formation of a collagenous intraretinal network, occurs in response to forces exerted upon this area, i.e. most likely vitreous movements and vitreoretinal tractional forces induced by eye movements (Hilding, 1954). In the light of eye mechanics, this remodelling could be aimed to decrease the effect of forces and strengthen the vitreous base. However, on ageing, connections between vitreous collagen and intraretinal collagen can make the (pre-)equatorial area more vulnerable to tearing and retinal detachment in the case of liquefaction and PVD. Whether Müller cells, which are closely related to the packages of vitreous collagen and the ILL, are involved in the synthesis and/or breakdown (e.g. phagocytosis) of this intraretinal collagen network, is currently under research.

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3

Collagen distribution in the human vitreoretinal interface

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Submitted

Abstract

Purpose

This study evaluates the presence of types I-VII, IX, XI, and XVIII collagen at the posterior pole, the equator and the pre-equatorial area in human donor eyes, since collagens are important macromolecules contributing to vitreoretinal adhesion at the vitreoretinal interface.

Methods

Freshly isolated human retinectomy samples from the equator were used for reverse transcriptase-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 above-mentioned collagens and evaluated by light microscopy (LM).

Results

Retinectomy samples express mRNA of all tested collagen types. By LM, vitreous cortex is positive for types II, V, IX, and XI collagen. In all three regions within the donor eyes and in the retinectomy samples, the internal limiting membrane (ILM) shows types IV, VI, and XVIII collagen, the retinal vasculature is positive for types I-VI and XVIII collagen in most specimens, and retinal layers show condensed spots of type VII collagen. In addition, type VII collagen increases in density and in distribution over the retinal layers towards the posterior pole.

Conclusions

Staining patterns of types I-V, IX, XI, and XVIII collagen are conform 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 could be involved in vitreoretinal attachment. Furthermore, the presence of collagen mRNA in human retinectomy samples might be an indication of postnatal collagen production by retinal cells.

Introduction

The vitreous body (or vitreous) of the human eye is the transparent extracellular matrix (ECM) located between the lens and the retina. The vitreous is the largest structure of the eye and consists of 98-99% 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] Collagen types present in the vitreous are types II,[7,8] V/XI,[9-11] VI,[11,20] and IX.[8,10-15]

The vitreous cortex is located 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 macula.[24] Furthermore, morphological studies revealed a regional variability in thickness of the ILM, consisting of an increase in thickness from the vitreous base towards the macular area with a thinning over the fovea, optic disc, and retinal blood vessels.[23,26-28] Finally, attachment plaques (i.e. hemidesmosomes) were 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 were found.[26,29,30] Immunohistochemical studies on ILM composition have shown the presence of the non-collagenous components laminin, fibronectin, proteoglycans, and several glycoconjugates[31,32] as well as types I, IV, and XVIII collagens.[33-35]

In the adult human retina, collagen types that have been described (starting from the photoreceptor layer to the ILM) are types I-VI[26,36,37--41] and type XVIII.[35] In retinas of non-pathological donor eyes, isolated packages of type II collagen were described in the pre-equatorial and equatorial area.[26] In several studies, type II collagen was also present in retinal blood vessels,[37,39,41] although this was not unambiguous.[38] It was also unclear whether there was preference for an anterior location as suggested in a histopathological pilot study on inherited rhegmatogenous retinal detachment.[41] Types I, III, IV, V, VI, and XVIII collagen were described as components of retinal vasculature.[35-40]

The present study focuses on the presence and distribution of types I-VII, IX, XI, and XVIII collagen 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 Transcriptase-Polymerase Chain Reaction (RT-PCR)

Four fresh retinectomy samples (70, 74, 86, and 87 years) were acquired from patients with exsudative 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 for this procedure.[42,43] Informed consent was obtained prior to surgery with the approval of the medical ethical committee in accordance with the ethical 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 from the samples was extracted by RNeasy Mini kit method (Qiagen) according to the manufacturer's instructions. To eliminate DNA contamination, RNA samples were treated with DNase treatment Ambion-kit (DNA-free). 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. 2 µg RNA was reverse transcribed into cDNA using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) according to 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 (Fermentas) and 16.25 µl milli-Q water. Finally, a total of 1 µl of the two specific flanking primers (50µM) was added (Table 1). The mixtures were initially denatured at 94°C for five minutes. The PCR consisted of 35 cycles at the following conditions: denaturation at 94°C for 0.5 minute, annealing at 55°C (for types I, II, III, V, and IX collagen) and 58°C (for types IV, VI, VII, XI, and XVIII collagen) for 1 minute, and an extension period at 72°C for 1 minute. These cycles were followed by a final extension period at 72°C of 10 minutes. 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 controls. No amplification was obtained from the water controls (data not shown).

Table 1 Primers used in the RT-PCR analyses.

Collagen	Forward Primer: 5'→3'	Reverse Primer: 5'→3'	Size
COL1A1	TCG GCG AGA GCA TGA CCG ATG GAT	GAC GCT GTA GGT GAA GCG GCT GTT	254 bps
COL2A1	GTG GAA GAG TGG AGA CTA CTG	TGT ACG TGA ACC TGC TAT TG	419 bps
COL3A1	ACC GAT GAG ATT ATG ACT TCA CT	CTG CAC ATC AAG GAC ATC TTC AG	369 bps
COL4A2	ATC GGC TAC CTC CTG GTG AA	GCT GAT GTG TGT GCG GAT GA	648 bps
COL5A1	GAC TAC GCG GAC GGC ATG GAA	CCT GCC AGG CCA CTG ACT GGT A	454 bps
COL6A1	GGA GCT CAA GGA AGC CAT CAA G	TCC TCC AGC AGC TCT GCA TAG T	342 bps
COL7A1	CCG AGG ACG AGA TGG TGA AGT TG	CTG GCT CCA GGT CCT GTG TCT AC	261 bps
COL9A1	GCC TCT GGT GAA GAA GGT GAA	TGC TGA TCT GTC GGT GCT CTA	245 bps
COL11A1	CAG CAG GCT CGG ATT GCT CTG A	GGC CAT CTA CAC CTG CCA TAC C	460 bps
COL18A1	TCT ACG TGG ACT GTG AGG AGT T	CTG CTC CTC GAC TTC TCC ACT T	380 bps

Paraffin embedding procedure

Three human eyes (3 donors) from 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, eyes were fixed by immersion within 36 hours post mortem in 2% paraformaldehyde (PF, Polysciences Inc., Warrington, U.K.) in phosphate buffered saline (PBS) overnight at 4°C. In order to achieve good penetration of the fixatives, washing, dehydration and infiltration steps were promoted by gently rotating the specimens. The eyes were washed in PBS and dehydrated by ethanols (50-100%). Finally, the specimens were embedded in paraffin. In addition, three retinectomy samples of 3 patients (74, 75(A), and 75(B) years) were put into 2% PF immediately after surgical removal and embedded in paraffin according to above-mentioned procedure.

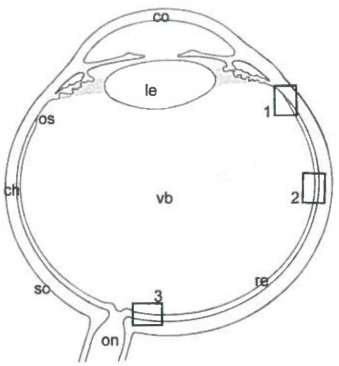

Figure 1 Schematic overview of an eye. Boxes 1, 2, and 3 indicate the pre-equatorial area, the equator and the posterior pole, respectively. Co: cornea, le: lens, sc: sclera, on: optic nerve, ch: choroid, re: retina, os: ora serrata, vb: vitreous body.

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). Slides were deparaffinised by the addition of xylene followed by short hydration steps with ethanols (100-50%). After washing with demiwater, 1% type XXIV protease (Sigma, St. Louis, USA) 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 (i) rabbit polyclonal antibodies against human types I, III, V (Abcam, Cambridge, UK), and XI collagen (a kind gift from J. Oxford, Boise State University, Boise, Idaho, USA) and against endostatin, the product of the C-terminal of type XVIII collagen (Abcam), (ii) biotinylated rabbit polyclonal antibody against human type VI collagen (Abcam), (iii) goat polyclonal antibodies against human types II and IV collagen (Southern Biotechnology Associates (SBA), Birmingham, USA), and (iv) mouse monoclonal antibodies against human types VII (Abcam) and IX collagen (USBiological, Massachusetts, USA). After washing, the peroxidised 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 washing with PBS, sections were stained with 3-amino-9-ethylcarbazole (AEC; Sigma) and hematoxylin.

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

Morphological analysis

The morphological 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 from 0 to 2 (0 was negative, 1 weakly positive, and 2 strongly positive).

Results

RT-PCR

The retinectomy samples expressed mRNA of all tested collagen types (Fig. 2). Amplimers were seen at the expected positions, which are for *COL1A1* at 254 base pairs (bps), for *COL2A1* at 419 bps, for *COL3A1* at 369 bps, for *COL4A2* at 648 bps, for *COL5A1* at 454 bps, for *COL6A1* at 342 bps, for *COL7A1* at 261 bps, for *COL9A1* at 245 bps, for *COL11A1* at 460 bps, and *COL18A1* 380 bps.

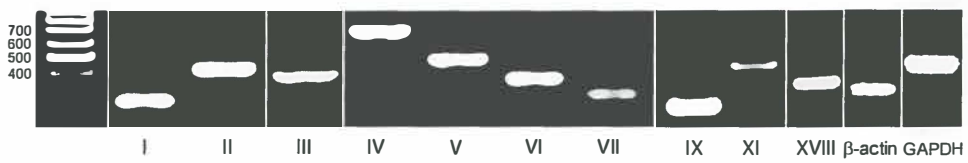

Figure 2 RT-PCR on an equatorial retinectomy sample (74 year-old patient). From left to right, bands indicating the positions of types I, II, III, IV, V, VI, VII, IX, XI, and XVIII collagen are depicted. At the left margin, a 100 bps DNA ladder is added. Human β-actin and GAPDH were positive.

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 adhering 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 only used positive or negative scores in our results. In the cases that the pre-equatorial area, the equator, and the posterior pole stained positive or negative, the intervening areas (not shown) were likewise 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 (Fig. 3B) collagen and variably for types V, IX, and XI collagen (Figs. 3E,G,H). The vitreous cortex showed no staining with the antibody against type VI collagen (Fig. 3F). The ILM was clearly positive for types IV, VI, and XVIII collagen in all three regions (Figs. 3D,F,I). The staining patterns of types IV and VI collagen at the ILM were the same. In the case of type II collagen, the ILM could not be discerned as a separate entity from the vitreous cortex (Figs. 3B,D). This was only possible at places with a local vitreous detachment. The retinectomy samples confirmed the presence of types IV and VI collagen and the absence of type II collagen in the ILM (not shown). Human corneas stained specifically for types IV and VI collagen (not shown).[44]

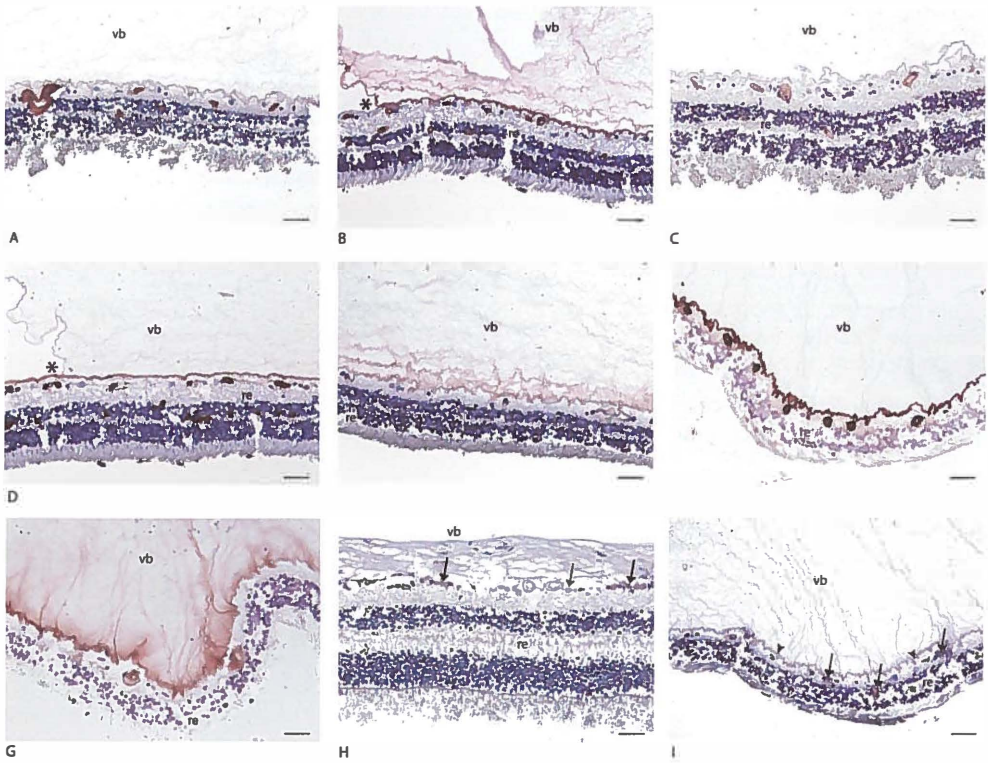

Figure 3 Immunohistochemical analyses at the equator of human donor eyes evaluated by LM. **A**: Type I collagen (55yr) is present in the smaller and larger retinal blood vessels. **B**: Type II collagen (74yr) is found in the vitreous cortex and in retinal blood vessels. The ILM shows no type II collagen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

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 types IV and VI collagen and positive for types I, II, III, and XVIII collagen and for type V collagen in two eyes (55 and 74 years; Figs. 3A-F,I). However, type IX collagen was only found 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 collagen which was not detected.

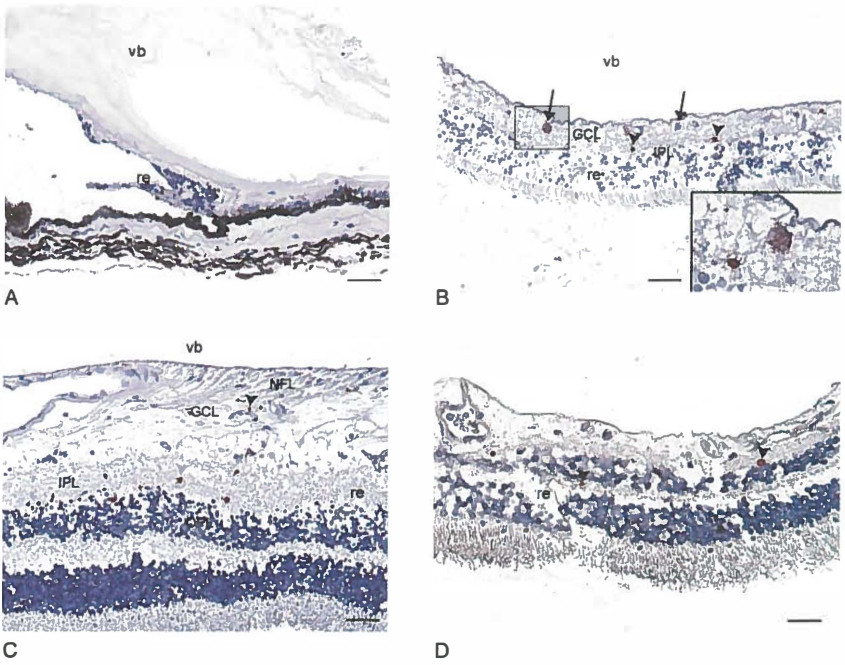

Figure 4 The distribution of type VII collagen in the 74- year-old donor eye (A-C) and in the equatorial retinectomy sample (D) is shown (results of the mouse monoclonal antibody are shown). In the pre-equatorial area (**A**), type VII collagen was not found. In the equator (**B**), type VII collagen is visible as small positive spots in the ganglion cell layer (GCL) and inner plexiform layer (IPL), whereas, in the posterior pole (**C**), type VII collagen is present in nerve fiber layer, the GCL, the IPL and the outer plexiform layer. Arrows indicate the dotted aspect and arrow heads the circular spots and the inlays show circular spots (1) and dotted spots (2) positive for type VII collagen. In the equatorial retinectomy sample (**D**), type VII collagen was also found in the outer plexiform and outer nuclear layers. Arrows indicate the spots with the dotted aspect and arrow heads the circular spots. Vb: vitreous body, re: retina. Bars = 50 µm.

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 collagen 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 collagen positive spots towards the posterior pole (Fig. 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, and INL, outer plexiform layer (OPL), and outer nuclear layer (ONL).

Discussion

By immunohistochemical staining and LM evaluation, we were able to detect 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 types II, V, VI, and XVIII collagen) and collagens which in other tissues are known to mediate anchoring of one tissue structure to another (e.g. type VII collagen). New findings in this study are that type VI collagen is located in the ILM (previously only described in retinal blood vessels)[39,45] and that type VII collagen is widely distributed in several retina layers with increasing density from the pre-equatorial area towards the posterior pole. Furthermore, type II collagen was present in human retinal vasculature and 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-VII, IX, XI, and XVIII collagen was 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,[46-49] equatorial retinal cells may be able to synthesize vitreous and ILM collagens. A comment should be made that only types II, III, VII, and XVIII collagen consist of three identical procollagens (α1) and thus types I, IV, V, VI, IX, and XI collagen may also require other chains to build up a functional triple helical molecule.[36]

As previously described, the vitreous cortex was positive for types II, V/XI, and IX collagen.[5] We did not find type VI collagen in the vitreous cortex, although this collagen

was previously been reported to be present in the vitreous.[20] The light microscopic absence of type VI collagen in the vitreous cortex and the variable presence of types V, IX, and XI might 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 to discriminate both structures is a known problem described in previous studies.[34,50] Based on specimens with a local vitreous detachment and on the retinectomy samples after vitrectomy, it was concluded that the ILM contained types IV, VI, and XVIII collagen, but not type II collagen.

Type VI collagen is essentially a glycoprotein which belongs to the non-fibril forming collagens and forms a (beaded) filamentous network in most ECMs.[51,52] It has a predominant role in linking cells and matrix macromolecules.[53,54] Type VI collagen showed specific interactions with (i) hyaluronan in calf skin, which is interesting since vitreous is also rich in hyaluronan,[55,56] (ii) the striated collagen fibers of the inner and outer layers of Bruch's,[40] (iii) types I, III, and V collagen within the scleral and the corneal stromal collagen network,[57-59] (iv) type IV collagen in Bowman's layer, [58,59] and (v) pericytes at the choroidal side of the choriocapillaris.[60] In human iris and ciliary body, type VI collagen 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.[61] From the present study, based on the widespread presence of type VI collagen throughout the ILM, we conclude that the entire vitreous is likely 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.[35,62] Based on mice studies, its function may be twofold: (i) part of an anchoring complex between the vitreous fibrillar collagens and the ILM, and (ii) responsibility for the disappearance of vitreous hyaloid vasculature in the embryonic period.[63] Thus, its presence could theoretically be associated with a protection against PVD or against vascular neovascularisation. It would therefore be interesting to study type XVIII collagen/endostatin on ageing and in diseases characterized by retinal neovascularisation.

Retinal blood vessels contained types I-VI and XVIII collagen, whereas types V and IX collagen were variably present, which is largely in agreement with previous studies on human retinal vasculature. Previous immunohistochemical studies could not uniformly confirm the presence of types II and IX collagen.[35,36,38-40] In our study, the presence of type IX collagen was very variable and needs further investigation. With regard to type

II collagen, one study[38] questioned its presence, which could be explained by the used antiserum, and another only found type II collagen in the peripheral vasculature.[41] However, Western Blot analysis on bovine retinal blood vessels, confirmed the presence of types I-V collagen, of which types II and IV collagen were prominently present.[37]

The presence of type II collagen is interesting in the light of (i) strong interconnections between vitreous and retinal vasculature[23] and (ii) the possible source of the type II collagen. As a consequence of the strong connection, vitreous hemorrhage can occur during a posterior vitreous detachment.[64] The presence of mRNA *COL2A1* in freshly isolated human retina could indicate the retina as a possible production place of type II collagen. The producing cell of type II collagen has still to be established, but Müller cells are good candidates, since they are attached to the retinal vasculature and ILM[65] and their endfeet are closely related to sublaminar intraretinal type II collagen.[34]

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).[66] The presence of type VII collagen 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),[67] 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 collagen.[68] 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.

Types VI, VII, and XVIII collagen 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.[69-72] When we focus on collagens: (i) subtypes of collagenases should be able to degrade specific collagens,[72] (ii) nattokinase can hydrolyze vitreous collagen fibers,[73] (iii) thrombin and plasmin can cleave type V collagen[74] and (iv) exogenous plasmin can activate matrix metalloproteinase-2,[75] which is capable to degrade types IV and VII collagen.[76] 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 will stop 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 (i) vitreoretinal adhesions and interface pathology, (ii) the process resulting in PVD, and (iii) the potential effects of enzymatic vitreolysis on the vitreoretinal interface and retina.

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4

In vitro phagocytosis of collagens by immortalised human retinal Müller cells

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Abstract

Purpose

This study is a first step to investigate phagocytosis of collagens by human retinal Müller cells, since Müller cells could be involved in remodelling of the vitreous and vitreoretinal interface in the human eye.

Methods

Müller cells in culture were exposed to 2.0 μm fluorescent latex beads coated with BSA and human types I, II, and IV collagen and to non-coated beads for 2, 12, 24, and 48 h. To influence phagocytosis, cytochalasin B and anti-integrin subunits (α1, α2, and β1) were added to the cells. Phagocytosis was evaluated by flow cytometry, transmission electron microscopy (TEM) and confocal microscopy.

Results

Müller cells preferred to phagocytose beads coated with type II collagen compared with type IV collagen-, BSA- and non-coated beads. Phagocytosis of type I collagen-coated beads was intermediate. TEM and confocal microscopic evaluation confirmed phagocytosis of the beads. No significant differences were observed in phagocytosis of type II collagen-coated beads in the case of addition of cytochalasin B and anti-integrin subunits. Immunohistochemical analyses revealed that Müller cells were positive, under all tested circumstances, for vimentin and CRALBP. Less than 5% of the cells tested were GFAP positive.

Conclusions

Our observations demonstrate that human Müller cells in culture prefer to phagocytose type II collagen. In contrast, the phagocytosis of type IV collagen is comparable with the control coatings. We speculate that the relatively limited collagen phagocytosis by Müller cells supports a possible role for Müller cells in the slow process of vitreoretinal remodelling in adult human eyes.

Introduction

The interface between the vitreous body and the retina of the human eye has been studied extensively to find agerelated changes that could explain vitreoretinal lesions of diverse cause. Centrifugally, the interface consists of a vitreous cortex (mainly type II collagen), retinal internal limiting lamina (ILL, mainly type IV collagen), and Müller cell endfeet (Fig. 1a,b). Foos [5] was the first to describe degenerative remodelling of the vitreoretinal juncture in the peripheral retina, which consisted of gaps in the ILL and penetration of vitreous fibrils into Müller cells. Gloor and Daicker [8] described atrophy of neuroglia in the same area, which caused intercellular gaps becoming filled with cords of collagen fibrils connecting vitreous and retina. Malecaze et al. [14] found widening of intercellular spaces filled with fibrils adhering to Müller cells in the middle part, but the lesions were smaller than described before and not associated with gaps in the ILL. More recently, Wang et al. [35] observed, mainly by scanning electron microscopy, progressive invasion of the innermost peripheral retina by bundles of collagen fibrils, initially as characteristic collagen fibres spreading out under the ILL and eventually as a dense mat of collagen in the elderly. Furthermore, collagen fibrils penetrated the ILL through localised defects and intertwined with those in the basal vitreous. Since the amount of collagen expanded on ageing, intraretinal synthesis of collagen fibrils could have occurred. Recently, our light microscopic (LM) and transmission electron microscopic (TEM) evaluation of the equatorial area gave comparable findings [24]. Intraretinal packages of vitreous collagen were closely related to Müller cells (Fig. 1c) and often associated with macrophages, cell debris and focal interruptions of the ILL. This was interpreted as the net result of a process of interactive remodelling, in which both phagocytosis and synthesis of vitreous and ILL collagens occur and in which Müller cells possibly play a role [24].

Müller cells are the principal glial cells in the retina, having similar functions to astrocytes, oligodendrocytes, and ependymal cells in other regions of the central nervous system [19]. They are radial macroglia that pass through the retina from its inner (vitreal) border to the distal end of the outer nuclear layer, and because of their cell processes, they surround neuronal cell bodies, axons and blood vessels. Müller cells have many local functions: they stabilise the retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space [19, 27]. They have also been found to phagocytose a variety of substances.

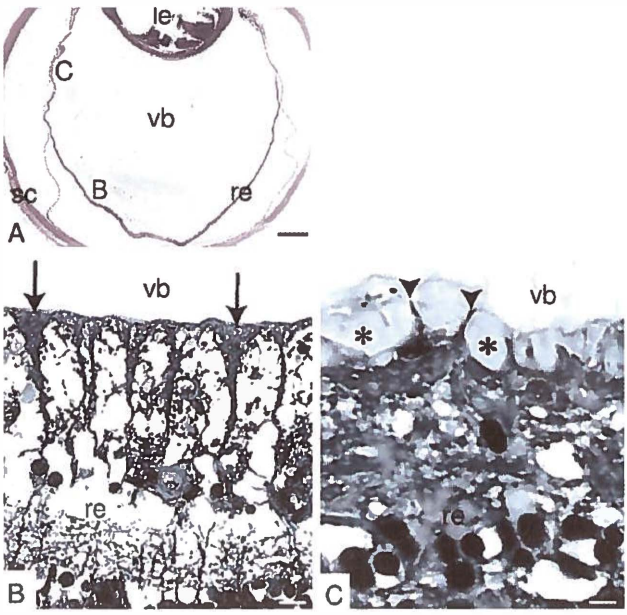

Figure 1 **a** Overview of a Technovit 8100-embedded donor eye. *Le* lens; *vb* vitreous body; *re* retina; *sc* sclera; *bar* 2.5 mm. **b** LM detail of an area in the posterior pole (B in 1a). *Arrows* indicate Müller cell endfeet subjacent to the internal limiting lamina. *Vb* vitreous body; *re* retina; *bar* 1 µm. **c** LM detail of the peripheral retina (C in 1a). Under the ILL, collagen packages (*) are located between the ILL and displaced Müller cell processes (*arrowheads*). *Vb* vitreous body; *re* retina; *bar* 1 µm.

Friedenwald and Chan [7] first described in vivo phagocytic activity by Müller cells more than 7 decades ago. Their histological study reported that Müller cells internalised pigment granules injected into the vitreous cavity of rabbits. Subsequently, it was shown that rabbit Müller cells in vivo were able to phagocytose copper, carbon, egg-lecithin-coated silicone, and erythrocyte particles [1, 18, 20, 26]. In the latter three, the ILL was thinned, interrupted or had disappeared so that their cell processes were able to make contact with the foreign bodies. Additionally, Müller cells could phagocytose both melanin granules in a rabbit model of experimental retinal detachment and erythrocytes in a rat model of subretinal haemorrhage [6, 12]. Cultured rabbit Müller cells were able to phagocytose latex beads [31]. In a goldfish model, Wagner and Raymond [34] demonstrated phagocytosis of latex beads by Müller cells in vitro, although no uptake was observed in vivo. In human fetal eyes, Müller cells phagocytosed cell debris derived from apoptotic cells in the developing retina [23]. In eyes with chalcosis, human intraretinal glial cells internalised copper particles [25]. Furthermore, they were capable of phagocytosis of cell debris, latex beads, and fluorescent carboxyl microspheres, in culture [15].

At present, it is not known whether Müller cells can phagocytose collagens in vivo

and in vitro. Since collagen phagocytosis is an important aspect in ECM remodelling under steady-state conditions [4] and might play a role in age-related vitreoretinal remodelling [24], we performed an in vitro study to test the capability of human retinal Müller cells to phagocytose collagens. The focus was on the main collagen types of the vitreous and the ILL, types II and IV collagen, respectively. The main goal was to determine whether human Müller cells can phagocytose the abovementioned collagens and if they have a preference for either one of them. Furthermore, we tried to influence the phagocytosis process.

Table 1 Overview of the Müller cell experiments[1]

Müller cells				
Characteristics (LM)		Flow cytometry	CM	TEM
General	Integrins	1) Time-related phagocytosis (2, 12, 24, 4 h); col II-, IV-, BSA-, non-coated beads *	Internalisation of col II-coated beads by Müller cells stained with anti-vimentin, anti-GFAP, and DAPI	Confirmation internalised col II, IV, BSA, non-coated beads
Anti-vimentin	Anti-α1	2) Phagocytosis of col I and II (24 h); col I-, II-, BSA-coated beads *		
Anti-CRALBP	Anti-α2			
Anti-GFAP	Anti-β1			
Cell viability	Controlcells: HBL-100 Fibroblasts	3) Blocking of phagocytosis (24 h): a) Cytochalasin B: col II-, BSA-coated beads b) Anti-integrins: col I-, II-, IV-, BSA-coated beads		

*[1]LM light microscopy; TEM transmission electron microscopy; CM confocal microscopy; Col collagen; and * : fibroblasts used as controls.*

Materials and methods

Culture of Müller cells

The human Müller cells, MIO-M1, were kindly provided by G.A. Limb (Moorfields/Institute of Ophthalmology, London, UK). The MIO-M1 cells were very well characterised as human retinal Müller cells [13]. The cells were cultured to confluence in Dulbecco's modification of Eagle's medium (DMEM) high glucose containing L-glutamax I (Life Technologies Inc., Rockville, USA), 10% fetal bovine serum (FBS; Life Technologies Inc.) and 1% penicillin/streptomycin. Table 1 gives an overview of all experiments performed with the Müller cells.

Culture of control cells

Human fetal lung fibroblasts (primary cell line) were used in most experiments as a control

for our phagocytosis model. The cells were cultured till confluence in DMEM containing 10% FBS, 1% glutamin, and 1% gentamicin.

Characteristics of Müller cells

The Müller cells were initially characterised during each passage, but after five passages with similar results, only every fifth passage was identified. Their characteristics were also evaluated after the different phagocytic assays. Cells were checked for their morphology and their main characteristics: expression of glial fibrillary acidic protein (GFAP), cellular retinaldehyde binding protein (CRALBP) and vimentin. To determine the presence of integrins, Müller cells were stained with antibodies against integrin subunits α1, α2, and β1. Cell viability was checked routinely after our experiments (see Phagocytosis assays).

Cells were seeded for 48 h in glass chamber slides. Müller cells were fixed, after washing with phosphate buffered saline (PBS), with 1:1 acetone/methanol for 10 min at –20°C. After fixation, cells were washed with PBS and preincubated for 30 min with 2% serum of the producer of the secondary antibody in PBS with 1% BSA (Sanquin, Amsterdam, the Netherlands), followed by incubation for 1 h with primary antibodies diluted 1:100 in PBS with 1% BSA. The primary antibodies included a rabbit polyclonal anti-CRALBP antibody (UW55, a kind gift from J.C. Saari, University of Washington, Seattle, Wash., USA) and mouse monoclonal antihuman antibodies against vimentin (DAKO, Glostrup, Denmark), GFAP (Sigma, St Louis, USA), and integrin subunits α1, α2, and β1 (Chemicon, Temecula, USA). After incubation and washing with PBS, endogenic peroxidases were blocked. In the case of integrin subunit staining, this step was followed by blocking steps with avidin and biotin. Secondary antibodies included a swineanti-rabbit peroxidase (SARPO; DAKO), a rabbit-anti-goat peroxidase (RAGPO; DAKO), a rabbit-anti-mouse peroxidase (RAMPO; DAKO) and a goat-anti-mouse-biotin (GAMbio, DAKO). Secondary antibodies were diluted 1:100 with PBS and 2% human serum and added for 1 h at room temperature. GAMbio was followed by incubation with ABC complex HRP (DAKO). Finally, cells were stained with 3-amino-9-ethylcarbazole (AEC; Sigma) and haematoxylin. The human fibroblasts were also tested for integrin expression. In preliminary studies, we tested the specificity of the anti-integrin subunits on human normal mammary cells, HBL-100 ([28]; a kind gift from A. Sonnenberg, NKI Institute, Amsterdam, The Netherlands). Negative control sections underwent the entire procedure except for substitution of the primary antibody. In addition, MOC31, a monoclonal biotinylated antibody against the human epithelial glycoprotein-2 and irrelevant to Müller cells, was used to determine antibody specificity [17].

Coating of beads

The latex beads used were 2.0 μm Fluoresbrite yellow-green microspheres (Polysciences, Warrington, Pa., USA). The coating of the beads was performed according to previously described methods [16, 22, 36]. Beads were incubated with (i) 0.20 μg/ml bovine serum albumin (BSA; Sigma) dissolved in 0.5 M acetic acid, (ii) the solution fluid 0.5 M acetic acid (non-coated) and (iii) 0.20 μg/ml human type I (Southern Biotechnology Associates (SBA), Birmingham, USA), type II (Biogenesis, Poole, UK), and type IV (SBA) collagens (dissolved in 0.5 M acetic acid), at 37°C for 75 min. After incubation, the beads were centrifuged for 5 min at 10,000 rpm and washed with PBS.

Collagen coatings were evaluated, at different time points, by immunohistochemical staining. The type I, II and IV collagen-coated beads were incubated with goat polyclonal antibodies against human types I, II, and IV collagen (SBA) diluted 1:50 in PBS with 1% BSA-c (Aurion, Wageningen, The Netherlands) at 37°C for 1 h. The beads were washed with PBS and incubated with a TRITC-labelled rabbit antigoat antibody (Sigma) diluted 1:100 in PBS with 1% BSA-c for 30 min at room temperature. Beads were evaluated with a Leica DC300F (Rijswijk, The Netherlands). Immunohistochemical analysis showed collagen coating of beads (Fig. 2).

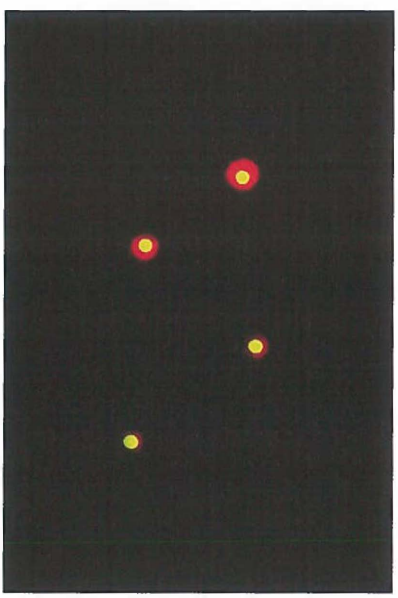

Figure 2 Immunohistochemical staining (*red*) of type II collagen-coated beads (*green*). All other collagen coatings gave similar staining patterns.

Phagocytosis assays

To study the phagocytosis of coated beads by human retinal Müller cells and fibroblasts, cells were seeded in 6-well plates (6.5×10^5 Müller cells/well and 9.0×10^5 fibroblasts/well) with medium containing 10% FBS for 24 h at 37°C. Thereafter, cells were washed and received medium with 5% FBS, which was replaced after 24 h by medium supplemented with 4.0 mg/ml BSA. After 24 h serum-free incubation, beads were vortexed and briefly sonicated to separate bead clumps in order to calculate the bead concentration with a Bürker-Türker counter. In every experiment, five beads per cell were added to each well. Müller cells were used in all assays mentioned below, while fibroblasts were only used in the experiments of time-related phagocytosis and phagocytosis of types I and II collagen.

Time-related phagocytosis

After incubation periods of 2, 12, 24, and 48 h with BSA-, non- and type II and IV collagen-coated beads, cells were washed 3 times with PBS and trypsinised with trypsin-EDTA (0.5% trypsin and 0.2% EDTA; Sigma) for 8 min. By this procedure, non-internalised beads were removed from the cell membrane [9, 16, 22]. The cells were then centrifuged for 5 min at 1,600 rpm. For flow cytometry analysis, cell pellets were dissolved, resuspended in 200 µl PBS, and measured. Cells were categorised into one of the following groups: cells with one, two, three or more than three beads.

Phagocytosis of types I and II collagen

After the time-related phagocytosis, we compared phagocytosis of beads coated with types I and II collagen with phagocytosis of BSA-coated beads after incubation for 24 h.

Blocking of phagocytosis

For blocking experiments with (i) cytochalasin B (concentrations up to 0.6 µg/ml) and (ii) antibodies against human integrin subunits $\alpha1$, $\alpha2$, and $\beta1$ (5.0 µg/ml as used by Zhao et al. [36]), Müller cells were seeded in 6-well plates (i) and in 24-well plates (ii), respectively, for 24 h. Beads were coated with type II collagen and BSA (i) and with BSA and types I, II, and IV collagen (ii), respectively.

Cell viability

In order to determine cell viability, Müller cells and their medium were harvested after passing the above-mentioned phagocytic assays with an incubation period of 24 h. A

well with 10% FBS served as a reference and was compared with a well with 0% FBS supplemented with 4.0 mg/ml BSA with or without addition of stimuli. Dead cells were stained with trypan blue, counted in a Bürker-Türker counter, and compared to viable cells.

Flow cytometry

In all assays, the quantity of phagocytosis was determined by flow cytometry using an Epics-Elite (Coulter), with 488 nm excitation (laser power 15 mW) and a 525 nm band-pass filter in the emission path. Forward and side scatter characteristics were used to gate the intact cells from dead cells, cell debris, and free beads. To set the gate in each experiment, untreated Müller cells and fibroblasts were used, and, in general, for a flow cytometry run at least 20,000 cells were gated and in the case of the blocking experiments at least 5,000 cells. Results are presented as percentage of cells containing beads.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to verify that Müller cells and fibroblasts had really phagocytosed the different beads. After culturing Müller cells and fibroblasts for 24 and 48 h in the presence of coated beads (BSA and types II and IV collagen) and noncoated beads, the medium was discarded and cells were washed with PBS. Cells were fixed for 24 h with 2% glutaraldehyde (GA, TAAB Laboratories, Aldermaston, UK) at 4°C. Then, cells were washed with PBS and 6.8% sucrose (pH 7.4), postfixed at 4°C in 1% osmiumtetroxide (OsO_4) dissolved in 1.5% potassium hexacyanoferrate(II) trihydrate (Merck, Darmstadt, Germany) in 0.1 M PBS, washed with distilled water, dehydrated through a graded series of ethanol, and embedded in EPON 812 (Serva Feinbiochemica, Heidelberg, Germany).

Ultrathin sections were cut on a Sorvall microtome (Sorvall, Newtown, Conn., USA) and contrasted with uranyl acetate and lead citrate and evaluated in a Philips 201 TEM (Philips, Eindhoven, The Netherlands) operated at 60 kV.

Confocal microscopy

Müller cells were exposed to beads coated with type II collagen for 24 h, fixed with methanol/acetone at –20°C for 10 min and stained with anti-vimentin and anti-GFAP antibodies (see Characteristics of Müller cells) for confocal microscopic evaluation. Trypsinisation to remove adherent beads was not possible in this experiment, since this would also detach the cells. The secondary antibody was a TRITC-labelled antimouse antibody (Sigma) and nuclei were visualised with 4′,6′-diamino-2-phenylindole (DAPI; Sigma). Cells were analysed

by a LEICA TCS SP2 3channel confocal laser scanning microscope, equipped with lasers providing 488 nm and 543 nm laser lines for FITC and TRITC visualisation, respectively. DAPI-stained nuclei were visualised with UV laser and corresponding UV optics. Stack sections were chosen to obtain a z-resolution of 0.5 µm or less.

Surface charge of coated beads

In order to evaluate an effect of surface charge, the zeta potential, a measure for surface charge, of coated and noncoated beads was determined at room temperature by particulate microelectrophoresis. Beads with different coatings were suspended in 30 ml medium with 0% FBS supplemented with BSA 4.0 mg/ml to a concentration between five and 10 million beads per ml. The electrophoretic mobilities were measured at 150 V in a Lazer Zee Meter 501 (PenKem, USA) and converted into apparent zeta potentials according to the Helmholtz-Smoluchowski equation [11].

Statistical analysis

Each assay was repeated at least 3 times. The percentage of cells that phagocytosed beads was globally analysed using analysis of variance (ANOVA) to study whether significant differences were present between the coatings (assays 1 and 2), between incubation times (assay 1), and between the different anti-integrin subunits (assay 3). The corresponding interactions were also taken into account in the various assays. In the case of a global significance, post hoc tests were performed to specify the differences between pairs of coatings, incubation times (for each coating separately), or anti-integrin subunits. For the time-related phagocytosis (assay 1), the number of internalised beads was tested by means of linear regression to evaluate whether the percentage of cells with a specified number of beads phagocytosed increased linearly with incubation time. This was done separately for one, two, three, or more than three beads per cell. Differences were considered significant if $P<0.05$.

Results

Light microscopy and immunohistochemistry

LM results revealed that the beads were coated with their respective collagens (not shown), that 90% of the Müller cells were viable under all conditions of this study, and that they preserved their morphology and characteristics in the different phagocytic assays.

Approximately 100% of the cells stained positive for vimentin and CRALBP and less than 5% of the cells were GFAP positive (Fig. 3a–c). All Müller cells were positive for integrin subunits α1 and α2 and weakly positive for subunit β1 (Fig. 3d–f). Fibroblasts were also positive for these integrin subunits (not shown). All control sections (i.e. without primary antibody and sections stained with MOC31) were negative (not shown).

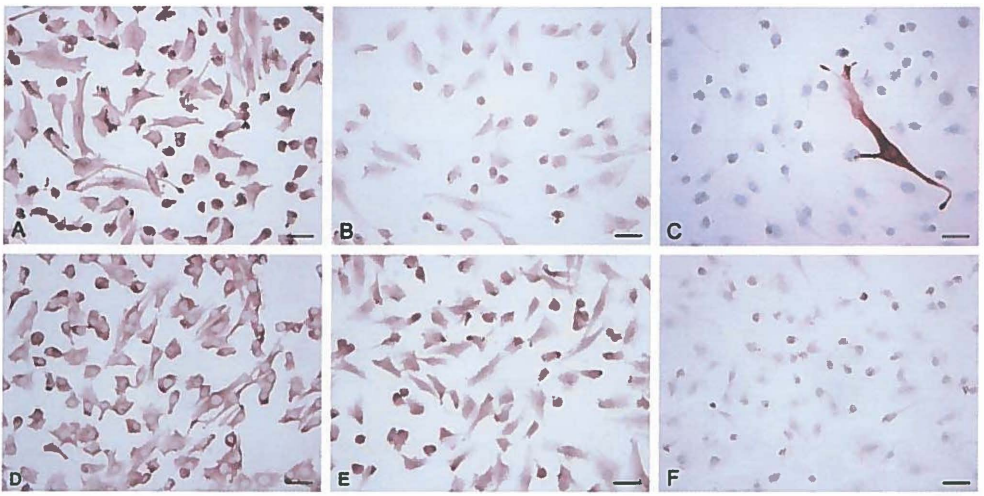

Figure 3 Immunohistochemical analysis of Müller cells shows positive staining for (**a**) vimentin, (**b**) CRALBP, (**c**) GFAP (<5%), (**d**) integrin subunit α1, (**e**) integrin subunit α2, and (**f**) integrin subunit β1. *Bars* 30 μm.

Phagocytosis assays

Time-related phagocytosis

Müller cells (Fig. 4a) and fibroblasts (Fig. 4b) were exposed to non-coated beads and beads coated with BSA or human types II and IV collagen for 2, 12, 24, and 48 h. The phagocytosis by fibroblasts is about 10 times higher compared with Müller cells. In the case of Müller cells, the phagocytosis of type II collagen-coated beads increased from an average of 1.9% (±SD of 0.5%) after 2 h to 6.5% (±1.4%) after 48 h, while the average of the other coatings, including type IV collagen, remained lower than 3% after 48 h. The percentage of phagocytosing cells significantly differed between incubation times for the collagen coatings ($P=0.004$ and $P=0.036$ for type II and type IV collagen, respectively) and suggestively for BSA ($P=0.054$), which is visible as an increase in time, and also between the coatings ($P<0.001$). Post-hoc tests on differences between phagocytosis of differently coated beads indicated that uptake of type II collagen-coated beads differed from type IV

collagen, BSA, and non-coated beads, but that no difference was observed between the type IV collagen, BSA, and non-coated beads. These findings imply that the difference between coatings was completely caused by type II collagen. Significant increases in phagocytosing cells incubated with type II collagen-coated beads were found between 2 and 24 h (P=0.007) and between 2 and 48 h (P=0.008).

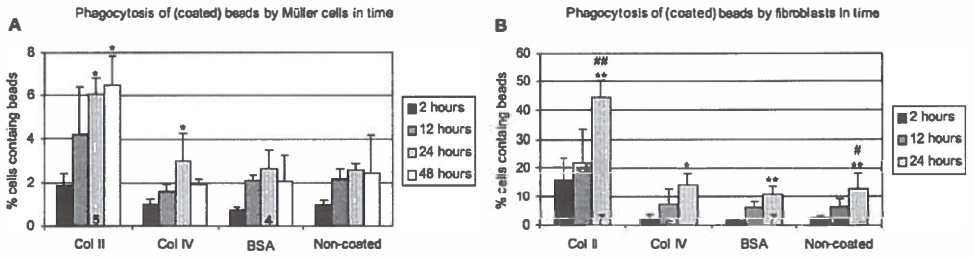

Figure 4 Percentages of cells containing beads coated with type II collagen, type IV collagen and BSA and non-coated beads per incubation time of 2, 12, 24, and 48 h. **a** Müller cells. **b** Fibroblasts. * $P<0.05$ and ** $P\leq0.005$ compared with 2 h and # $P<0.05$ and ## $P<0.005$ compared with 12 h. *Col* collagen. *Bars* indicate the SD of three measurements unless indicated separately with a number in the bar. Note scale difference between **a** and **b**.

Fibroblasts (Fig. 4b) also showed a significant increase in phagocytosis up to 24 h: from an average of 16.0% (±7.8%) after 2 h to 44.6% (±5.8%) after 24 h for type II collagen coated beads (P=0.001). Because fibroblasts died of serum deprivation after 48 h, we excluded that time-point. Phagocytosis of beads coated with type IV collagen was 14.2% (±4.0%) after 24 h and comparable with BSA- and non-coated beads with 10.7% (±3.2%) and 13.0% (±2.7%), respectively.

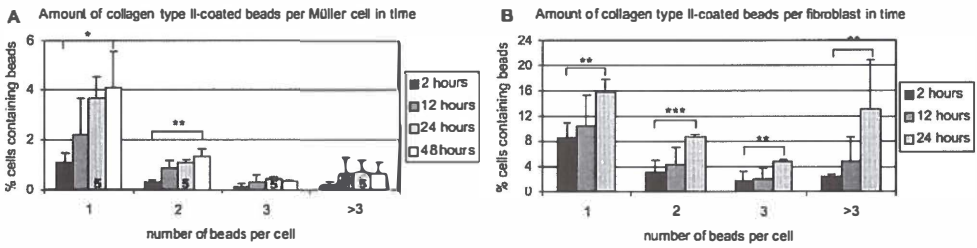

Figure 5 Percentages of cells containing type II collagen-coated beads divided in groups with 1, 2, 3, or >3 beads per cell per incubation time of 2, 12, 24, and 48 h (latter only for Müller cells). **a** Müller cells. **b** Fibroblasts. * P<0.05; ** $P<0.005$; and *** $P<0.0005$. *Bars* represent the SD of three measurements unless indicated separately with a number in the bar. Note scale difference between **a** and **b.**

From the spikes (representing the number of beads per cell) obtained by the flow cytometry (not shown), the number of internalised type II collagen-coated beads

per cell were evaluated. Most cells contained one bead and with ongoing incubation time, we observed an increasing percentage of Müller cells with internalised beads for one and two beads per cell and of fibroblasts for one, two, three, and more than three beads per cell (Fig. 5a,b).

Phagocytosis of types I and II collagen

This second assay was performed to evaluate whether the Müller cells (Fig. 6a) and fibroblasts (Fig. 6b) had a preference for types I or II collagen. Beads coated with types I and II collagen were compared with BSA-coated beads and the cells were analysed after an incubation period of 24 h. Müller cells phagocytosed 4.6% (±1.0%) type I collagen-, 6.0% (± 1.1%) type II collagen- and 3.0% (±1.0%) BSA-coated beads. Significant differences between the coatings were demonstrated globally (P=0.001) and between BSA and type II collagen coating (P=0.001) specifically (between BSA and type I collagen coating P=0.067 and between type I collagen and type II collagen coating, P=0.10). As far as fibroblasts are concerned, 67.9% (±8.3%) of these cells phagocytosed type I collagen-, 73.3% (±3.5%) type II collagen-and 18.5% (±1.2%) BSA-coated beads. Between the two collagen coatings, no significant difference was observed, but BSA-coated beads differed significantly from type I collagen (P<0.001) and type II collagen (P<0.001). These data showed that phagocytosis of beads by fibroblasts proved to be 8–12 times higher than by Müller cells.

Fibroblasts showed a higher phagocytosis for type II collagen and BSA in this assay than in the time-related phagocytosis assay, but the ratio between type II collagen and BSA remained comparable at 3.96 and 3.63, respectively (Figs. 4 and 6).

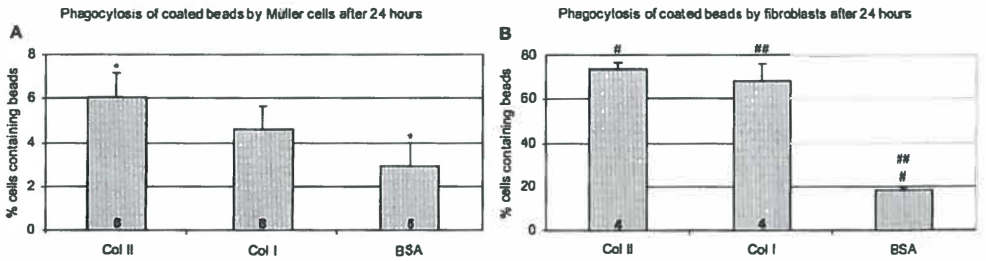

Figure 6 Percentages of cells containing BSA-, type I collagen-, and type II collagen-coated beads after an incubation time of 24 h. **a** Müller cells. * *P*=0.001. **b** Fibroblasts. # *P*<0.001 and ## *P* <0.001. *Col* collagen. *Bars* represent the SD of three measurements unless indicated separately with a number in the bar. Note scale difference between **a** and **b**.

Blocking of phagocytosis

In the two blocking assays, no significant differences were observed. In the case of anti-integrin subunits, percentages of phagocytosis were low in combination with relatively large standard deviations (not shown). In the case of cytochalasin B, the suggested dose for an effect (2.0– 5.0 µg/ml [3, 22]) was very toxic in our model. The highest concentration not affecting Müller cell viability was 0.6 µg/ml. However, at this concentration, phagocytosis was not notably influenced (not shown).

Transmission electron microscopy and confocal microscopy

TEM analyses showed beads internalised in Müller cells (Fig. 7) and fibroblasts. Beads were found within membrane-bound vacuolar structures in the cytoplasm.

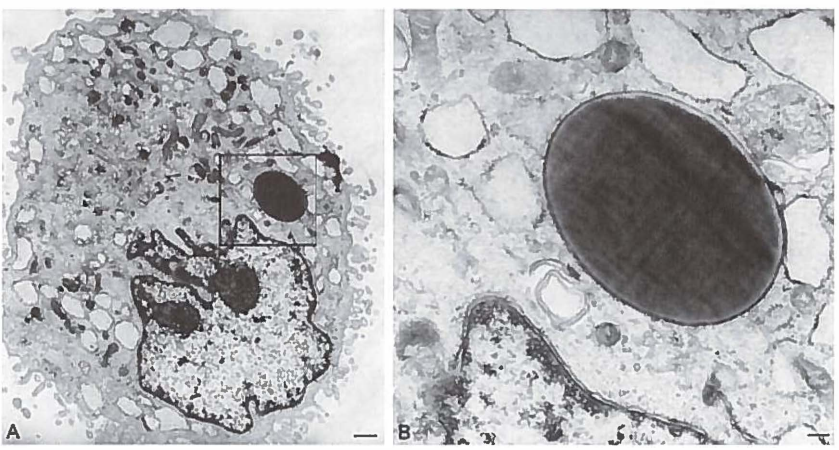

Figure 7 **a** Transmission electron micrograph showing a phagocytosed type II collagen-coated bead in the cytoplasm of the Müller cell after 24 h incubation. *Bar* 1 µm. **b** Detail: the bead is surrounded by an electron dense membrane, indicating its position inside a phago-lysosome. *Bar* 200 nm.

For confocal microscopy, Müller cells were fixed in situ and stained according to the above-mentioned protocol. Washing without trypsinisation as used in this experiment caused most of the beads to remain in situ lying on the cell surfaces. In all, 100% of the Müller cells were positive for vimentin and less than 5% positive for GFAP. To visualise whether beads had also been taken up by the cells, cells positive for vimentin within a (random) microscopic field (50 cells) were closely examined for the possible internalisation of beads by scanning from the top of the cells facing the medium to the bottom of the well (Fig. 8a–h). Within this field, two cells contained one intracellular bead each and

one cell contained two. Only five cells positive for GFAP were found and analysed. The GFAP-positive cells seemed to be detaching from the bottom of the well. These cells contained no beads.

Surface charge of coated beads

BSA-coated beads had the most negative zeta potential around –27 mV. All Collagen coatings were comparable in the range from –5to –10 mV.

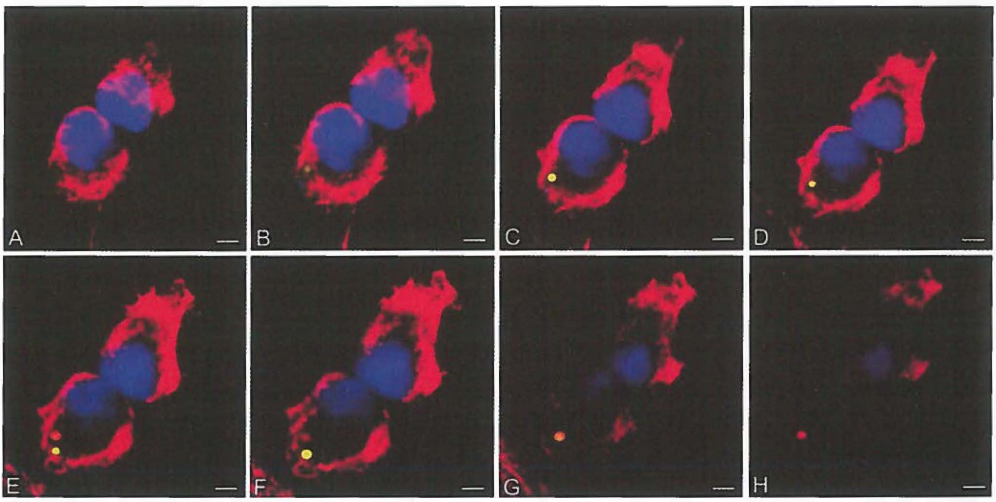

Figure 8 Confocal microscopic pictures (**a–h**) of two human Müller cells incubated for 24 h with type II collagen-coated beads (*yellowgreen*). The cytoplasm is stained with anti-vimentin (*red*) and the nucleus with DAPI (*blue*). Scanning through the cell (**a**=cell surface and **h**=cell bottom) two internalised beads will appear. First, one bead is visible (**b–d**), then both (**e** and **f**), and finally only the second bead and its scattering (**g** and **h**). *Bars* 5 µm.

Discussion

This study shows phagocytosis of type II collagen by human retinal Müller cells in a fluorescent latex bead model. Comparing all experiments, cultured Müller cells phagocytose beads coated with type II collagen better than beads coated with BSA or types I and IV collagen and noncoated beads. These findings may be adduced in support of the hypothesis that Müller cells could be involved in the slow process of vitreoretinal remodelling, which has been described as interactive or degenerative remodelling, predominantly in the peripheral retina, [5, 8, 14, 24, 33, 35] and which is very slowly progressive on ageing [33, 35]. In

a lifetime, the width of the vitreous base increases only with approximately 2 mm [35]. Vitreous liquefaction has been described from the age of 4 [2], and O'Malley [21] reported that 45% of people between 60 and 69 years old had at least 50% liquefaction.

A phagocytic model for ECM components was chosen, because phagocytosis is important during remodelling processes under steady-state conditions [4]. In our model, Müller cells preserved their immunohistochemical and morphological characteristics, as described by Limb et al. [13], although serum was withdrawn from the medium. Previously, the model with collagen-coated beads had successfully been used in phagocytosis studies with retinal pigment epithelial cells and fibroblasts [16, 22, 36]. In the present study, collagens attached well to the fluorescent latex beads as observed by immunohistochemistry. Phagocytic experiments were reproducible both quantitatively by flow cytometry and qualitatively by TEM and confocal microscopy. By TEM, membranes in the cytoplasm of the Müller cell were clearly visible around beads, indicating that they are located within phagosomes. When results of the confocal microscopy and flow cytometry were compared, the effect of trypsin on detaching adherent beads was obvious. Without trypsin treatment as in the confocal microscopic evaluations, almost every Müller cell showed beads attached to the cell membrane, but only three cells (out of 50) had internalised beads. After trypsinisation, flow cytometry showed a comparable 6% phagocytosis, confirming that effective removal of cell membrane adherent beads had taken place and that thus our results were not disturbed by adherent beads.

The phagocytosis of all types of beads by human Müller cells is about 10 times lower compared with human fibroblasts. One of the main functions of fibroblasts is phagocytosis of ECM components to remodel ECMs [4, 32]. The clear difference in phagocytosis between the two cell types indicates a lower phagocytic capacity of Müller cells. The latter might not be unexpected since the highest amount of intracellular collagen is found in those tissues, which are characterised by a rapid collagen turnover (i.e. the periodontal ligament and gingiva [4]). Lower levels are found in tissues with a slower turnover such as skin [32]. In the case of vitreoretinal remodelling, the intracellular collagen level is not known, but is probably even lower. Currently, the rate of phagocytosis by Müller cells in vivo is not known. Three in vivo studies describe phagocytosis by Müller cells after 1 week: two following particle injection in the vitreous [18, 20] and one after retinal detachment [6].

Fibroblasts in the assay of phagocytosis of types I and II collagen showed a higher phagocytosis for type II collagen and BSA than in the time-related phagocytosis assay, although the ratio between type II collagen and BSA remained comparable. Because a primary cell line of fibroblasts with a limited number of passages was used, these cells

were defrosted several times to acquire new cells which probably led to differences in the amount of phagocytosis. This phenomenon was not seen in our experiments with Müller cells, which came from a immortalised cell line [13].

The assays of the time-related phagocytosis and the phagocytosis of types I and II collagen showed that Müller cells preferentially phagocytosed type I and II collagen-coated beads. We should, however, remark that the difference between phagocytosis of type I collagen- and BSA-coated beads was only suggestive in our data set (P=0.067). These results led to the hypothesis that the phagocytosis was receptor mediated. Integrins were a likely candidate since different integrin subunits (α1, α2, and β1) can be involved in binding of cells to types I, II, and IV collagen [29, 30]. In addition, Müller cells expressed the integrin subunits α1, α2, and β1, which is in accordance with human Müller cells described by Guidry et al. [10]. Unfortunately, we were not able to show significant differences in phagocytosis by blocking integrin subunits α1, α2, and β1 by adding adequate concentrations of anti-integrins [36] and thus not able to prove the involvement of integrins. Furthermore, cytochalasin B, which can interfere with microfilament polymerisation, had no effect. Cytochalasin B was lethal to Müller cells in the previously published doses [3, 22]. Possibly, its effect diminished by decreasing the concentration. Finally, the difference between the phagocytosis of BSA-coated and collagen-coated beads by both Müller cells and fibroblasts can be explained by differences in surface charge. BSA-coated beads are more negatively charged than the collagen-coated beads, which may cause repulsion between the beads and the also negatively charged cells. The difference in the phagocytosis between the different collagen-coated beads cannot be explained by the zeta potentials because they are similar. In conclusion, the exact mechanism for the differences in collagen phagocytosis still needs to be established.

We speculate that the relatively low percentage of type II collagen phagocytosis by Müller cells observed in our in vitro study may support a role of Müller cells in the slow process of vitreoretinal remodelling in adult human eyes. Whether these cells also prefer phagocytosis of type II collagen in vivo, needs further confirmation.

Acknowledgements

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5

Human retinal Müller cells synthesize collagens of the vitreous and vitreoretinal interface in vitro

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Abstract

Purpose

To investigate the capacity of cultured Müller cells to synthesize collagens, since previous studies indicated that Müller cells could be involved in collagen remodeling at the vitreoretinal border in adult human eyes.

Methods

Spontaneously immortalized cultured human Müller cells were analyzed for the presence of mRNA of types I-VII, IX, XI, and XVII collagen by RT–PCR. Furthermore, Müller cells were immunocytochemically stained for light microscopic (LM) evaluation of these collagens and their main characteristics. Finally, cell extracts and culture medium were evaluated by western blot (WB) analysis using anticollagen antibodies.

Results

Cultured Müller cells contained mRNA for types I-VII, IX, and XI collagen, but not for type XVII collagen. LM and WB confirmed the intracellular expression of all the above-mentioned collagens with the exception of type XVII. Collagen secretion into the medium was established for types I-VII, IX, and XI collagen.

Conclusions

Cultured Müller cells can synthesize internal limiting lamina and vitreous collagens. Possible collagen production by Müller cells could explain and expand on previous in vivo morphological findings in the embryonic and postnatal period and in pathologic conditions.

Introduction

Müller cells are radially oriented macroglia that traverse the retina from its inner (vitreal) border to the outer limiting membrane. These cells have many local functions: they stabilize the retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space [1,2]. In vivo and in vitro, Müller cells can produce and express several cytokines, growth factors, and receptors [2]. Other features of Müller cells are the expression of cellular retinaldehyde binding protein (CRALBP), vimentin, and, on activation, glial fibrillary acidic protein (GFAP) [3,4]. A possible role in the production of vitreous macromolecules during growth and in adulthood has been suggested [5,6], but little is known about their capability to produce vitreous and internal limiting lamina (ILL) collagens postnatally. Whether Müller cells are capable of producing basement membrane components is a matter of debate. Some studies find evidence hereof [7-12], while others fail to confirm it [13-15].

Recently, turnover and remodeling of vitreous collagen was described in human donor eyes. Evidence for collagen breakdown in matrix areas bordering liquefied spaces was found in the human vitreous [16]. In addition to collagen breakdown, a study on vitreous collagens and two studies on the vitreoretinal interface found evidence of postnatal collagen synthesis in the human eye. The first detected type II procollagens in the vitreous [17] and the latter two described intraretinal fibers and isolated packages of vitreous collagen (type II) [18,19]. On aging, intraretinal collagen fibers expanded under the ILL at the vitreous base into networks and made contact with the basal vitreous, leading to the formation of vitreoretinal collagen connections [18]. We observed comparable intraretinal packages of vitreous collagen, often associated with surrounding Müller cell processes, focal interruptions of the ILL, and the presence of macrophages and cell debris. These findings could be consistent with a process of interactive remodeling with a net synthesis of vitreous collagens. Because of their close proximity to collagen packages, Müller cells may be involved in this process of matrix remodeling [19].

The present study evaluates the in vitro capacity of the human Müller cell line, MIO-M1 [4], to synthesize (1) known vitreous collagens (i.e., types II, V/XI, VI, and IX) [20], (2) ILL collagens (types IV and VI; unpublished data of our group) [19,21], (3) type VII collagen [22] (which appears to be present in the human retina by immunohistochemical staining; unpublished data of our group), (4) collagens described in epiretinal and vitreoretinal membranes (types IV) [23,24], and (5) a collagen not related to the vitreoretinal interface

(the hemidesmosomal transmembrane type XVII collagen found in basement membranes of stratified and pseudostratified epithelia) [25]. In this in vitro model, we demonstrate the capacity of Müller cells for collagen production, cytoplasmic expression of collagens, and their secretion into the cell medium.

Table 1 Overview of all primers used in the RT-PCR analysis

Collagen mRNA	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
COL17A1	ATG GAG CTG CTC ATC ATG AC	AGG AGT AGC AGC CAG GTG AG	364

COL1A1 indicates mRNA of the α1 chain of type I collagen.

Methods

Culture of cells

The spontaneously immortalized human Müller cell line MIO-M1 (a kind gift of G.A. Limb, Moorfields/Institute of Ophthalmology, London, UK) has all the characteristics of human retinal Müller cells [4]. The cells were cultured to confluence in Dulbecco's modification of Eagle's medium (DMEM) high glucose containing L-glutamax I (Life Technologies Inc., Rockville, MD), 10% fetal bovine serum (FBS; Life Technologies Inc.) and 1% penicillin/streptomycin.

For western blot (WB) analyses of the supernatant, Müller cells were cultured in DMEM high glucose containing L-glutamax I without FBS and supplemented with 1% G5 (Life Technologies Inc.), 0.2 mM ß-aminopropionitrilefumurate salt (ß-APN; Sigma, St. Louis, MO), 0.2 mM ascorbic acid (Sigma), and 1% penicillin/streptomycin, since 10% FBS caused clotting of the medium after our concentration procedure. The serum-free medium with supplements was introduced after 24 h to allow uniform attachment of the Müller cells. Ascorbic acid promotes the intracellular hydroxylation of prolyl and lysyl residues during collagen synthesis [26], whereas ß-APN inhibits the enzyme lysyl oxidase in the extracellular space thus preventing collagen cross-link formation [27].

Reverse transcriptase-polymerase chain reaction

Total RNA from the Müller cells was extracted by RNeasy Mini kit method (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. To eliminate DNA contamination, we treated RNA samples with DNase treatment Ambion-kit (DNA-free). RNA concentration and purity were determined on a spectrophotometer (Nanodrop, Isogen, Maarssen, the Netherlands) by calculating the ratio of optical density at wave lengths of 260 and 280 nm. Two µg RNA was reverse transcribed into cDNA using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) according to manufacturer's protocol for a total reaction of 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 (Fermentas) and 16.25 µl milli-Q water. 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 min. The PCR consisted of 35 cycles at the following conditions: denaturation at 94 °C for 0.5 min, annealing at 55 °C (for types I, II, III, V, and IX collagen) and at 58 °C (for types IV, VI, VII, XI, and XVII collagen) for 1 min, and an extension period at 72 °C for 1 min. These cycles were followed by a final extension period at 72 °C of 10 min. PCR products were analyzed by agarose gel electrophoresis (1%) with 500 ng/ml ethidium bromide. Keratinocytes were used as positive control for type XVII collagen.

Immunocytochemistry

By light microscopy (LM), Müller cells were identified by their morphology and by their expression of CRALBP, vimentin, and GFAP. The expression of cellular characteristics was measured in at least three microscopic areas at a magnification of 10 times. To determine the intracellular expression of collagens, we specifically stained Müller cells with antibodies against human types I-VII, IX, XI, and XVII collagen.

For immunocytochemical staining, cells were seeded for 48 h in glass chamber slides. After fixation with 1:1 acetone/methanol for 10 min at -20 °C, the slides were washed with phosphate buffered saline (PBS) and pre-incubated for 30 min with 3% serum of the producer of the secondary antibody in PBS with 2% BSA (Sanquin, Amsterdam, the Netherlands), followed by incubation for 1 h with primary antibodies diluted 1:50 in PBS with 1% BSA. In the case of types VI, VII, IX, and XI collagen, the latter step was preceded by blocking steps with avidin and biotin. The primary antibodies included the following: rabbit polyclonal antibodies against CRALBP (UW55, a kind gift from J.C. Saari, University of Washington, Seattle, WA) and human types I, III, V

(Abcam, Cambridge, UK), and XI collagen (a kind gift from J. Oxford, Boise State University, Boise, Idaho); a biotinylated rabbit polyclonal antibody against human type VI collagen (Abcam); goat polyclonal antihuman antibodies against types II and IV collagen (Southern Biotechnology Associates, Birmingham, AL); and mouse monoclonal antihuman antibodies against vimentin (DAKO, Glostrup, Denmark), GFAP (Sigma), and types VII (Abcam), IX (USBiological, Swampscott, MA), and XVII collagen (1A°C [28]; a kind gift from K. Owaribe, Nagoya University, Japan). Subsequently, cells were washed with PBS and endogenous peroxidases were blocked. Secondary antibodies included a swine-antirabbit peroxidase (DAKO), a biotinylated goat-antirabbit (GARbio, DAKO), a rabbit-antigoat peroxidase (DAKO), a rabbit-antimouse peroxidase (DAKO), a biotinylated goat-antimouse IgG1 (GAMbio, SBA), and a biotinylated goat-antimouse IgG2a (GAMbio, SBA). Secondary antibodies were diluted 1:100 with PBS containing 2% human serum was derived from a pool of human volunteers and added for 1 h at room temperature. Biotinylated type VI collagen, GARbio, and GAMbio (IgG1 and IgG2) were followed by incubation with ABC complex horse radish peroxidase (DAKO) for 20 min. Finally, cells were stained with 3-amino-9-ethylcarbazole (AEC; Sigma) and hematoxylin. Negative controls underwent the entire procedure, except for the substitution of the primary antibody.

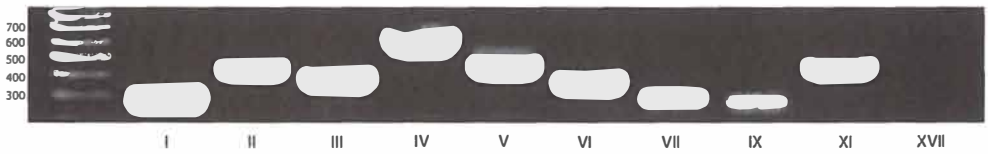

Figure 1 RT–PCR on Müller cell extracts. From left to right, bands indicating the positions of types I, II, III, IV, V, VI, VII, IX, XI, and XVII collagen are depicted. At the left margin, a 100 bp DNA ladder has been added.

Isolation of cell extract and supernatant concentration

Cells were harvested in sodium dodecyl sulfate PAGE (SDS–PAGE) denaturation buffer (10 mM Tris-HCl, pH 7 containing 1 mM EDTA, 2.5% SDS, 5% 2 mercaptoethanol, and 10% glycerol). The extract was heated for 5 min at 100 °C to unfold the collagen helices into separate α-chains.

After dead cells and cellular debris were removed by centrifuging at 1,600 rpm for 5 min, the supernatant was concentrated by ultrafiltration with an Amicon membrane (100,000 kDa cut-off; Millipore, Billerica, MA) and, in the case of collagen XI, with a Vivaspin 0.5 ml concentrator (30,000 kDa cut-off; Vivascience, Hannover, Germany).

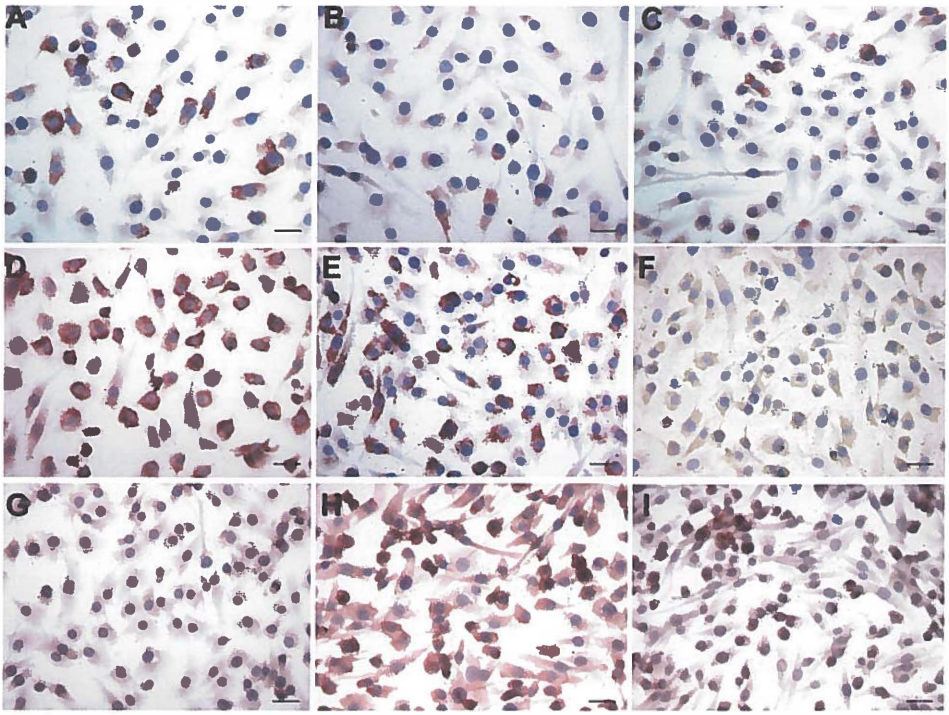

Figure 2 Immunocytochemical analyses of cultured Müller cells in medium with fetal bovine serum. **A**: Type I collagen shows a granular staining with a variable intensity between cells. **B**: Type II collagen is seen as a faint staining in the cytoplasm. **C**: Type III collagen is positive in all cells. **D**: Type IV collagen is visible as a strong granular cytoplasmic staining. **E**: Type V collagen shows mainly staining in the cytoplasm. **F**: In the case of type VI collagen, the cells are predominantly stained in the cytoplasm. **G**: Type VII collagen is faintly positive in the cytoplasm. **H**: Type IX collagen is also present in the cytoplasm. **I**: Type XI collagen is primarily seen in the cytoplasm. Bars in all panels equal 50 µm.

Table 2 Overview of the western blot results

Collagen	Molecular weight of collagen bands in Müller cells	Molecular weight of collagen bands in culture medium	Molecular weight of collagen bands after type VII collagenase
Type I	140 kDa	140 kDa	None
Type II	150 kDa	150 kDa	None
Type III	180 kDa	180 kDa	None
Type IV	130 and 210 kDa	130 and 210 kDa	75, 90, 110, and 250 kDa
Type V	260 kDa	260 kDa	None
Type VI	120 and 230 kDa	120 and 230 kDa	150 kDa
Type VII	270 kDa	270 kDa	130, 135, and 140 kDa
Type IX	200 kDa	150 and 200 kDa	None
Type XI	100, 150, and 200 kDa	150 kDa	None
Type XVII	None	None	None

By SDS–PAGE, collagen secretion into the medium was established for types I-VII, IX, and XI collagen. The specific collagen antibodies detected the collagen bands (in kDa) obtained before and after treatment with type VII collagenase. The results of both Müller cell extracts and medium with G5 are shown. The results after type VII collagenase concern Müller cell extracts and confirm the collagen nature of the bands as seen by WB. "None" indicates no collagen bands were detected.

Immunoblotting procedure

Polyacrylamide SDS electrophoresis was performed according to the method of Laemmli [29], using 3.9% and 5% slab gels and a 72-mm wide 2D gel comb in the Bio-Rad Mini Protean II electrophoresis apparatus (Bio-Rad, Hercules, CA). After separation, the gel was blotted to nitrocellulose using the Mini Protean II blotting unit (Bio-Rad) with 22 mM Tris, 168 mM Glycine, 0.05% SDS, and 20% methanol as a transfer buffer. After the transfer, the nitrocellulose was blocked for 1 h in TBS-buffer (20 mM Tris-HCl and 500 mM NaCl; pH 7.5) containing 3% BSA. Primary antibodies (Table 2) were diluted 1:500 in TBS and added to the blot. After incubation overnight, the blot was washed with TBS containing 0.05% Tween-20 (TTBS), and then secondary antibodies (Table 2) diluted 1:500 in TTBS were added. After 1 h incubation, the blot was washed with TTBS and incubated with alkaline phosphatase (AP) conjugated tertiary antibody (Table 2) diluted 1:250 in TTBS for another hour. After washing with TTBS and AP buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5), the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in AP buffer. All incubation and washing steps were performed at room temperature.

Collagenase digestion

Cell extracts were mixed with $CaCl_2$ to a final concentration of 10 mM to inactivate EDTA. Type VII collagenase (high purity grade, Sigma) was added in increasing concentrations (0–30 units/ml) to 60 µl cell extract and incubated for 1 h at 37 °C. Previously, the absence of nonspecific proteases in this collagenase batch had been confirmed [30]. To confirm that type VII collagenase specifically cleaves collagen, we exposed Müller cell extracts to this collagenase, after which vimentin and CRALBP expressions were checked. After incubation, samples were mixed with SDS–PAGE sample buffer to inactivate collagenase activity.

Cell viability

To determine cell viability, Müller cells and their medium supplemented with 1% G5, 0.2 mM β-APN, and 0.2 mM ascorbic acid were harvested and compared to a well with 10% FBS, which served as a reference, after an incubation period of 48 h. Dead cells were stained with trypan blue, counted in a Bürker-Türker counter (W. Schreck, Hofheim, Germany), and compared to viable cells.

Results

Reverse transcriptase polymerase chain reaction

Müller cells expressed mRNA of all tested collagen types, except for type XVII collagen (Figure 1). Amplimers were seen at the expected positions, which are for *COL1A1* at 254 bps, for *COL2A1* at 419 bps, for *COL3A1* at 369 bps, for *COL4A2* at 648 bps, for *COL5A1* at 454 bps, for *COL6A1* at 342 bps, for *COL7A1* at 261 bps, for *COL9A1* at 245 bps, and for *COL11A1* at 460 bps. *COL17A1* gave no product band as expected. Keratinocytes contained *COL17A1* (figure not shown).

Immunocytochemistry

LM results revealed that Müller cells preserved their morphology and characteristics under the culture method with FBS. They were all positive for vimentin and CRALBP, and less than 5% of the cells were GFAP positive (pictures not shown). In the case of collagen staining, the cytoplasm of all Müller cells was positive for all collagens with the exception of type XVII collagen (Table 3; Figures 2A-I). The pattern of the cytoplasmic staining varied in intensity and had a granular to fibrillary aspect. Types V, VI, and XI collagen also stained positively outside the cell - e.g., as small granules and fibers. All negative controls showed no staining (not shown).

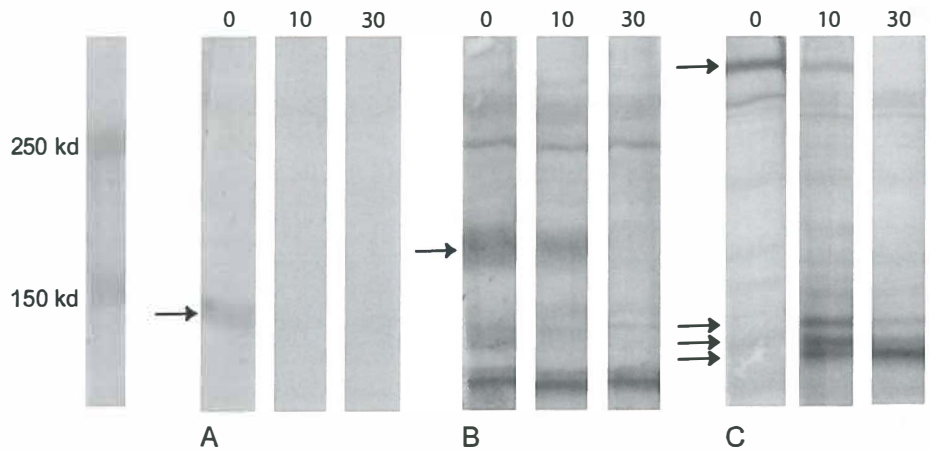

Figure 3 Examples of western blot analyses: Müller cell extracts with the addition of type VII collagenase (0, 10, and 30 units/ml). **A:** The specific band of type II collagen is shown at 150 kDa and disappears without formation of new bands when type VII collagenase is added. **B:** At 180 kDa, the specific band of type III collagen disappears gradually on the addition of type VII collagenase. **C:** Addition of type VII collagenase to type VII collagen results in the gradual disappearance of the specific band at 270 kDa and the appearance of breakdown products at 130, 135, and 140 kDa. Specific bands are indicated with arrows.

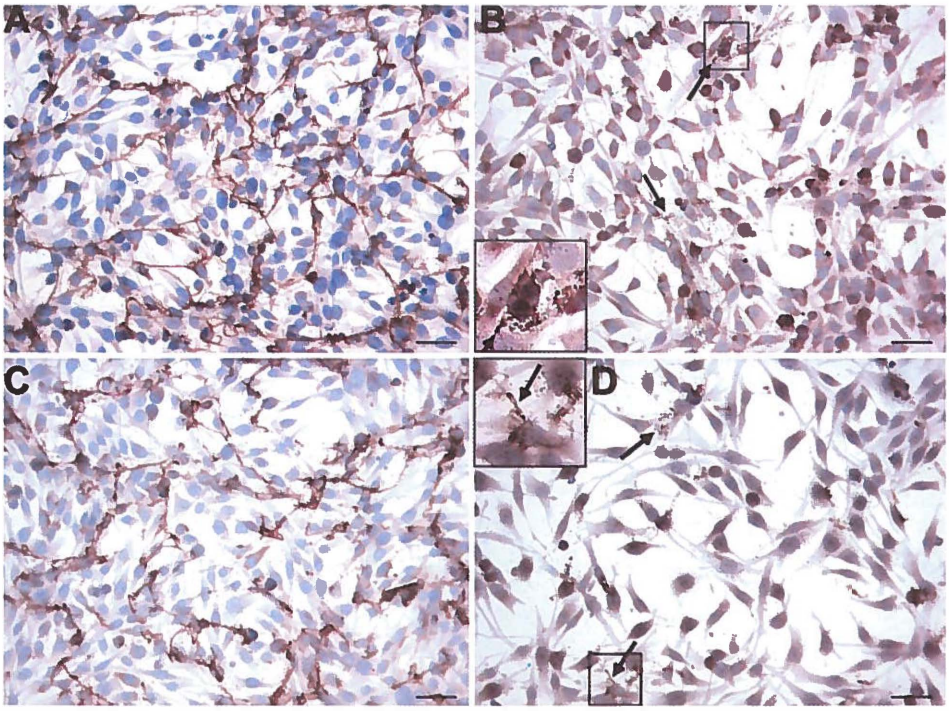

Figure 4 Immunocytochemical analyses of cultured Müller cells in medium with G5. Types I (**A**) and V (**C**) collagen show clear extracellular fibrillar threads and less intracellular staining compared to Figures 2A and 2E, respectively. Types II (**B**) and XI (**D**) collagen show some fine extracellular threads and small granules (arrows) and decreased intracellular staining compared to Figures 2B and 2I, respectively. In the inlays of Figures 4B and 4D, the extracellular collagen is magnified two times. Bars in each panel equal 50 µm.

Western blot

Cell extracts of Müller cells were immunoblotted for the presence of types I-VII, IX, XI, and XVII collagen and showed specific collagen bands (Table 2; Figures 3A-C), which disappeared on treatment with increasing doses of type VII collagenase, except for type XVII collagen which showed no band. In the case of types IV, VI, and VII collagens (Figure 3C), new product bands were detected after collagenase treatment. The collagenase had no effect on the bands of CRALBP and vimentin (not shown).

Analysis of conditioned growth medium with G5

Without FBS but with G5, Müller cells preserved their immunocytochemical characteristics, although, morphologically, they appeared a little stretched. Cell viability remained above 95% with a slightly diminished proliferation rate compared to conditions with 10% FBS. In comparison with cells grown in the medium with FBS, the RT–PCR results were similar.

LM results were comparable but showed less intense intracellular and more extracellular staining for types I, II, V, and XI collagen. Types I and V collagen were visible outside the cell as fibrillar threads and types II and XI collagen also, but to a lesser extent and they had a more granular aspect (Figures 4A-D). In the medium, we found specific collagen bands (Table 2) similar to those detected in the cell extracts except for type IX collagen where in addition to the 200 kDa band a weak band at 150 kDa was present. For type XI collagen, only the 150 kDa band was detected in the medium.

Table 3 Immunocytochemical analysis of human retinal Müller cells

Collagen	Cytoplasm staining		Extracellular staining		Cytoplasm aspect	
FB S	G5	FBS	G5	FBS	G5	
Type I	++	+	n.d.	++	Granular	Granular
Type II	+	+	n.d.	+	Diffuse	Diffuse - granular
Type III	+	+	n.d.	n.d.	Diffuse	Diffuse
Type IV	++	++	n.d.	n.d.	Granular	Granular
Type V	++	+	Granules	++	Granular - fibrillar	Diffuse
Type VI	+	++	Granules - fibers	n.d.	Granular	Granular
Type VII	+	++	n.d.	n.d.	Diffuse	Granular
Type IX	++	++	n.d.	n.d.	Diffuse	Diffuse
Type XI	++	+	Small granule s	++	Diffuse	Diffuse
Type XVI I	-	-	n.d.	n.d.	-	-

Müller cells were fixed on glass chamber slides and stained with specific antibodies against types I-VII, IX, XI, and XVII-collagen. LM confirmed the intracellular expression of all the above-mentioned collagens except type XVII collagen. The aspectof the collagen staining differed in aspect (diffuse, granular, or fibrillar) and intensity. In the table, the following symbols andabbreviations are used: strongly positive (++), positive (+), negative (-), not detected (n.d.), medium with 10% fetal bovi-neserum (FBS), and medium with 1% G5 (G5).

Discussion

This study shows collagen synthesis by human retinal Müller cells in vitro. Müller cells expressed mRNAs coding for typesI-VII, IX, and XI collagen. At the protein level, these collagens were demonstrated by immunocytochemical staining and shown to be present in the cytoplasm with LM. WB analysis of the cell extracts and of the medium in which the cells had been cultured confirmed the intracellular production and demonstrated that types I-VII, IX, and XI collagen were also secreted into the medium. The detected collagen bands could be procollagen chains as well as collagen chains, but we did not analyze this. Müller cells did not express type XVII collagen, a basement membrane protein that was recently demonstrated near photoreceptor synapses and its outer segments [25]. Apparently, Müller cells synthesize those collagens that are found in their natural vicinity (vitreous, ILL, and retina).

Although the spontaneously immortalized Müller cells have been well characterised [4] and shown to keep their main characteristics, cell models in general have as a major limitation that they are artificial in vitro systems unlike an in vivo model. Cultured Müller cells therefore might display somewhat deviant behavior because the cells are not in their natural surrounding, since they are growing on medium and moreover have shown spontaneous immortalization. To confirm our findings, primary isolated Müller cells could provide additional information, but in vivo data would be preferable.

For the WB experiments with Müller cells, ascorbic acid and ß-APN were added to stimulate collagen synthesis and prevent extracellular collagen cross-linking, respectively [27,31,32]. The differences in collagen staining observed by LM - the increased extracellular staining for types I, II, V, and XI collagen paralleled by a decreased intracellular staining - were most likely the effect of ascorbic acid. The digestion experiments with collagenase confirmed the collagen nature of the bands as seen by WB.

We hypothesize that the in vitro capability of Müller cells to produce the aforementioned collagens might (1) adduce support to previously described morphological findings in the embryonic period [5,6], (2) contribute to the stable level of postnatal vitreous collagen [33], and (3) explain, in part, the origin of epiretinal membranes in pathology (see below) [7,8,34-36].

In the embryonic vitreous, the neural retina and sometimes specifically Müller cells are indicated as possible sources of vitreous and ILL collagens. In chicken embryos, retina was involved in collagen (e.g., type II) synthesis [37,38]. In the developing mouse neural retina, mRNA of types II and IX collagen has been detected [39-41]. In the human embryo, Müller cells seemed continuous with the vitreous fibrils (primarily collagen type II) present at the vitreal side [6,42,43]. The production of vitreous fibrils during embryonic growth of the eye was ascribed to Müller cells and other cell types [5,6]. Also, Müller cells were supposed to contribute to the formation of the ILL in the human embryo [6].

The postnatal vitreous has long been regarded as an almost inert extracellular matrix, in which hardly any production or breakdown of its macromolecular components occurs [20,33,44]. Recent studies question the inertness of the vitreous body and suggested turnover of vitreous components [16,18,19,45-52]. Currently, two hypotheses on vitreous aging are postulated. The first is a concept of vitreous destabilization on aging because synchisis (liquefaction) and syneresis (aggregation of vitreous matrix components) lead to the formation of spaces and aggregated collagens; the second is a view that extracellular breakdown of vitreous matrix [16] (synchisis) would coincide with production of vitreous collagen [53-55], leading to an increase in optically dense

structures over time. In both views, the total amount of collagen in the human vitreous appears to remain stable during life [33], which, for the latter theory, may indicate that collagen synthesis and collagen breakdown are in equilibrium. The in vitro capacity of the Müller cell to synthesize vitreous collagens suggests a possible role in postnatal vitreous collagen synthesis. In addition, our in vitro results support the possible role of Müller cells in the formation of sublaminar vitreoretinal collagen complexes expanding on aging [11,18, 19].

Müller cells are found in epiretinal membranes in pathological circumstances such as massive retinal gliosis, preretinal macular fibrosis, idiopathic epiretinal membranes, and retinal injuries or degeneration [7,8,34-36]. These membranes can contain types I-V collagen [23,24]. In morphological studies, Müller cells appear to contribute to the formation of pathological membranes [7,8,34]. However, they may not be the only type of cell involved since glial cells, astrocytes, fibrocytes, and retinal pigment epithelium have also been observed in epiretinal membranes [24,56-59].

In summary, the finding that immortalized human Müller cells synthesize collagens in vitro indicates that they might also be involved in this process in vivo. Collagen synthesis by Müller cells could explain and expand on previous morphological findings in the embryonic and postnatal period as well as in pathologic conditions. In vivo experiments will be necessary to validate our results.

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The mature enzymatic collagen cross-links hydroxylysylpyridinoline and lysylpyridinoline in the aging human vitreous

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Submitted

Abstract

Purpose

The vitreous body of the human eye undergoes progressive morphological changes with aging. Since the enzymatic collagen cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are known to be important for the integrity of the collagen matrix, we studied their presence in the vitreous upon aging.

Methods

Vitreous bodies (VBs; n=143) from 119 donors (age 4-80 years; mean 54.3 ± 17.0 years) were carefully dissected. After weighing and freeze-drying, all samples were analyzed by high performance liquid chromatography. Left and right eyes of 24 donors were compared and, for age-related phenomena, 119 single eyes were used.

Results

Within one donor, no significant differences are found between left and right eyes. Upon aging, VB wet weight (4.42 ± 0.84 g) raises until 35 years and decreases thereafter. Collagen content (0.30 ± 0.14 mg), HP per triple helix (TH; 0.55 ± 0.18), and (HP plus LP)/TH (0.61 ± 0.19) increase until 50 years followed by a decrease, whereas LP/TH (0.057 ± 0.018) accumulates until 50 years and remains constant thereafter. The ratio between HP and LP (range 0.42 – 31.0, median 10.0) is constant over time.

Conclusions

The accumulation of enzymatic collagen cross-links until 50 years is an indication of collagen maturation and possible collagen synthesis in the human vitreous body. The decline of collagen cross-links after 50 years can be caused by collagen breakdown. Furthermore, the decline itself can contribute to the instability of the collagen network resulting in the increase of morphological changes in the elder VB.

Introduction

The vitreous body of the human eye is the transparent and highly hydrated (98-99% water) extracellular matrix (ECM) located behind the lens and surrounded by and attached to the retina. Its structure is maintained by heterotypic collagen fibrils, which contain collagen types II, V/XI, and IX, with type II predominating.[1] Types II, V, and XI collagen belong to the family of the fibril-forming collagens that assemble into fibrils and can form stable cross-links; type IX collagen belongs to the family of the fibril-associated collagens that is covalently linked to the surface of collagen fibrils.[2] Collagen fibrils in their turn can aggregate into collagen fibers. Enzymatic collagen cross-links are essential for the physical and mechanical properties of the collagen fibers.[3]

The formation of enzymatic collagen cross-links is preceded by collagen synthesis (Fig. 1).

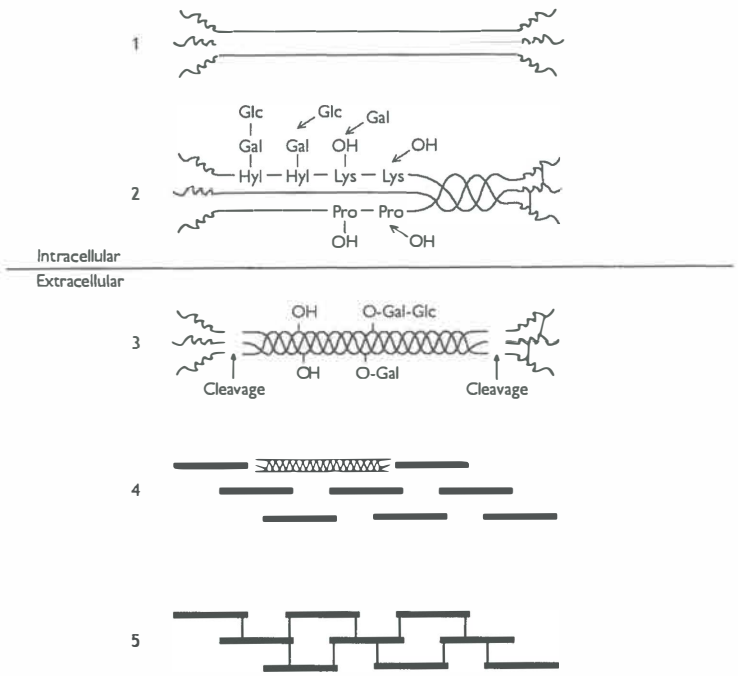

Figure 1 The synthesis of collagen. 1. Collagen is synthesized as pre-pro-α-chains. Following translocation, the signal peptides are removed and the individual procollagen α-chains will associate through the C-peptides. 2. Procollagens undergo multiple posttranslational modifications such as the hydroxylation of specific lysine (Lys) and proline (Pro) residues as well as the glycosylation of hydroxylysyl residues. 3. The procollagen is excreted and is converted extracellularly into collagen by cleaving the propeptides. 4. Subsequently, collagen molecules assembly into ordered fibrils. 5. These are finally stabilized by the formation of intra- and/or inter-molecular cross-links. (Reprinted with permission from A.J. van der Slot-Verhoeven[4])

Synthesis of fibril-forming collagens (e.g. type II) starts with the transcription of the gene within the cell nucleus followed by its translation. After translation, procollagens are formed which undergo multiple posttranslational modifications (e.g. the hydroxylation of specific proline and lysine residues and the glycosylation of hydroxylysine residues) before their secretion into the ECM. The hydroxylation of lysine residues within the triple helix as well as the C- and N-telopeptides is catalyzed by lysyl hydroxylases.[4] In the ECM, the C- and N-terminal propeptides are removed by proteinases, enabling the molecules to aggregate into fibrils.[5-8] Subsequently, collagen fibrils are stabilized by the formation of enzymatic intermolecular and/or intramolecular cross-links. The formation of cross-links starts with the oxidative deamination of the ε-amino group of specific lysine and hydroxylysine residues within the C- and N-terminal telopeptides leading to the formation of reactive aldehydes. The conversion of lysine and hydroxylysine into the respective aldehydes allysine and hydroxyallysine is catalyzed by the enzyme lysyl oxidase. The reactive aldehyde condensates either with hydroxylysine or lysine within an adjacent collagen molecule to form the stable intermolecular cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).[4,7,9,10]

With aging, the human vitreous undergoes a progressive increase in liquefied spaces (synchisis)[11-14] and an increase in optically dense structures (syneresis).[15,16] The first evidence of liquefaction is described at the age of 4 years.[13] Synchisis and syneresis progress slowly and these processes can be followed by a posterior vitreous detachment (PVD), which is a separation between the vitreous cortex and the retina.[11,12,14,17] Post-mortem studies reported that 45% of persons aged 60-69 years had at least 50% liquefaction,[14] that PVD is first seen in the sixth decade, and that 50-60% of persons aged 80-90 years had a PVD.[17] Posterior vitreous detachment in itself is not a serious condition, although it may lead to local interference with the passage of light and cause symptoms referred to as 'mouches volantes' or floaters. However, it may induce more serious pathology, such as retinal tears, retinal detachment, intravitreal hemorrhage, and cystoid macular edema.[18] The (patho)physiological mechanisms underlying synchisis and syneresis have not yet been clarified. Currently, two possible mechanisms are discussed in the literature. Generally, synchisis is supposed to start with changes in the noncollagenous components of the matrix and to result in an aggregation of collagen fibrils.[1,16,19-22] Following this theory, synchisis and syneresis are the structural manifestations of a destabilization of the vitreous matrix.[13,16,19] More recent studies find evidence of an alternative hypothesis, in which a breakdown of the vitreous matrix leading to synchisis[23] would coincide with the synthesis of vitreous collagen[24-29] leading to an increase in optically dense structures upon aging

(syneresis). In this theory, synchisis and syneresis can occur at different locations within the matrix and by different physiological and pathophysiological mechanisms.

In this study, we measured the contents of both HP and LP cross-links in whole human vitreous with aging, since the role of enzymatic collagen cross-links has not specifically been studied in the aging process of the vitreous. We show the presence of HP and LP cross-links, which appear to reach their maximum before the general onset of liquefaction.[14]

Methods

Vitreous preparation

Human eyes (n=143) from 119 donors (80 men and 39 women) with ages varying between 4 and 80 years (mean 54.3 ± 17.0 years) and with no known ophthalmic disorders were obtained from the Cornea Bank (Amsterdam, The Netherlands). Twelve donors (18 eyes) had diabetes mellitus and only one donor (75 years) had a complete PVD, defined as complete posterior detachment of the vitreous cortex from the retina to the vitreous base. Vitreous bodies (VBs) were prepared under a dissection microscope within 1-14 days post-mortem (mean 5.6 ± 2.6 days) according to a standard protocol previously described by Worst.[30] In short, eyes were placed in an eye holder filled with sodium chloride 0.9% and remained below the surface. Sclera, choroid, lens and iris were removed. After blunt cleaving, almost all retina and ciliary body parts were dissected from the vitreous except for the strong interconnections around the pars plana, which were initially left in place in order to prevent damage to the vitreous cortex. Then, the lens capsule was carefully dissected with most fibers of the zonula from the vitreous base. The final step was the dissection of pars plana remnants (PPR) consisting largely of ciliary body fragments still adhering to the vitreous.[31] Some vitreous base could have been removed during the latter procedure. All VBs and PPRs were weighed and stored at –20 °C before freeze-drying. In this study, VBs and PPRs were divided in 24 pairs of left en right eyes and 119 single eyes.

Freeze-drying

To reduce vitreous volume, VB and PPR samples were freeze-dried by a Christ Alpha 1-4 freeze-dryer (Salm en Kipp, Breukelen, The Netherlands). Before the drying process, the samples were put in liquid nitrogen. The lyophilization was performed using a shelf temperature of –30 °C, a condenser temperature of –53 °C and a pressure of 0.220 mbar

for 18 hours. Then, the shelf temperature and pressure were gradually raised to 20 °C and 0.520 mbar, respectively, during 6 hours. Finally, the drying process was continued for another 20 hours under these conditions. In a separate pilot analysis, we confirmed by repetitive freeze-drying cycles that there was no loss of 'dry weight' sample (data not shown).

HPLC analyses

Analyses of HP, LP, and amino acid content were performed by high performance liquid chromatography (HPLC) as described previously.[32,33] The HPLC system (Separations, Hendrik Ido Ambacht, The Netherlands) consisted of a Gynkotek Model 480 multisolvent delivery system, a Sparks Holland Triathlon autosampler, a Jasco Model 821-FP fluorimeter, and a Lab-Quatec Model Gastorr Gt-103 degasser. Calibration of amino acids was performed with the amino acid standard for collagen hydrolysates (A-9531; Sigma, St. Louis, MO, USA).

Amino acids (hydroxyproline and proline) and cross-links were determined after acid hydrolysis, as described previously.[33] All freeze-dried samples were hydrolyzed in 6 M HCl at 110°C for 20-24 hours. After drying (Speed Vac SC 110; Savant, Farmingdale, USA), the specimens were dissolved in 200 µl water containing 10 µM pyridoxine (Sigma; internal standard for cross-link analysis) and 2.4 mM homoarginine (Sigma; internal standard for amino acid analysis).

For cross-link analysis, the samples were diluted in 0.5% (v/v) heptafluorobutyric acid (HFBA; Buchs, Switzerland) in 10% (v/v) acetonitrile (Walkerburn, Scotland). Separation was performed on a 4.6 mm x 150 mm Micropak ODS-80TM (Varian, Sunnyvale, CA, USA). The column was equilibrated with 0.15% (v/v) HFBA in 24% (v/v) methanol (solvent A). Elution of pyridinolines and the internal standard pyridoxine was achieved at ambient temperature at a flow-rate of 1.0 ml/min in two isocratic steps: time 0-17 min solvent A; time 17-30 min 0.05% (v/v) HFBA in 40% methanol (solvent B). The column was washed with 0.1% (v/v) HFBA in 75% (v/v) acetonitrile (solvent C) for 10 min and equilibrated for 10 min with solvent A, resulting in a total analysis time of 50 min per sample. Fluorescence was monitored with a programmable fluorimeter: 0-22 min 295/400 nm (pyridoxine and pyridinolines).

For amino acid analysis, aliquots of the hydrolyzed samples were diluted in 0.1 M sodium borate buffer, pH 8.0, and derivatized at room temperature for 5 min with 6 mM 9-fluorenylmethyl chloroformate in acetone. Termination of the reaction and removal of excess reagent and acetone was performed by extraction with 600 µl pentane. After two

additional extractions, 400 µl 25% acetonitrile in 0.1 M borate buffer, pH 8.0, was added. A 50 µl aliquot of the diluted sample was injected into the HPLC system, after which separation was performed on the above-mentioned reversed-phase column. Chromatography was carried out at a column temperature of 40 °C; fluorescence was monitored at 254/630 nm. Solvent composition and the ternary gradient have been described in detail, previously.[32]

Collagen cross-links are expressed as mol per mol collagen, assuming 300 hydroxyproline residues per triple helical collagen molecule (TH).[34]

Statistical analysis

The HPLC results were analyzed by Student's *t*-tests for differences between two groups. Age-related phenomena were studied by linear regression analysis and by curve estamination, a form of non-linear regression using a quadratic model by which a reversal or top of the curve can be determined (an increase followed by a decrease for example). The age at the reversal point (top of the curve) was used as a cut off point in the subsequent non-linear regression analyses. Right and left eyes were compared by paired Student's *t*-tests. All analyses were performed with SPSS version 14.0 for Windows (SPSS, Chicago, IL, USA). When needed, the data were normalized using log transformation. $P < 0.05$ was considered to represent statistically significant differences.

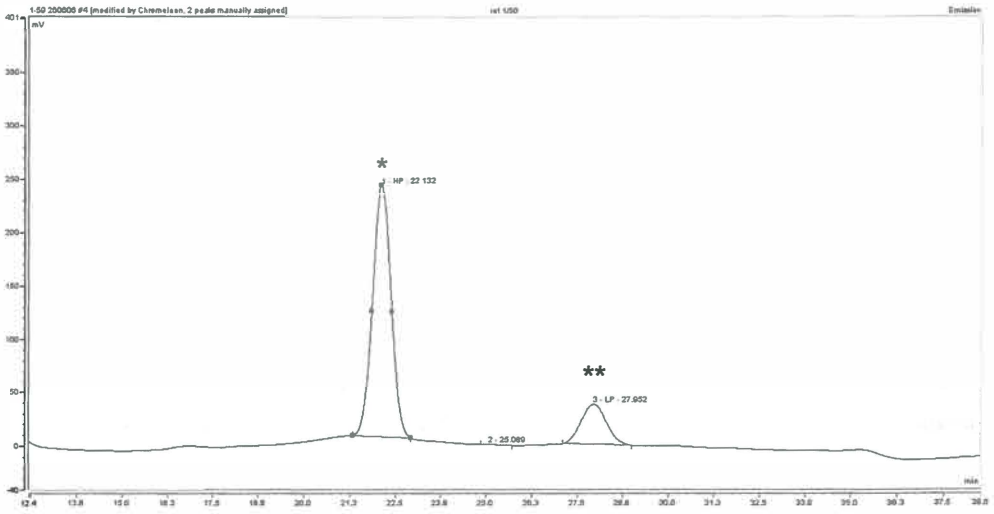

Figure 2 This is an example of HPLC output. The first peak represents HP (*) and the second LP (**).

Results

Right versus left eye

The 24 right and left VBs (Table 1) showed no significant differences between right and left eyes in all test variables: (dry)weight, hydroxyproline per proline (Hyp/Pro), percentage of collagen, mg collagen in total VB, HP/TH, LP/TH, (log) HP/LP, and (HP plus LP)/TH. Thus, VBs of one donor showed a high correspondence between right and left eyes. Therefore, only one (randomly chosen) eye of each donor was used in further analyses.

Table 1 Paired Student's *t*-test of right and left vitreous body (VB). Left and right eyes show no significant differences.

	Right	Left	*P*-value
Weight (g)	4.21 ± 0.68	4.18 ± 0.59	0.600 (n=24)
Dry weight (mg)	36.4 ± 7.0	37.2 ± 7.0	0.434 (n=24)
Hyp/Pro ratio	0.16 ± 0.06	0.16 ± 0.06	0.924 (n=24)
Collagen % (%)	0.76 ± 0.40	0.84 ± 0.46	0.384 (n=24)
Collagen weight (mg)	0.27 ± 0.14	0.30 ± 0.16	0.215 (n=24)
HP/TH (mol/mol)	0.51 ± 0.14	0.51 ± 0.14	0.880 (n=23)
LP/TH (mol/mol)	0.046 ± 0.02	0.048 ± 0.02	0.612 (n=23)
HP/LP	12.6 ± 5.7	11.6 ± 4.1	0.421 (n=23)
(HP+LP)/TH (mol/mol)	0.56 ± 0.15	0.56 ± 0.16	0.937 (n=23)

Hyp = hydroxyproline; Pro = proline; collagen % = collagen percentage; HP = hydroxylysylpyridinoline; LP = lysylpyridinoline; TH = triple helix; n = number.

VB of single eyes

In order to detect age-related phenomena, single eyes of 119 donors were analyzed with linear regression and curve estimation followed by non-linear regression (Figs. 3A-F). Macroscopically, elder VBs appeared much smaller. By curve estimation analysis, vitreous wet weight (mean 4.42 ± standard deviation 0.84 g; Fig. 3A) appeared to increase until 35 years ($P = 0.048$) and to decrease thereafter ($P < 0.001$), while dry weight (40.1 ± 9.8 mg) and VB collagen content (0.30 ± 0.14 mg) only declined significantly after 35 years and 50 years, respectively (both $P < 0.001$; not shown). By linear regression analysis, the Hyp/Pro ratio (0.17 ± 0.069; Fig. 3B), which is the ratio between collagenous and non-collagenous proteins, diminished markedly with aging ($P < 0.001$), while the percentage of collagen (percentage of the dry weight; 0.75 ± 0.33 %; Fig. 3C) remained constant over

time ($P = 0.111$).The ratio between HP and LP (range 0.42-31.0, median 10.0) did not change significantly (log transformed data: $P = 0.087$). Curve estimation analysis showed reversal points for HP/TH (0.55 ± 0.18; Fig. 3D), LP/TH (0.057 ± 0.018; Fig. 3E) and (HP plus LP)/TH (0.61 ± 0.19; Fig. 3F) around 50 years. Both HP/TH and (HP plus LP)/TH accumulated until 50 years (both $P < 0.001$) and decreased significantly thereafter ($P = 0.020$ and $P = 0.010$, respectively). LP/TH increased until 50 years ($P = 0.003$), and remained constant thereafter ($P = 0.355$). In our dataset, a few outliers were found and we did not remove them, since they had no effect on our results (not shown).

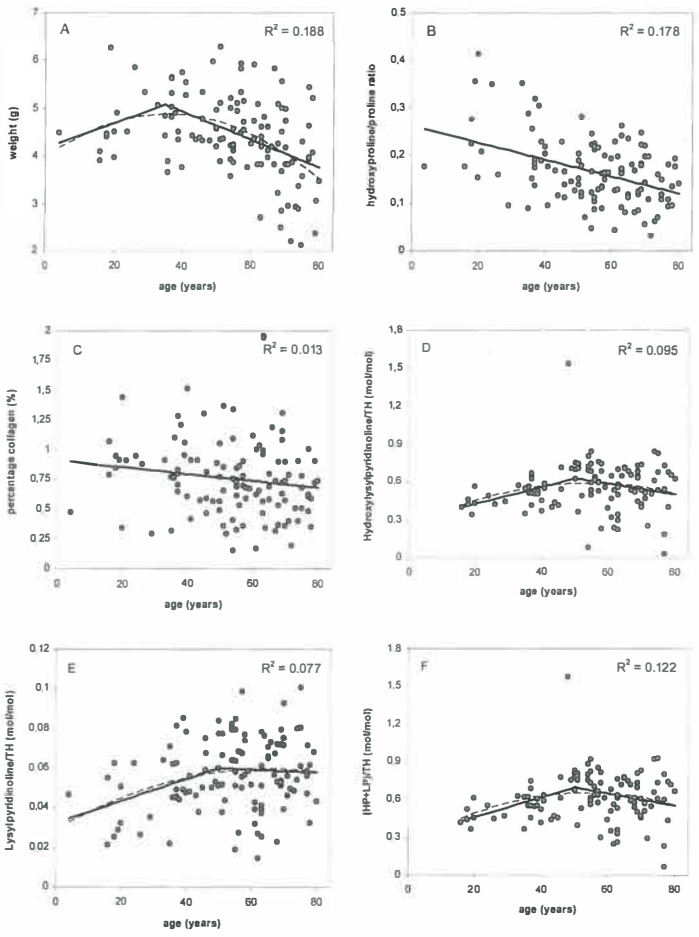

Figure 3 The effect of age on the vitreous body (VB). A. Until 35 years, VB Weight increased ($P = 0.048$) and decreased thereafter ($P < 0.001$). B. On aging, the hydroxyproline/proline (Hyp/Pro) ratio declined ($P < 0.001$). C. The collagen percentage remained constant over time ($P = 0.111$). D. Until 50 years, hydroxylysylpyridinoline (HP) per TH rose ($P < 0.001$), while it decreased thereafter ($P = 0.020$). E. Lysylpyridinoline per TH (LP/TH) increased until 50 years ($P = 0.003$) followed by a constant level ($P = 0.355$). F. Up to 50 years, (HP plus LP)/TH showed an increase ($P < 0.001$) and diminished thereafter ($P = 0.010$).

VBs showed no significant differences in the enzymatic collagen cross-links in our sub analysis of (i) diabetics (n=12) versus non-diabetics (n=107), (ii) causes of death (chronic alcohol intoxication (n=5), vascular cause (n=73), pulmonary cause (n=13), malignancy (n=19), and trauma (n=9)), and (iii) time interval (1-12 days) between death and preparation (data not shown). Finally, possible differences between gender (80 men and 39 women) were studied, since female gender is a risk factor for the development of PVD,[12,35,36] but no clear differences were found between men and women (data not shown).

PPR of single eyes

Linear regression analysis of the 119 PPRs (48.5 ± 17.0 mg; prepared from the 119 VBs used in the analysis of single eyes) showed an increasing percentage of collagen (14.5 ± 4.5 %) on aging ($P < 0.001$). Curve estimation analyses showed an estimated reversal point around 50 years for HP/TH (0.79 ± 0.30), LP/TH (0.074 ± 0.026) and (HP plus LP)/TH (range 0.12-2.09; median 0.84). An increase in enzymatic collagen cross-links was noticed until 50 years ($P < 0.001$, $P = 0.005$ and $P < 0.001$, respectively), while the cross-links remained constant thereafter (data not shown). The ratio between HP and LP (11.5 ± 4.0) remained constant over time ($P = 0.953$; data not shown).

Because VB and PPR are tightly interconnected,[31] we evaluated whether "PPR" was in fact concentrated VB. This seemed unlikely because all parameters except the HP/LP ratio proved to be significantly different (Table 2).

Table 2 Paired Student's *t*-test of the vitreous body (VB) and its pars plana remnant (PPR). All parameters except HP/LP differ significantly. For abbreviations see Table 1.

	VB	PPR	*P*-value
Weight (mg)	4,420 ± 840	49 ± 17	< 0.001 (n=119)
Dry weight (mg)	40.1 ± 9.8	2.9 ± 0.9	< 0.001 (n=119)
Hyp/Pro ratio	0.17 ± 0.069	0.63 ± 0.11	< 0.001 (n=117)
Collagen % (%)	0.75 ± 0.33	14.6 ± 4.4	< 0.001 (n=117)
Collagen weight (mg)	0.30 ± 0.14	0.42 ± 0.16	< 0.001 (n=117)
HP/TH (mol/mol)	0.56 ± 0.18	0.81 ± 0.29	< 0.001 (n=113)
LP/TH (mol/mol)	0.057 ± 0.018	0.075 ± 0.025	< 0.001 (n=112)
HP/LP	10.6 ± 4.47	11.5 ± 4.1	0.071 (n=110)
(HP+LP)/TH (mol/mol)	0.61 ± 0.19	0.90 ± 0.29	< 0.001 (n=110)

Discussion

This study shows an increase in the mature enzymatic collagen cross-links, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), in the human vitreous body from childhood until 50 years followed by a decline or stabilization thereafter, respectively. For the VB cross-link composition, the influence of LP appears to be limited, since HP is the most abundant collagen cross-link and the HP/LP ratio does not change significantly on aging. Overall, the enzymatic cross-links (HP plus LP) per TH increase until 50 years and decline thereafter.

The pyridinoline cross-links form the last enzymatic step in collagen maturation. They provide physical and mechanical strength to the collagen network and thus contribute to its integrity.[3] The currently described collagen cross-links should not be confused with the aggregation or fusion of collagen fibrils and collagen fibers described upon aging of the VB.[15,16,37,38] This latter form of "cross-linking" of collagens is, in fact, variably used to describe the age-related morphological changes and is thus easily interchanged with collagen cross-linking in the case of normal collagen maturation (formation of enzymatic connections between collagen molecules). The relationship between enzymatic cross-links and morphological changes with aging has not been studied in the VB.

In the VB, we find an increase in the enzymatic cross-links per TH until 50 years. A possible explanation for the increase could be the formation of enzymatic cross-links from the processing of type II procollagen present in the VB.[28] These procollagens dilute the amount of collagen cross-links per TH since procollagens do not contribute to enzymatic collagen cross-links formation. As the procollagens develop into new collagen molecules, they become available to cross-link formation and thus the amount of collagen cross-links could rise. At this moment, it is not known whether the amount of procollagens in the vitreous changes with aging. Furthermore, the presence of procollagens may indicate that collagen synthesis and thus cross-link formation continuously take place upon aging. This latter hypothesis is supported by the finding of immature cross-links in adult bovine vitreous.[26] In older cartilage and bone, immature cross-links decline in parallel with a raise in mature enzymatic cross-links.[39,40] In this study, we were only able to measure the mature enzymatic cross-links. Therefore, a direct relationship between increasing mature and decreasing immature cross-links could not be demonstrated. In nonmineralized tissues (such as cartilage and vitreous) maturation of immature collagen cross-links is probably a quick process taking only one to four weeks.[39,41-43] This is in contrast to mineralized ECMs (e.g. bone and dentine) in which maturation of enzymatic cross-links is a slower process

because of the abundant presence of mineral.[44]

Besides the increase in enzymatic cross-links per TH until 50 years, we found a decline thereafter. This decrease occurs at an age at which morphological changes in the VB become more prominent.[14,17] A reasonable explanation could be a breakdown or loss of collagen cross-links, which is supported by the decrease in collagen content after 50 years and the morphological presence of collagen fragments near liquefied spaces.[23] By analogy, the increasing percentage of collagen and the increasing amount of collagen cross-links in the PPR is in agreement with the morphological finding that intraretinal collagen in the vitreous base expands posteriorly with aging.[45] Another explanation might be that an older individual has less cross-linked collagens because of diminished cell-regulated activity of lysyl oxidase or lysyl hydroxylase-2 with age. Results found in the VB are globally in agreement with other human ECMs (bone, cartilage, meniscus, and intervertebral disc) in which the maximum amount of enzymatic cross-links is often reached in adolescence or midlife.[10,40,46-49]

The presence of HP and LP is confined to tissues not exposed to ultraviolet radiation. It is absent from skin, cornea, and basal membranes and mainly present in cartilage and bone.[39,50] The lens of the human eye is largely responsible for the blockage of UV-light[51] and, thus, it would be interesting to look at a possible shift in enzymatic collagen cross-links in aphakic eyes and longstanding pseudophakia to evaluate the possible relationship of these cross-links with the earlier appearance of liquefaction in these eyes. A significant presence of LP is largely confined to mineralized tissues (e.g. bone, dentin, and hypertrophic cartilage).[39,52,53] The vitreous calcium level is equal to aqueous and plasma.[54] In humans, the HP/LP ratio in bone is around 3-4:1,[40,52,55] in dentin 4:1,[52] in tendon 15:1,[52] in nonarthritic meniscus 28:1,[48] in articular cartilage 30-50:1,[8,55] and in synovium 25:1.[55] Our study found a mean HP/LP ratio in human VB of 11:1 and in PPR of 12:1, which remained constant upon aging.

At this moment, the only study concerning HP and LP in the human VB and thus our only reference for mature enzymatic cross-links is based on vitrectomy samples.[56] However, this study failed to detect age-related changes in HP and LP cross-links. Possible explanations for this difference with our study include: (i) a limited age range (38-77 years versus 4-80 years), (ii) the use of vitrectomy samples (sampling error since the VB is not a homogenous structure[1,15,17,57]), and (iii) the expression of cross-links in ng per ml versus amounts per triple helix.

In the present study, the apparent increase in VB weight and VB collagen weight until 35 and 50 years, respectively, and the significant decrease thereafter was partly in

agreement with a previous study which showed a maximum total VB weight around 40 years and a constant VB collagen content from the third decade onwards preceded by a possible increase.[13] Because we found higher amounts of total vitreous collagen weight (mean 0.30 mg versus 0.22 mg by estimation[13]), it was not likely that we lost collagens during preparation. The increase followed by the decrease could be explained by a net collagen synthesis followed by a net collagen breakdown (and removal from the VB). In our opinion, it is impossible to explain this phenomenon by the assumption that collagen once formed never changes and only aggregates with aging. Our hypothesis can be supported by morphological studies, which showed an age-related loss of type IX collagen[37] and found evidence of collagen fragmentation near liquefied spaces.[23]

The Hyp/Pro ratio showed a significant decrease with aging, implying a higher increase in the amount of non-collagenous proteins than in the amount of collagens. Non-collagenous proteins in the vitreous include glycoproteins (GPs such as opticin), proteoglycans (PGs e.g. chondroitin sulphate), and other structural proteins (e.g. fibrillin). Since GPs and PGs are the most abundant non-collagenous proteins of the VB, the change in Hyp/Pro ratio could reflect an increase in these proteins. Alternatively, an increase in total protein concentration with aging has been described and found to be related to a progressive leakage of serum proteins into the VB.[58]

Our results on enzymatic collagen cross-links can contribute to the insight in the age-related processes synchisis and syneresis in the concept of collagen turnover. The accumulation of collagen cross-links until 50 years is an indication of (ongoing) collagen maturation, which in its turn can be the result of collagen synthesis. At the age of 50 years when striking morphological changes in the VB are evident,[14,17] the enzymatic collagen cross-links start to diminish. This decline can be caused by collagen breakdown, but more importantly the decline itself can contribute to the instability of the collagen network resulting in an increase in morphological changes in the elder VB.

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Remodeling of the human vitreous and vitreoretinal interface – a dynamic process –

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Abstract

The highly hydrated, almost acellular vitreous body of the human eye consists of only 0.1% macromolecules of which collagens are the most important for its matrix structure. With aging, the human vitreous undergoes a slowly progressive remodeling characterized by a gradual formation of collagenous condensations and liquefied spaces in the gel structure. The former can be the result of collagen synthesis, while the latter is an indication of collagen breakdown. This review describes the embryological and postnatal remodeling of the human vitreous matrix. Possible sites involved in the remodeling of vitreous extracellular matrix components, e.g. the ciliary body and the retina, are discussed.

I. Introduction

The vitreous body (or vitreous) of the human eye is an almost acellular, highly hydrated (98-99% water) extracellular matrix located between the lens and the retina. It consists of just 0.1% macromolecules.[1] The most important macromolecules (see biochemistry) are the collagens[2-10] and the glycosaminoglycan, hyaluronan (HA).[1,11,12] A collagen network of heterotypic fibrils (types II, V/XI, and IX) presumably maintains the gel structure and HA fills the spaces between these collagen fibrils and stabilizes the gel.[2,13,14] Other non collagenous macromolecules can also be involved in spacing, binding and linking of extracellular matrix (ECM) components.[13,15] Since collagens are held responsible for the preservation of the gel structure,[13] these macromolecules are of primary interest.

With aging, two morphological changes in the vitreous have been described: a progressive increase in fluid-filled areas (synchisis)[16-18] and an increase in optically dense structures (syneresis).[19-24] As these morphological alterations progress, they may locally interfere with the passage of light and cause symptoms referred to as 'mouches volantes' or floaters. Finally, these processes may be followed by a posterior vitreous detachment (PVD), a separation between the vitreous cortex and the retina. In most eyes, PVD is not a serious condition, but it may induce real pathology, such as retinal tears, retinal detachment, intravitreal hemorrhage, and cystoid macular edema.[25-27] Liquefaction is characterized by the replacement of vitreous gel by liquefied vitreous and generally starts in the central vitreous.[22] The liquefied part is typically free of collagen fibrils[28] and surrounded by optically dense structures or condensations.[22] The (patho)physiological mechanisms underlying the morphological changes have not yet been clarified. In general, liquefaction is supposed to start with changes in the noncollagenous components of the matrix and to result in an aggregation of collagens.[13,14,22,28-30] In one theory, synchisis and syneresis are interpreted as expressions of matrix destabilization.[14,16,22] Based on more recent studies, an alternative hypothesis is postulated, in which breakdown of vitreous matrix (synchisis)[31] would coincide with synthesis of vitreous collagen[32-37] leading to the formation of dense structures (syneresis). This hypothesis is also in line with adult ECMs other than the vitreous (e.g. cartilage), in which collagens are synthesized and broken down during matrix remodeling.[38-41]

This review will discuss the morphological changes of the vitreous and vitreoretinal interface of the human eye from a dynamic point of view. We will start with the embryologic development of the vitreous (2). Subsequently, observations on the postnatal vitreous (3) will be discussed. Finally, we discuss regions, cells, and processes possibly involved in lifelong vitreous remodeling (4,5).

2. The embryonic vitreous

In the earliest stages of the human embryo (4-5 mm stage or third to fourth week), evidence for vitreous formation is found when the neural ectoderm separates from the surface ectoderm in the optic vesicle. Fibrils or proteoplasmic protrusions, fill the narrow space between the lens vesicle and the inner retinal layer: the primordial vitreous.[42]

In the lentiretinal space of the optic cup, the primary vitreous develops along with the hyaloid vasculature in the 10-13 mm stage (fifth week). It consists of ectodermal components, which are the fibrils produced by the inner layer of the future sensory retina and the posterior side of the lens vesicle. At this stage, it is a matter of debate whether the primary vitreous also consists of mesenchymal components, which enter posteriorly with the hyaloid vessels and anteriorly through the space between the anterior rim of the optic cup and the lens vesicle.[1,11,42-45] The cells of the inner layer of the cup differentiate into an inner and outer neuroblastic layer, which both will develop into retinal neurons and glia whereas the outer layer will also form rods and cones.[46-48]

Together with the closure of the optic fissure around the 14 mm stage (end of the sixth week), the development of the secondary vitreous (avascular vitreous) begins.[1,11,42,44] In one theory, the secondary vitreous appears on the inside of the sensory retina pushing the primary vitreous and the hyaloid system forward and inward, except at the periphery and optic disc.[1,11,42,44] In contrast to this theory of strict spatial separation, morphological evidence has been found that retracting hyaloid vessels may act as a scaffold along which fibers of the definite vitreous organize themselves.[24,49-53] Both in human and rabbit vitreous, the course of vitreous fibrils and lamellae can be retranslated into the course of the hyaloid blood vessels of the primary vitreous.[49-52] This latter theory implies a gradual remodeling of the primary into the secondary vitreous.

The secondary vitreous consists primarily of type II collagen and HA.[11,44,54,55] At the retina, processes of Müller cells start to unite and form the internal limiting membrane (ILM), which begins in the posterior pole. The ILM is not a surface of separation yet and Müller cells seem continuous with the vitreous fibrils.[1,11,42] The foot-plates of the Müller cells are supposed to be concerned in the synthesis of vitreous fibrils during the growth of the eye.[42] Mesenchymal cells accompanying the hyaloid vessel, which grows in the vitreous as an arteriole giving off a complicated system of branches (vasa hyaloidea propria and tunica vasculosa lentis), may also contribute to the formation of vitreous.[42] Around 40-48 mm (ninth week) the maximum size of the hyaloid system is reached, the atrophy of the posterior vasa hyaloidea propria has begun and the primary vitreous has ceased to

grow. This is followed by degeneration of the tunica vasculosa lentis.[1,11,42] The hyaloid artery persists much longer and elongates with the growth of the whole eye, because it is attached to the lens and to the center of the optic disc. At the place where primary vitreous meets with the secondary vitreous, possible transformations can be seen: the supposed canal of Cloquet or other central canals.[42,44,56-58] Between 70-100 mm (fourth month), the tertiary vitreous or zonular system is produced at the level of the developing ciliary body by surface ectoderm and fibroblasts.[11,42] Around 240 mm (seventh month) the blood flow in the hyaloid artery ceases, followed by glycogen and lipid deposition in the endothelial cells, and, finally, cells in the vessel wall become atrophic and are phagocytozed by mononuclear phagocytes.[1,11,44,59] In general, at birth, all blood vessels have disappeared from the vitreous. The eye itself is then almost completely developed except for the macula, which reaches its adult configuration at 45 months.[44,60-63]

During the embryological period, the human vitreous body is an active ECM that undergoes matrix remodeling - breakdown and synthesis of vitreous components - which results in the avascular and transparent vitreous at birth.

3. The postnatal vitreous

A. Aging of the human vitreous

At birth, the human vitreous has an ultrasonic length of 10.48 mm and 10.22 mm for males and females, respectively. Through the years, this difference is preserved. After fast growth in the first three postnatal years (around 3.5 mm), growth slows down and the vitreous is almost full-grown at the age of 13 years with measurements of 16.09 mm for males and 15.59 mm for females.[64,65] Within the vitreous body, (i) the basal vitreous, (ii) the vitreous cortex, (iii) the intermediate vitreous, and (iv) the central vitreous are recognized. The vitreous base is a three dimensional annular zone composed of dense bundles of collagen fibrils, which firmly adhere to the retina and to the non-pigmented ciliary epithelium. The cortex surrounds the central matrix and adjoins the retina. From the cortex to the central vitreous, the concentration of collagen fibrils decreases: from a high density in the cortex via an area with intermediate density to a loose-meshed network in the center.[19,23] The central vitreous is the largest part and could still contain the canal of Cloquet, which represents the remnant of the embryonic hyaloid vasculature.[1,13] However, the existence of the canal of Cloquet as described above is doubtful. Several ink injection studies show a distinct morphology of transvitreal channels in the adult eye, i.e. central canal(s) which end

behind the ciliary body.[56-58] Furthermore, evidence is found that the embryonic and early postnatal Cloquet's canal disappears around the third postnatal month.[23]

During postnatal development, vitreous remodeling continues. This affects overall matrix structure and also vitreoretinal attachments at the vitreous base area. From the age of four onwards, the first evidence for synchisis is described[16] and simultaneously, syneresis occurs.[19-23] Both processes progress slowly with aging and it was reported in a post-mortem study that 45% of the persons between 60-69 years old had at least 50% liquefaction.[18] As a consequence, spaces and condensations have been found. Most tissue condensations found in the vitreous cortex follow a course parallel to the retina, while some follow a course perpendicular to the retina.[23,66] In the intermediate vitreous, condensations come from the vitreous base. They are changeably described as tracts,[19,67] septa,[56,68] lamellae,[23,69,70] membranes,[19,23,71] and fiber bundles.[72,73] These condensations appear to be collagenous in origin.[58,73] With aging, they become more noteworthy and increasingly irregular.[22,67] Furthermore, in the anterior vitreoretinal interface, increasing intraretinal collagen connected to the vitreous collagen and widening of the vitreous base are described.[66,74,75] At the same moment, posterior vitreoretinal attachments weaken.[76] Finally, all these processes may be followed by a PVD, which is particularly associated with senescence and the amount of liquefied spaces,[17,20] and less with myopia, which mainly causes an earlier onset,[25,77,78] female gender,[17] ischemia of Müller cells,[79] and several metabolic disturbances, e.g. diabetes mellitus.[79-81]

B. Biochemistry of the vitreous

The vitreous has a low percentage of macromolecules (0.1%) which consist of collagens,[2-10] glycosaminoglycans (GAGs: such as HA),[12] proteoglycans (PGs: such as versican),[30,82,83] glycoproteins (GPs: such as opticin),[13,84,85] and other noncollagenous structural proteins (e.g. fibrillin).[13,84,86,87] Collagens form a network of heterotypic fibrils (types II, V/XI, and IX) which presumably maintain the gel structure.[2,13,14] Collagen types present in the vitreous are types II,[2,3] V/XI,[4-6] VI,[6,88] and IX.[3,5-10] The general functions of GAGs, PGs, GPs, and noncollagenous structural proteins are spacing, binding, and linking of ECM components.[13,15] All GAGs, except HA, are synthesized attached to a protein core and this results in a PG. The most important GAG in the human vitreous is HA, while chondroitin sulphate proteoglycans (such as versican and type IX collagen) and heparan sulphate proteoglycans represent minor components (agrin).[13,83] Opticin an ECM glycoprotein and member of the family of the ECM small leucine-rich repeat protein/PGs is able to bind GAGs and collagens, which suggest a role in stabilizing vitreous gel structure and in maintaining

vitreoretinal adhesion.[13,89] Another GP is link protein that stabilizes the binding of versican to HA.[83] Important non-collagenous macromolecules are: (i) fibrillin-1, which is de major component of fibrillin-containing microfibrils present as a third type of fibrillar structure (together with heterotypic and type VI collagen fibrils),[13,15] (ii) VIT1, which is a collagen binding macromolecule and is an important structural molecule in the vitreous, and (iii) cartilage oligomeric matrix protein.[13]

C. Cells in the vitreous and vitreoretinal interface

The adult vitreous body contains only a small number of cells predominantly located in the vitreous cortex. Hyalocytes represent approximately 90% of the cells and fibroblasts the other 10%. In the vitreous base, hyalocytes have the highest cell density, followed by the posterior pole and the lowest density at the equator.[90,91] There is also a preferential location of cells along retinal blood vessels.[90] Hyalocytes are 10-15 µm in diameter with a lobulated nucleus and depending on their activity and location, these cells can be round, oval, spindle-shaped, or flattened.[92,93] These cells contain a well-developed Golgi complex, a moderate amount of mitochondria, basophilic Periodic Acid Schiff (PAS)-positive granules (0.2-2.0 µm), lysosomes, and smooth and rough endoplasmatic reticulum phagosomes.[1,92] In hyalocyte granules, several substances are found: GAG, GP, nucleic acid, acid phosphatase, beta-glucuronidase, and alkaline phosphatase.[94,95]

In the posterior vitreous cortex, laminocytes are found.[96] These cells are predominantly present at the ILM and absent from the rest of the vitreous. They could be involved in maintaining the adhesion between the posterior vitreous cortex and the ILM.

Fibroblasts are localized in the cortex at the ciliary processes, vitreous base and adjacent to the optic disc.[97,98] They differ form hyalocytes by the presence of longer extensions (with a maximum length of 260 µm) and the absence of PAS-positive granules.[90,99,100]

Müller cells are radially oriented macroglia that traverse the retina from the outer limiting membrane to the vitreal border, the ILM, where they adhere with their endfeet. They have many local functions: they stabilize the retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space.[101,102] In vivo and in vitro, Müller cells can produce and express several cytokines, growth factors and receptors.[102-104] It has been suggested that they are responsible for the production of vitreous and ILM collagens during growth, in adulthood and in culture.[2,42,47,105-110] Furthermore, Müller cells are also able to phagocytoze several substances such as pigment and melanin granules, erythrocytes, foreign particles and type II collagen.[111-115]

D. The vitreoretinal interface

The vitreoretinal interface is the area of contact between the vitreous body and the retina. Centrifugally, the interface consists of vitreous cortex, retinal ILM, and Müller cell endfeet. The posterior vitreous cortex is thinned over the optic disc and over the macula.[13,24,99] A prepapillary hole can sometimes be visualized clinically when the posterior vitreous is detached from the retina (Weiss' ring). Strong vitreoretinal adhesions have been described at the equator,[68] at the macula,[116] at the optic disc,[116,117] and over retinal blood vessels.[118] In the anterior vitreoretinal interface, the strong vitreoretinal adhesions extend themselves posteriorly with aging. Newly formed sublaminar collagen packages are responsible for this.[66,74,75] Müller cells could play a role in their formation.[110]

4. Remodeling of the vitreous matrix

In the case of collagen remodeling in other ECMs than the vitreous (e.g. gingiva and cartilage) collagens undergo turnover (synthesis and breakdown).[38-41] Synthesis of fibril-forming collagens (e.g. type II) occurs intracellularly and in general procollagen molecules are secreted into the ECM, where the C- and N-terminal propeptides are removed, collagen fibrils assemble and collagen cross-link formation occurs.[40,119,120] Conversely, collagen breakdown under physiological conditions follows mainly an intracellular route.[40] Under pathological conditions, when large amounts of collagen have to be broken down in a relatively short interval (i.e. inflammation), extracellular breakdown is more important with a main role for matrix metalloproteinases (MMPs).[121,122]

In adult human and mammalian vitreous, the finding of procollagens, immature collagen cross-links, and a late maximum of mature enzymatic collagen cross-links indicate an ongoing collagen synthesis with aging.[32-36,123] In addition, two recent morphological TEM-studies speculate about the involvement of extracellular enzymes in breakdown of vitreous matrix.[31,124] The first describes collagen fragments near liquefied spaces in the absence of cells.[31] The other hypothesizes that the age-related loss of type IX collagen from the surface of vitreous fibrils is caused by MMP-2.[124]

Extracellular enzymes, such as MMPs, are able to degrade collagens, although their role in physiological steady state conditions remains unclear.[40] In human nonpathologic vitreous, several MMPs have been demonstrated: MMP-1 (collagenase 1), (pro-) MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-8 (collagenase 2) and MMP-9 (gelatinase B).[125-128] MMP-1 and MMP-8 are able to cleave type II collagen and MMP-2 can cleave types V and

XI collagen and type IX collagen fragments.[129] Another extracellular enzyme plasmin(ogen) which has also a potential proteolytic activity in human vitreous increases significantly with aging.[130]

Besides extracellular enzymes, reducing sugars, oxoaldehydes, oxidized lipids and reactive carboniles can react with long-lived vitreous proteins and form advanced glycation end products (AGEs).[131] AGEs in the presence of iron are able to degrade HA by free radical formation and thus contribute to the degradation of vitreous matrix.[81] These AGEs may thus be important with aging and even more so in patients with diabetes mellitus.

Both collagen breakdown and synthesis may be in equilibrium since the total amount and percentage of collagen in the human vitreous remain stable during life.[16,123] Since the accumulation of pentosidine in time can be used to estimate a collagen turnover rate, with a higher pentosidine accumulation indicating a lower turnover rate, we hypothesize that the aging human vitreous has a higher collagen turnover than articular cartilage.[132,133] Over a time period of 50 years, the estimated increase of pentosidine in cartilage is about six times, while vitreous pentosidine increases approximately two times.[132,133] Collagen turnover in cartilage is an established fact with an estimated half-life of cartilage collagen of 117 years.[132] In the case of a much lower half-life of vitreous collagen, this is a very strong indication of collagen turnover in the vitreous.

Besides collagens, the amount of non-collagenous proteins (e.g. GPs and PGs) increase at higher rate than the amount of collagens.[123] An increase in total protein concentration with aging can be related to a progressive leakage of serum proteins into the VB.[2] The concentration of HA increases during growth till 20 years, remains fairly constant till 70 years, when the concentration again rises.[16]

To recover sites involved in collagenous matrix remodeling in the vitreous, we extracted possible sources of synthesis and breakdown of collagens and other macromolecular components from literature: (I) lens,[37,134-137] (II) optic disc,[136,137] (III) ciliary body,[71,84,86,134,136-143] (IV) retina,[42,66,75,85,108,110,115,134,136,137,139,142,144-153] (V) cells in the vitreous,[29,93,95,97,144,154-157] (VI) stem cells or progenitor cells,[158-176] and (VII) extraocular factors.[130,133,177-179]

5. Possible sites involved in collagenous matrix remodeling

I. Lens

In the mouse lens, type II collagen mRNA is expressed in the embryonic and adult period.[134] In the same animal, high mRNA levels of type IV collagen were found in the embryonic lens.[135] In the fetal lens of the chick, mRNA of several basal membrane proteins (nidogen, perlecan, and type IV collagen) was detected.[37,136,137]

II. Optic Disc

During chick embryogenesis, types IV and XVIII collagen, agrin, and laminin γ1 originate form cells of the optic disc and optic nerve.[136,137]

III. Ciliary Body

Smith[71] was probably the first to describe that the fluid of the vitreous humor was mainly secreted by the ciliary body. Since then, this region is often discussed as a producer of vitreous macromolecular components in the embryonic and postnatal period.[138] In the embryonic chick, mRNA for type II collagen is initially present in the presumptive retina as seen with in situ hybridization. By day 7 it becomes also localized in the presumptive ciliary body, but as soon as the ciliary body has formed it is only limited to that area.[142] Type IX collagen mRNA is predominantly found in the tissue that forms the ciliary epithelium and in the ciliary body.[142,143] Other macromolecules of which mRNA is expressed in that area during chick embryogenesis are type XVIII collagen, nidogen, agrin, and laminin β1 and γ1.[136,137] In both the embryonic and adult mouse eye, type IX collagen mRNA is mainly found in the ciliary body region.[134] Type II collagen mRNA is also localized at that place in the developing and young mice, but type IV collagen mRNA only in the embryonic period.[135,139] Several studies demonstrated synthesis of GPs in the ciliary body region of adult rabbit eyes.[84,86,140,141] Via in situ hybridization in the adult mouse, expression of opticin is specifically localized in the nonpigmented ciliary epithelium.[134]

Production of macromolecular components of the ILM (such as type XVIII collagen, nidogen, and laminin) by the ciliary body implies that ILM components traverse the vitreous cavity to assemble on the foot-plates of the Müller cells. At the time when the chick retina has its highest growth rate, mRNA of these matrix proteins is expressed in the ciliary body region and, at the same time, these matrix proteins have their highest concentration in the vitreous.[37] Shortly after reaching peak concentrations, the concentrations drop markedly and the lowest levels are seen in the adult vitreous body.[37]

IV. Retina

In the human embryo, Müller cells are supposed to be concerned with the synthesis of vitreous fibrils during growth of the human eye, although mesenchymal cells can also contribute to the formation of vitreous.[42] In the early embryonic stages, chick retina synthesizes large quantities of vitreous collagen, which is diminished in later development.[142,144] Synthesis of type II collagen by developing chick retina is also confirmed in vitro.[145] In the same animal, short, transient mRNA expression of type IV collagen and laminin γ1 and a continuous expression of agrin was found.[136] In the neural retina of mouse embryos and neonatal and adult canine retina, expression of type II collagen mRNA is observed.[146,147] In the mouse, type II collagen mRNA is found in the ganglion cell layer of the embryonic retina as well as the young retina.[139] COL9A1 expression is seen in both the embryonic and adult mouse retina.[134,152] MRNA of type IV collagen is only weakly detected in embryonic mice.[135] Apart from collagens, agrin mRNA is continuously present in ganglion cells of the embryonic chick retina.[137] The human embryonic retina, probably its Müller cells, produces HA from 12 week's gestation onwards.[148] In adult eyes, opticin may also be produced by the human retina, because it expresses opticin mRNA.[85]

Furthermore, there are strong indications that the retina synthesizes (postnatal) collagens by: (i) the repair and production of the ILM, (ii) a disturbed growth of the vitreous after retinal damage, (iii) the presence of collagen mRNA, and (iv) the presence of intraretinal type II collagen. Firstly, retinal Müller cells might be able to synthesize type IV collagen (an important component of the ILM).[108,149] Secondly, the synthesis of vitreous matrix by retinal cells is disturbed in sheep after photocoagulation of the retina, which is characterized by the absence of vitreous growth over the damaged retinal areas.[150] In rabbits with retinal ablation, destruction of vitreous producing cells in the retina could also be the cause of a diminished eye growth.[151] In addition, freshly isolated human retinectomy samples from the equatorial area contain mRNA of types I-VII, IX, XI, and XVIII collagen.[153] Finally, in the peripheral retina, a net synthesis of vitreous collagen has been described with a possible role for Müller cells.[66,75]

The role of the retinal Müller cell can be twofold in the interactive process of a net accumulation of intraretinal collagen. On the one hand, these cells can be involved in the production of type II collagen.[110] On the other hand, Müller cells can be capable of specific phagocytosis and thus breakdown of type II collagen.[115]

V. Cells in the vitreous

Hyalocytes may be involved in turnover of vitreous components. On the one hand, these

cells synthesize embryonic chick vitreous collagen in the late stages of development.[144] In addition, hyalocytes are indicated as postnatal producers of HA.[29,156] Cultured pig hyalocyte-resembling cells synthesize HA and express type II collagen.[157] On the other hand, these cells are also held responsible for the degradation of GAGs, GPs and nucleic acids.[95,154] Hyalocytes of guinea pigs could have phagocytic and synthetic functions of several substances.[93]

In general, fibroblasts are important in synthesis and degradation of collagenous proteins in soft connective tissues.[180] In the vitreous, fibroblasts -only 10% of the vitreous cells- could be the synthesizing cells of early postnatal collagen.[155] In young and adult eyes, collagens were found at the membrane and in the cytoplasm of vitreous fibrocytes.[97] The presence of collagens was attributed to both catabolic and synthetic mechanisms.

VI. Stem cells or progenitor cells

The presence of stem cells in or near the vitreous is interesting because of the phenotypic plasticity of these cells, which means that these cells have the potential to differentiate upon the right stimulus into cells with more specialized functions. In potency, these cells can become matrix-synthesizing cells.

In the last decades, the interest in retinal stem cells and progenitor cells has grown to increase knowledge about development, regeneration and potential therapies. In general, stem cells are pluripotent and capable of self-renewal, which means that these cells have phenotypic plasticity before and after cell division and that daughter cells must eventually differentiate, losing their identity as stem cells.[173] From this point of view, the finding of stem cells and progenitor cells in organ tissues may indicate a site for local postnatal regeneration (repair and remodeling) and for therapeutic intervention (transplantation).

In fish and amphibians, the retina continues to grow along with the eye throughout life by adding new cells of all types from the ciliary marginal zone (CMZ), a region at the peripheral edge of the retina. The retinal margin contains a gradient with the most primordial cells residing in more anterior regions compared with more mature cells that reside more posteriorly, adjacent to the retina.[169] Other possible retinal stem cells in fish and amphibians are the retinal pigment epithelial cells, the rod progenitors and the Müller cells.[167] In chickens, non-pigmented cells in the ciliary body are capable of producing neurons in vivo in response to intraocular growth factors. [170,172] In addition, chick Müller cells can also transdifferentiate into retinal progenitor cells under certain conditions and become a source of neural regeneration.[164-166]

In mammals, the retina is almost completely formed at birth in contrast to fish and amphibians.[160] In the adult rat[161,175] and mouse eye,[159] the capacity of (pigmented) cells from the ciliary body to generate new neurons in vitro was described in response to fibroblast growth factor-2 (FGF2) and other growth factors. In the postnatal rat retina, similar mitotically quiescent cells respond proliferatively to growth factors and these cells are capable of differentiation along neuronal and glial lineages.[158] However, after a longer postnatal time these cells undergo a progressive decrease in number and proliferative potential probably due to a temporal decline of proliferative signaling (e.g. FGF2). The ciliary epithelium of adult rabbits contains also retinal progenitor (stem) cells and fate-restricted progenitor cells (glial progenitor).[163] After optic nerve transection, progenitor-like cells of the ciliary body respond in the mouse,[168] while Müller cells express nestin, an intermediate filament marker for neural progenitor cells, in the rat.[171] Rat Müller cells can regenerate retinal tissue under neurotoxic conditions.[176] In the adult human retina and in epiretinal membranes, some neurons and glia cells stain positively for nestin. Nestin positive cells are found in the anterior retina at the ora serrata and, in large numbers, in epiretinal membranes. The clear presence of nestin positive cells suggests that a population of progenitor cells from normal adult human retina may differentiate to form retinal scar tissue.[162] In culture, human Müller cell lines exhibit neural stem cell characteristics.[174]

VII. Extraocular factors

Extraocular factors may be involved in the process of vitreous liquefaction, such as: (i) high molecular weight fraction of serum, (ii) light, and (iii) female gender. Firstly, the high molecular weight fraction of serum can induce gel contraction as seen in PVD under pathological circumstances (e.g. proliferative diabetic retinopathy) possibly caused by altered collagen-collagen interactions.[177] In addition, vitreous liquefaction could be induced by light as a result of active oxygen species, which likely affect HA.[179] Exposure to light in combination with AGEs can also decrease the molecular weight of HA.[178] Finally, female gender is a risk factor for the development of PVD.[17] Whether the higher increase in pentosidine (an AGE) in female compared to male vitreous is involved, needs further evaluation.[133]

6. Overall conclusion

The highly hydrated, almost acellular vitreous consists of only 0.1% macromolecules of which collagens are the most important building blocks. Remodeling of the human vitreous is a slowly progressive process characterized by a gradual formation of collagenous condensations and liquefied spaces in the gel structure. Together with the strong vitreoretinal attachments in the anterior vitreoretinal interface and the weakening of the posterior vitreoretinal attachments, these processes could eventually lead to PVD. In this review, we provide evidence for the dynamic process of vitreous matrix remodeling, in which collagen production leads to the formation of collagen condensations,[32-36] while at the same moment matrix degeneration causes the formation of liquefied spaces.[31] The results on enzymatic collagen cross-links with aging confirm the dynamic latter theory.[123]

For postnatal collagen synthesis, the retina and the ciliary body are the most likely candidate sources of human vitreous collagen. Both regions display potential to synthesize macromolecules at different time points in different animals. The peripheral human retinal cells, probably Müller cells, are able to produce vitreous collagen.[66,75,110] However, the exact location still needs to be determined.

For postnatal vitreous collagen breakdown, extracellular enzymes (e.g. MMPs and plasmin) are likely candidates, since cells are virtually absent from the vitreous and not found at all near liquefied spaces. Extracellular enzymes can be synthesized locally. Extracellular enzymes (e.g. MMPs) can be present in an inactive form (e.g. pro-MMPs), which needs stimulation for activation. In addition, proteolytic enzymes can be balanced by neutralizing substances (such as tissue inhibitors of metalloproteinases). A shift or misbalance in the level of inactive versus active extracellular enzymes can result in a net breakdown of matrix. The influence of extraocular factors and the effect of AGEs on collagen breakdown still need to be determined.

Knowledge about structures, cells and enzymes involved in the remodeling process of the human vitreous and vitreoretinal interface could lead to possible interventions in this process in the future. In a high risk population for PVD and retinal breaks (such as people with high myopia), selective inhibition of collagen producing (Müller) cells could diminish the amount of or weaken peripheral vitreoretinal attachments. In addition, by stimulation of extracellular enzymes involved in breakdown of vitreous collagen, collagen breakdown could be enhanced and thus liquefaction induced. The latter could result in an earlier PVD and thus ease a possible vitrectomy in young people.

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8

Summary

Chapter 1 is the introduction of this thesis.
The vitreous body (or vitreous) of the human eye is an almost acellular, transparent loose-meshed connective tissue consisting mainly of water (99%) and of just 0.1% macromolecules, such as glycosaminoglycans (e.g. hyaluronan), proteoglycans, glycoproteins (such as opticin), collagens, and noncollagenous structural proteins (e.g. fibrillin). The most important macromolecules are the collagens, which form a network of heterotypic fibrils (types II, V, IX, and XI) and presumably maintain the gel structure. Collagen types present in the vitreous are types II, V, VI, IX, and XI. Vitreous structure has been studied in some detail, but there is no absolute distinctness in anatomy in the literature, which might be explained by (i) the high water content, (ii) the use of different preservation methods, and (iii) the variable visualization techniques. The presence and course of intravitreal structures (e.g. lamellae, channels, and cisterns) are still discussed.

During embryonic development, the vitreous matrix is continuously processed. Postnatally, the vitreous is subjected to progressive morphological changes: a progressive change in the volume of liquefied spaces (synchisis) and an increase in optically dense structures (syneresis). The precise mechanisms underlying the age-related changes and posterior vitreous detachment (PVD) have still to be elucidated. Currently, two (patho)physiological mechanisms are described. In the first theory, synchisis and syneresis are the structural manifestations of a destabilization of the vitreous matrix, while, in the other theory, a continuous remodeling is taking place consisting of breakdown of vitreous matrix (synchisis) coinciding with synthesis of vitreous matrix components (syneresis).

This thesis tries to find and add evidence to the dynamic view of collagen turnover (synthesis and breakdown) in the adult and aging human vitreous by studying the vitreoretinal interface and the vitreous. We study (i) the vitreous and its collagenous interaction with the vitreoretinal interface (Ch. 2, 3), (ii) retinal Müller cells to determine their possible role in collagen synthesis and breakdown (Ch. 4, 5), and (iii) enzymatic collagen cross-links with aging (Ch. 6).

In **chapter 2**, the vitreoretinal interface, which is the area of contact between the vitreous body and the retina, was evaluated in the pre-equatorial and equatorial area. This region is clinically important, because the majority of retinal defects induced by PVD are located there. Whether a PVD will induce interface pathology depends on the nature of vitreoretinal adhesions and the thickness of the ILL (internal limiting lamina). In the vitreous base area, which is known for its extremely strong attachments, several variations in the vitreoretinal interface have been described. These consist of (i) direct insertions

of vitreous fibrils into and between adjacent Müller cells and (ii) sublaminar collagenous fibers expanding into collagen networks upon aging.

By immuno-transmission electron microscopy (immuno-TEM), we found intraretinal collagen packages positive for type II collagen. The distance of these packages to the ILL ranged from 0 to 44 µm. In most donor eyes, we found retinal Müller cells in close vicinity of the type II collagen packages. Furthermore, macrophages were present in the vitreous cortex and retina adjacent to these packages. We also observed a large variability in the structure of the vitreoretinal interface. We found vitreous lamellae perpendicular and parallel to the ILL, vitreous lamellae fusing with the ILL, direct vitreoretinal adhesions without ILL interpositioning, and focal interruptions in the ILL. Our findings expanded on previous scanning electron microscopy and TEM studies on the vitreous. Immuno-TEM revealed the nature of intraretinal collagen (type II) and of cells (macrophages) located near cell debris and demonstrated interruptions in the ILL (type IV collagen) and the location of retinal Müller cell processes.

The occurrence of collagen turnover in this area of the vitreoretinal interface resulting in a net synthesis of sublaminar collagen networks has been made plausible by others. Our data are in support of this theory, even though we are not able to show age-related phenomena due to our limited sample size. It is unclear what causes this remodeling at the vitreous base and equator. The net result of intraretinal collagen synthesis could occur in response to forces exerted upon this area, i.e. most likely vitreous movements and vitreoretinal tractional forces induced by eye movements, in order to strengthen this area.

We hypothesize that this remodeling gradually results in the formation of tighter vitreoretinal adhesions over time. Paradoxally, the anterior vitreoretinal interface will thus become more vulnerable for retinal tearing and detachment in the case of a PVD. The possible role of Müller cells in the synthesis and/or breakdown of intraretinal collagens will be discussed in chapters 4 and 5.

Chapter 3 is an immunohistochemical light microscopy (LM) study on the presence and distribution patterns of various collagens in the human retina at the posterior pole, the equator and the pre-equatorial area, since collagens are important macromolecules contributing to vitreoretinal adhesion and the vitreoretinal interface. Furthermore, the information about the distribution of collagens can contribute to our understanding of the (patho)physiology of a spontaneous, mechanical, or enzymatically induced PVD. We used four fresh equatorial retinectomy samples to detect mRNA of types I-VII, IX, and XI

collagen, which appeared to be present in all retinectomy samples.

By immunohistochemistry, the vitreous cortex was clearly positive for type II collagen and variably for types V, IX, and XI collagen. The ILM (internal limiting membrane) was clearly positive for types IV, VI, and XVIII collagen. Retinal blood vessels contained types I-VI and XVIII collagen, whereas types V and IX collagen were variably present. We found multiple circular and dotted spots positive for type VII collagen in several retinal layers. Type VII collagen positive spots were seen to increase in number and distribution area towards the posterior pole.

New findings were the presence of type II collagen in human retinal vasculature, the presence of type VI collagen in the ILM, and the presence of type VII collagen in several retina layers. The presence of type II collagen is interesting in the light of (i) strong interconnections between vitreous and retinal vasculature and (ii) the possible source of the type II collagen. The presence of mRNA *COL2A1* in the human retina could indicate the retina as a possible production place of type II collagen. The producing cell of type II collagen has still to be established, but Müller cells are good candidates, since they are attached to the retinal vasculature and ILM and their endfeet are closely related to sublaminar intraretinal type II collagen (Ch. 2). Furthermore, these cells are able to produce type II collagen in vitro (Ch. 5).

Type VI collagen has a predominant role in linking cells and matrix macromolecules. Based on the ubiquitous presence of type VI collagen at the ILM, we conclude that the entire vitreous is likely surrounded by this type of collagen, which thus could mediate an overall anchoring between the ILM and vitreous cortex. Type XVIII collagen can also act as part of an anchoring complex between the vitreous fibrillar collagens and the ILM. In addition, we found a condensed appearance of type VII collagen, an anchoring fibril, in the retina. This is a completely new finding and its intraretinal location and distribution pattern are surprising. Its function at this site has yet to be established.

In conclusion, the currently described distribution patterns of different collagen types in the human vitreoretinal interface emphasize the possible role of collagens in connecting the vitreous cortex to the retina.

Chapters 4 and 5 discuss the role of human retinal Müller cells in breakdown (phagocytosis) and synthesis of collagens in vitro, respectively. Müller cells are radially oriented macroglia that traverse the retina from its inner (vitreal) border to the outer limiting membrane. In addition, these cells are the principal glial cells in the retina and have many local functions such as stabilization of the retinal architecture, metabolic support to surrounding cells,

prevention of photoreceptor migration, phagocytosis of several substances, and production of cytokines, growth factors and receptors. Based on our previous immuno-TEM findings (Ch. 2), Müller cells could be involved in collagen synthesis and/or breakdown. A possible role in the production of vitreous macromolecules was suggested during growth and in adulthood based on morphological studies, while it was unknown whether these cells had a role in phagocytosis of collagens. To study their possible role in collagen turnover, we used a cell culture model.

In **chapter 4**, spontaneously immortalized human retinal Müller cells (MIO-M1) were exposed to collagen-coated fluorescent latex beads to study their role in phagocytosis of collagens. The focus was on collagen types of the vitreous and the ILL, types II and IV, respectively. Latex beads were coated with types I, II, and IV collagen and bovine serum albumin. Human fibroblasts served as control cells. All cells were exposed to coated latex beads for 2, 12, 24, and 48 hours. In order to influence the process of phagocytosis, we added cytochalasin B and anti-integrin subunits α1, α2, and β1 to Müller cells. Phagocytosis was evaluated by flow cytometry, TEM, and confocal microscopy. We found that Müller cells kept their main characteristics in vitro. Comparing all experiments, cultured Müller cells phagocytozed beads coated with type II collagen better than beads with another coating. TEM and confocal microscopy showed internalized beads. The phagocytosis of all types of beads by Müller cells is about 10 times lower compared with fibroblasts. In our model, we could not influence phagocytosis of type II collagen-coated beads by the addition of cytochalasin B and anti-integrin subunits. Based on the low phagocytic capacity of this Müller cell line, we speculate that Müller cells are possibly involved in the slow process of vitreoretinal remodeling in adult human eyes.

Chapter 5 evaluates the in vitro capacity of the human Müller cell line (MIO-M1) to synthesize types I-VII, IX, XI, and XVII collagen. Type XVII collagen is not related to the vitreoretinal interface and was used as a negative control. In this study, we demonstrated the capacity of Müller cells for collagen production by RT-PCR, their cytoplasmic expression of collagens by immunocytochemistry and their capacity to secrete collagens into the cell medium by Western Blotting.

Cells were cultured to confluence and fixed on the culture plate or harvested and analyzed. Cultured Müller cells kept their main characteristics and were capable of collagen synthesis, except for type XVII collagen. Müller cells expressed mRNAs coding for types I-VII, IX, and XI collagen. At the protein level, these collagens were present in the cytoplasm as detected by immunocytochemistry with LM. WB analysis of the cell extracts and of the medium in which the cells had been cultured confirmed the intracellular production and

demonstrated that types I-VII, IX, and XI collagen were also secreted into the medium.

Thus, Müller cells could synthesize those collagens that are found in their natural vicinity (vitreous, ILL, and retina). In theory, the in vitro capability of Müller cells to produce the above-mentioned collagens might adduce support to previously described morphological findings in the embryonic period consisting of Müller cells' capacity to form collagens of the vitreous and ILL. Furthermore, the collagen production by these cells could fit the theory of turnover of postnatal vitreous collagen. Finally, Müller cells can contribute to the formation of epiretinal membranes, since they represent one of the cell types observed in these membranes and collagens identified therein are consistent with those found in our study.

Chapter 6 is concerned with the role of the mature enzymatic collagen cross-links, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), in the adult and aging human vitreous body (VB). The pyridinoline cross-links form the last enzymatic step in collagen maturation. Briefly, collagen is synthesized intracellularly and secreted into the ECM as procollagen. After removal of the C- and N-propeptides, the molecules can aggregate into fibrils, which are stabilized by the formation of enzymatic intermolecular and/or intramolecular cross-links. They provide physical and mechanical strength to the collagen network and thus contribute to its integrity.

In this study, we used human eyes (n=143) from 119 donors with no known ophthalmic disorders. All VBs were freeze-dried before high performance liquid chromatography analysis. In the analysis of paired eyes (n=24), right and left eyes showed a high correspondence in all tested variables.

Age-related phenomena were analyzed in single eyes (n=119). Vitreous wet weight appeared to increase until 35 years and to decrease significantly thereafter, while VB collagen content only declined significantly after 50 years. The Hyp/Pro ratio showed a significant decrease with aging, implying a relatively higher increase in the amount of non-collagenous proteins compared to collagens. The percentage of collagen remained constant over time. Mature enzymatic collagen cross-links showed an increase until 50 years and a decline thereafter. This increase should be attributed primarily to HP, since this is the most abundant collagen cross-link and the HP/LP ratio does not change significantly with aging.

The increase in the enzymatic cross-links per TH could be explained by the formation of enzymatic cross-links from the processing of type II procollagen present in the VB. The decline after 50 years occurs at an age at which morphological changes in

the VB become more prominent. This could be clarified by breakdown or loss of collagen cross-links, which is supported, in this study, by the decrease in collagen content after 50 years.

In conclusion, our results on enzymatic collagen cross-links can contribute to the insight in the age-related processes synchisis and syneresis in the concept of collagen turnover. The accumulation of collagen cross-links until 50 years is an indication of (ongoing) collagen maturation, which in its turn can indicate an ongoing collagen synthesis. At the age of 50 years when striking morphological changes in the VB are evident, the enzymatic collagen cross-links start to diminish. This decline can be caused by collagen breakdown, but more importantly the decline itself can contribute to the instability of the collagen network resulting in an increase in morphological changes in the elder VB.

In **Chapter 7** we discuss the relevance of our results in a review on remodeling of the human adult vitreous and vitreoretinal interface as a dynamic process.

Our results support the concept of collagen synthesis and breakdown in the aging vitreous and vitreoretinal interface. Furthermore, we describe the presence and location of several different collagen types in the human retina. Some of these collagens (such as type VI, VII, and XVIII) are possibly involved in the vitreoretinal attachment.

With aging, the anterior vitreoretinal interface is remodeled by an increase in sublaminar type II collagen deposition and an increase in vitreoretinal adhesions. Retinal cells, presumably Müller cells, are likely involved in this process. In the mean time, the vitreous matrix differentiates with the formation of dense collagen structures, while a process of matrix degeneration results in liquefied spaces. Our results on mature enzymatic collagen cross-links support this theory by the finding of collagen maturation till 50 years and collagen breakdown thereafter.

9

Nederlandse samenvatting

Hoofdstuk 1 vormt de introductie van het proefschrift.
Het glasachtig lichaam (glasvocht) van het menselijk oog is een heldere gel, die de ruimte tussen de lens en het netvlies (retina) vult (*zie "vb" in figuur 1 uit hoofdstuk 1*). Het glasvocht is een transparant losmazig bindweefsel, dat voornamelijk bestaat uit water (98-99%), zouten en kleine moleculen (1%) en slechts 0.1% bouwstenen in de vorm van grote moleculen. Voorbeelden van deze grote moleculen zijn proteoglycanen (bijv. hyaluronan; HA), glycoproteïnen (bijv. opticine), collagenen en overige niet-collagene structurele eiwitten (bijv. fibrilline). De collagenen, lijmvormende eiwitten die een zeer belangrijk bestandsdeel zijn in humane bindweefsels, zijn waarschijnlijk verantwoordelijk voor de structuur van de glasvochtgel. Collageen type II is het meest voorkomende type gevolgd door type IX, V, XI en VI.

Over de exacte structuur van het glasvocht is nog geen eenduidigheid in de literatuur. Dat kan verklaard worden door (i) het hoge watergehalte, (ii) de verschillende technieken om het glasvocht te bewaren en (iii) de verschillende technieken om het glasvocht af te beelden. De aanwezigheid en het verloop van glasvochtstructuren, zoals lamellen, kanalen en cisternen, worden nog steeds bediscussieerd.

Tijdens de embryonale ontwikkeling is het glasvochtweefsel constant aan verandering onderhevig. Van collageenvezels tussen de embryonale lens en de retina gaat het over in een gel met bloedvaten, die vervolgens bij de geboorte weer zijn verdwenen. Na de geboorte ondergaat het glasvocht progressieve structurele veranderingen die al vanaf het vierde jaar waarneembaar zijn. Er is een progressieve toename van vervloeide holten (synchisis) en een toename van optische verdichtingen (syneresis). De onderliggende mechanismen die leiden tot deze leeftijdsgerelateerde veranderingen en een achterste glasvochtmembraanloslating (posterior vitreous detachment; PVD) zijn tot op heden niet bekend. Er zijn twee theorieën, waarbij de eerste theorie synchisis en syneresis benadert als een structurele uiting van de destabilisatie van de glasvochtstructuur. De andere theorie gaat uit van een constante afbraak (synchisis), die gepaard gaat met aanmaak van glasvocht onderdelen (syneresis).

Het huidige proefschrift ondersteunt de tweede theorie op het gebied van collageenturnover (aanmaak en afbraak) in het volwassen en verouderende humane glasvocht. Ditzal blijken uit onze onderzoeksresultaten van het glasvocht zelf en de overgang van het glasvocht en de retina (vitreoretinale grenslaag). Hoofdstuk 2 en 3 beschrijven de collageeninteractie van het glasvocht en de vitreoretinale overgang. Vervolgens wordt de rol van de retinale Müller cel in collageenafbraak en collageenproductie besproken (hoofdstuk 4 en 5). Hoofdstuk 6 beschrijft wat er gebeurt met de enzymatische collageenverbindingen

tijdens veroudering. Enzymatische collageenverbindingen ontstaan als laatste in de ontwikkelingsfase van het collageen molecuul, zijn zeer stevig en dragen bij aan de structuur van een bindweefsel. Tot slot worden onze bevindingen geplaatst in een review over het verouderende humane glasvocht (hoofdstuk 7).

In **hoofdstuk 2** beschrijven wij het onderzoek van de vitreoretinale grenslaag, het contactgebied van het glasvocht en de retina, aan de voorzijde van het humane oog (donorogen). Dit gebied is klinisch van belang omdat de meerderheid van de netvliesbeschadigingen als gevolg van een PVD daar ontstaat. Of een PVD leidt tot een netvliesbeschadiging hangt af van de glasvocht-retina aanhechtingen in de grenslaag en de dikte van de ILM (internal limiting membrane of membrana limitans interna, de laag tussen glasvocht en retinacellen). Voorin het oog vlak achter de lens bevindt zich de glasvochtbasis, waar extreem sterke aanhechtingen in de grenslaag gevonden worden. In dat gebied worden verschillende variaties beschreven in de grenslaag. Voorbeelden hiervan zijn (i) directe verbindingen tussen glasvochtstrengen en retinale Müller cellen en (ii) glasvochtachtige collageenvezels in de retina die uitbreiden in collageen netwerken met het toenemen van de leeftijd.

Met behulp van immunohistochemische kleuringen hebben we door middel van transmissie elektronenmicroscopie (TEM) aangetoond dat het collageen in de retina type II betreft. Pakketjes collageen reiken van 0 – 44 micrometer diep in de retina. In de meeste donorogen worden retinale Müller cellen dicht in de buurt van de collageenpakketten gezien. Daarnaast hebben we veel variatie in het verloop van de grenslaag gevonden, zoals (i) glasvochtstrengen loodrecht en parallel aan de ILM, (ii) glasvochtstrengen die fuseerden met de ILM, (iii) directe aanhechtingen van glasvocht aan de retina zonder aanwezigheid van de traditioneel aanwezige ILM en (iv) lokale onderbrekingen in de ILM. Bovendien hebben onze resultaten eerdere EM- bevindingen versterkt. Wij hebben het - tot dan toe onbekende - type collageen in de retina (type II) en de positie van retinale Müller cellen bepaald. We hebben cellen (macrofagen) naast celrestanten gevonden en discontinuïteit van collageen type IV in de ILM aangetoond.

Uit een andere studie naar de grenslaag kwam naar voren dat bij veroudering de collageenpakketten in de retina toenamen in grootte en zich verspreidden richting de achterkant van het oog. Dit is een direct bewijs van collageenturnover met een netto collageenproductie. Onze studie ondersteunt deze theorie. Op dit moment is het echter niet duidelijk wat deze verandering in de grenslaag in gang zet. De netto productie van collageen in de retina kan een reactie zijn op krachten die in deze regio aanwezig zijn om

dit gebied te verstevigen; het meest voor de hand liggend zijn glasvochtbewegingen en vitreoretinale trekkrachten als gevolg van oogbewegingen.

We speculeren dat deze geleidelijke veranderingen in de grenslaag met het stijgen van de leeftijd leiden tot de vorming van stevige vitreoretinale aanhechtingen. In het geval van een PVD zal de vitreoretinale grenslaag in de glasvochtbasis paradoxaal genoeg kwetsbaarder zijn voor netvliesbeschadigingen. De mogelijke rol van retinale Müller cellen in aanmaak en afbraak van collagenen zal verder besproken worden in hoofdstuk 4 en 5.

Hoofdstuk 3 bevat een immunohistochemische, lichtmicroscopische (LM) studie naar de aanwezigheid en het verdelingspatroon van verschillende collagenen in de humane retina aan de achterkant (achterpool), het middengedeelte (equator) en de voorzijde van het oog (glasvochtbasis).

We hebben specifiek naar collagenen gekeken vanwege hun rol in de vitreoretinale adhesie en vitreoretinale grenslaag. Het verdelingspatroon van collagenen kan bijdragen aan de ontrafeling van het mechanisme van een spontane, mechanische, of enzymatische PVD.

Voor dit onderzoek hebben we zowel donorogen als verse stukjes retina uit het equator gebied gebruikt. In de laatste hebben we aanwijzingen, d.w.z. mRNA van collageen typen I-VII, IX en XI, gevonden die wijzen op collageenaanmaak. Door analyse met specifieke antilichamen bleek het glasvocht positief voor collageen type II en in wisselende mate voor collageen typen V, IX en XI. De ILM was positief voor collageen typen IV, VI en XVIII. De retinale bloedvaten bevatten collageen typen I-VI en XVIII, maar ook wisselend typen V en IX. Bovendien vonden we meerdere ronde en gespikkelde structuren positief voor collageen type VII in verscheidene lagen van de retina. Het aantal structuren dat positief was voor collageen type VII nam toe in aantal en verdeling over de retinale lagen richting de achterkant van het oog.

Deze studie geeft duidelijk inzicht in de verdelingspatronen van de verschillende typen collageen in de vitreoretinale grenslaag. De nieuwe bevindingen in deze studie waren de aanwezigheid van (i) collageen type II in de humane retinale bloedvaten, (ii) collageen type VI in de ILM en (iii) collageen type VII in verscheidene lagen van de retina. De aanwezigheid van collageen type II in de retina is interessant vanwege de sterke aanhechtingen tussen glasvocht en retinale bloedvaten en vanwege de mogelijke oorsprong van het collageen type II. Op basis van het voorkomen van collageen type II mRNA in de retina zouden retinacellen een mogelijke bron van synthese kunnen zijn. Dit zouden de retinale Müller cellen kunnen zijn, omdat zij met de bloedvaten en ILM in contact staan

en hun celuitlopers zich dicht in de buurt bevinden van de genoemde collageenpakketten in de retina (Hoofdstuk 2). Bovendien hebben we laten zien dat Müller cellen in kweek collageen type II produceren (Hoofdstuk 5).

Verder speelt collageen type VI een belangrijke rol in het verbinden en koppelen van cellen en grote matrix moleculen. Gezien de prominente aanwezigheid van collageen type VI in de ILM zou dit molecuul kunnen bijdragen aan de hechting van glasvochtvezels aan de retina, maar collageen type XVIII zou ook betrokken kunnen zijn. Een geheel nieuwe bevinding tot slot is, dat collageen type VII (een bekende hechtingsvezel) met een bijzonder spreidingspatroon in de retina aanwezig is. De functie hiervan is echter nog niet vastgesteld.

Wij kunnen concluderen dat er verschillende typen collageen in de humane grenslaag aanwezig zijn en dat deze een mogelijke rol hebben bij de verbinding van het glasvocht met de retina.

In de **hoofdstukken 4 en 5** beschrijven wij de rol van humane retinale cellen in afbraak (fagocytose) en aanmaak van verschillende collagenen in kweek. Müller cellen zijn cellen die de gehele breedte van de retina overbruggen van de ILM tot aan de fotoreceptoren. Deze cellen zijn de belangrijkste glia cellen in de retina en hebben vele lokale functies zoals: stabilisatie van de retinale architectuur, metabole ondersteuning van omringende cellen, voorkomen van verplaatsing van fotoreceptoren, opruimen van (afval-)stoffen en productie van celregulerende stoffen (bijv. groeifactoren). Op basis van onze eigen TEM bevindingen (Hoofdstuk 2) kunnen Müller cellen betrokken zijn bij afbraak en/of productie van collageen. In eerdere studies is meerdere malen gesuggereerd dat Müller cellen betrokken kunnen zijn bij de productie van glasvocht- en ILM-collageen. Hun rol bij de afbraak van collagenen is onbekend. Voor onderzoek naar de rol van Müller cellen bij collageenturnover hebben we een kweekmodel gebruikt.

Hoofdstuk 4 laat zien dat we humane retinale Müller cellen (MIO-M1; spontaan geïmmortaliseerde Müller cellen) hebben blootgesteld aan fluorescerende latex bolletjes gecoat met collageen om hun rol in fagocytose van collagenen vast te stellen. De focus lag op de belangrijkste typen collageen van het glasvocht (type II) en de ILM (type IV). De fluorescerende latex bolletjes werden gecoat met collageen type I, II en IV en een controle eiwit (BSA). We gebruikten humane fibroblasten (cellen die bekend staan om hun fagocytosecapaciteit) als controle. Alle cellen werden gedurende 2, 12, 24 en 48 uur blootgesteld aan gecoate latex bolletjes. Het fagocytose-proces probeerden we te beïnvloeden door remmende stoffen aan de Müller cellen toe te dienen (cytochalasine B

en anti-integrines). We evalueerden fagocytose met flow-cytometrie, TEM en confocale microscopie.

In kweek behielden de Müller cellen hun belangrijkste celeigenschappen, die we met specifieke antilichamen controleerden middels LM. Hieruit bleek dat gekweekte Müller cellen latex bolletjes gecoat met collageen type II het best fagocyteerden. In vergelijking met fibroblasten fagocyteerden Müller cellen 10 keer zo weinig (niet-)gecoate latex bolletjes. In dit model waren we echter niet in staat om fagocytose van collageen type II door de bovengenoemde remmende stoffen te beïnvloeden. De lage fagocytose capaciteit van gekweekte Müller cellen suggereert een mogelijke rol van deze retinale cellen bij de geleidelijke collageenverandering in de humane grenslaag.

In **hoofdstuk 5** onderzoeken we de capaciteit van de gekweekte humane retinale Müller cellijn (MIO-M1) om collageen typen I-VII, IX, XI en XVII te produceren. Collageen type XVII is een collageen type dat niet gerelateerd is aan de vitreoretinale grenslaag en is gebruikt als een negatieve controle. In deze studie tonen we aan (i) met behulp van RT-PCR (reverse transcriptase-polymerase chain reaction) dat Müller cellen mRNA van collageen bevatten, (ii) met behulp van immunohistochemie met specifieke antilichamen (LM) dat collageen in hun cellichamen aanwezig is en (iii) met behulp van Western Blot dat deze cellen collageen kunnen uitscheiden in het celmedium.

Müller cellen bleken alle bovengenoemde typen collageen te kunnen synthetiseren met uitzondering van collageen type XVII. Müller cellen brachten mRNA voor collageen typen I-VII, IX en XI tot expressie. Bovendien waren deze collagenen, die met specifieke antilichamen werden aangekleurd, aanwezig in de cellichamen. De Western Blot analyse op het celmedium, waarin de cellen gekweekt werden, bevestigde andermaal dat Müller cellen collageen produceerden en dat collageen typen I-VII, IX en XI werden uitgescheiden in het celmedium.

We kunnen concluderen dat Müller cellen collagenen kunnen produceren die gevonden worden in hun natuurlijke omgeving (glasvocht, ILM en retina). In theorie kan de productiecapaciteit van bovengenoemde collagenen door de Müller cellen in kweek bijdragen aan eerder beschreven morfologische bevindingen in de embryonale periode, waarin Müller cellen aangewezen werden als mogelijke bron van glasvocht- en ILM-collageen. Daarnaast past de collageenproductie door deze cellen in de theorie van collageenturnover van volwassen glasvochtcollageen. Tot slot zouden Müller cellen kunnen bijdragen aan de vorming van (pathologische) collageenachtige laagjes (van genoemde collageen typen) op de retina (epiretinale membranen), omdat deze cellen hierin worden gevonden.

Hoofdstuk 6 beschrijft de rol van volgroeide enzymatische collageenverbindingen (cross-links), hydroxylysylpyridinoline (HP) en lysylpyridinoline (LP), in het volwassen en verouderende humane glasvocht. De pyridinoline cross-links vormen de laatste enzymatische stap in de collageenontwikkeling. Collageen wordt intracellulair geproduceerd en uitgescheiden in de extracellulaire matrix als collageen voorloper (procollageen). Na het verwijderen van de pro-groepen kunnen de moleculen samengaan en dunne vezels (fibrillen) vormen. Deze fibrillen worden gestabiliseerd door cross-links tussen de collageenmoleculen gevormd door een enzymatische reactie. Deze collageen-cross-links zorgen voor fysische en mechanische kracht van het collageennetwerk en zo dragen ze bij aan de netwerkintegriteit.

Voor deze studie hebben we 143 humane donorogen zonder bekende oogheelkundige pathologie geanalyseerd van 119 donoren. Alle glasvochten zijn gevriesdroogd voor de analyse middels high performance liquid chromatograpy (HPLC) om het volume te verkleinen.

De analyse van 24 gepaarde ogen toonde een grote overeenkomst tussen rechter- en linkerogen in alle geteste variabelen. We gebruikten daarom een willekeurig oog van één donor (n=119) in de verdere analyses naar leeftijdsgerelateerde veranderingen.

Het glasvocht bleek in gewicht toe te nemen tot de leeftijd van vijfendertig jaar en daarna daalde het significant, terwijl het gewicht van glasvochtcollageen significant daalde na het vijftigste levensjaar. De verhouding tussen glasvochtcollageen en niet-collagene eiwitten in het glasvocht ging omlaag met het toenemen van de leeftijd. Dit zou veroorzaakt kunnen worden door een relatief hogere stijging van de niet-collagene eiwitten in vergelijking met collagenen. Het collageenpercentage bleef constant tijdens veroudering. De enzymatische collageen-cross-links stegen tot het vijftigste levensjaar en daalden daarna. Deze stijging kon voornamelijk toegeschreven worden aan de stijging van HP, omdat dit de meest aanwezige collageen-cross-link was en de verhouding tussen HP en LP niet veranderde met het stijgen van de leeftijd.

De stijging van deze collageen-cross-links per collageen molecuul zou verklaard kunnen worden door de nieuwe vorming van enzymatische cross-links uit procollageen type II, dat in het humane glasvocht is gevonden. De daling na het vijftigste levensjaar valt samen met de leeftijd waarop de structurele veranderingen in het glasvocht meer op de voorgrond komen. Dit zou verklaard kunnen worden door afbraak of verlies van collageen-cross-links, wat ondersteund wordt door de afname van de hoeveelheid collageen na het vijftigste levensjaar.

De onderzoeksresultaten van de enzymatische collageen-cross-links kunnen

dus inzicht geven in de leeftijdsgerelateerde processen synchisis en syneresis binnen het concept van collageenturnover. De stijging van collageen-cross-links tot het vijftigste levensjaar is een indicatie van (continue) collageenuitrijping, dat kan wijzen op een continue collageenaanmaak. Als er in het glasvocht grote structurele veranderingen plaatsvinden na het vijftigste levensjaar, beginnen de enzymatische collageen-cross-links te dalen. Deze afname kan het gevolg zijn van collageenafbraak, maar - belangrijker nog - de afname zelf kan ook bijdragen aan de instabiliteit van het collageennetwerk leidend tot een toename van structurele veranderingen van het oudere glasvocht.

In het laatste **hoofdstuk 7** presenteren wij alle nu bekende resultaten in een overzicht over de collageenveranderingen van het humane volwassen glasvocht en de vitreoretinale grenslaag als een dynamisch proces.

Onze resultaten ondersteunen het concept van collageenaanmaak en -afbraak in het glasvocht en de vitreoretinale grenslaag tijdens het toenemen van de leeftijd. We leveren nieuw bewijs voor collageenturnover. Bovendien beschrijven wij de aanwezigheid en de locatie van verschillende typen collageen in de humane retina. Deze verschillende typen collageen hebben een mogelijke rol bij de verbinding van het glasvocht met de retina.

Met het stijgen van de leeftijd is de vitreoretinale grenslaag in het gebied van de glasvochtbasis onderhevig aan verandering door toename van collageen type II in de retina en door toename van het aantal collagene vitreoretinale adhesies. Retinacellen, in het bijzonder Müller cellen, zijn waarschijnlijk betrokken bij dit proces. Gelijktijdig ontwikkelen zich in het glasvocht dichte collagene glasvochtstructuren, terwijl een afbraakproces van glasvochtstructuren leidt tot vervloeide holtes. Onze onderzoeksresultaten van de volwassen enzymatische collageen-cross-links in het glasvocht ondersteunen deze theorie door de bevinding van collageenuitrijping tot het vijftigste levensjaar en collageenafbraak daarna.

List of abbreviations

Dankwoord

Curriculum Vitae

List of abbreviations

AEC	3-**A**mino-9-**E**thyl**c**arbazole
AGE	**A**dvanced **G**lycation **E**nd product
ANOVA	**An**alysis **O**f **Va**riance
β-APN	β-**A**mino**p**ropio**n**itrile fumurate salt
BPS	**B**ase **P**air**s**
BSA	**B**ovine **S**erum **A**lbumin
CB	**C**iliary **B**ody
Col I	Type **I** **Col**lagen
CRALBP	**C**ellular **R**etin**al**dehyde **B**inding **P**rotein
cDNA	**C**omplement **DNA**
DAPI	4',6-**Dia**midino-2-**p**henyl**i**ndole dihydrochloride
DMEM	**D**ulbecco's **M**odification of **E**agle's **M**edium
DNA	**D**eoxyribo**n**ucleic **A**cid
ECM	**E**xtra**c**ellular **M**atrix
EDTA	**E**thylene**di**amine**te**traacetic **A**cid
FBS	**F**etal **B**ovine **S**erum
FLF-92	**F**oetal **L**ung **F**ibroblast-92
GA	**G**lutar**a**ldehyde
GAG	**G**lycos**a**mino**g**lycan
GAMbio	**G**oat-**a**nti-**m**ouse-**bio**tin
GARPO	**G**oat-**a**nti-**r**abbit **P**er**o**xidase
GCL	**G**anglion **C**ell **L**ayer
GFAP	**G**lial **F**ibrillary **A**cidic **P**rotein
GP	**G**lyco**p**rotein
HA	**H**yaluronan
HFBA	**H**epta**f**luoro**b**utyric **A**cid
HBL-100	**H**uman Caucasian **B**reast Cancer Cell **L**ine (epithelial like)
HP	**H**ydroxylysyl**p**yridinoline
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
Hyp	**Hy**droxy**p**roline
ILL	**I**nternal **L**imiting **L**amina (TEM description)
ILM	**I**nternal **L**imiting **M**embrane (LM description)
INL	**I**nner **N**uclear **L**ayer

IPL	**I**nner **P**lexiform **L**ayer
LM	**L**ight **M**icroscopy
KDa	**K**ilo**d**alton
LP	**L**ysyl**p**yridinoline
MIO-M1	**M**oorfields **I**nstitute of **O**phthalmology-**M**üller **1** (Müller cell line)
MMP	**M**atrix **M**etallo**p**roteinase
mRNA	**m**essenger-**R**ibo**n**ucleic **A**cid
NFL	**N**erve **F**iber **L**ayer
ONL	**O**uter **N**uclear **L**ayer
OPL	**O**uter **P**lexiform **L**ayer
PAS	**P**eriodic **A**cid **S**chiff
PBS	**P**hosphate **B**uffered **S**aline
PF	**P**ara**f**ormaldehyde
PG	**P**roteo**g**lycan
PPR	**P**ars **P**lana **R**emnants (predominantly of the ciliary body)
Pro	**Pro**line
PVD	**P**osterior **V**itreous **D**etachment
RAGPO	**R**abbit-**a**nti-**g**oat **P**ero**x**idase
RAMPO	**R**abbit-**a**nti-**m**ouse **P**ero**x**idase
RT	**R**oom **T**emperature
RT-PCR	**R**everse **T**ranscriptase-**P**olymerase **C**hain **R**eaction
SAPO	**S**trept**a**vidin **P**ero**x**idase
SARPO	**S**wine-**a**nti-**r**abbit **p**ero**x**idase
SDS-PAGE	**S**odium **D**odecyl **S**ulfate-**P**olyacryl**A**mide **G**el **E**lectrophoresis
T8100	**T**echnovit 8100
TEM	**T**ransmission **E**lectron **M**icroscopy
(T)TBS	**T**ris **B**uffered **S**aline (containing 0.05% **T**ween-20)
TH	**T**riple **H**elix
UV	**U**ltra**v**iolet
VB	**V**itreous **B**ody
WB	**W**estern **B**lot

Dankwoord

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Tot slot wil ik graag mijn familie en vrienden bedanken voor hun steun en interesse in mijn onderzoek. Lieve vader en moeder, dankzij jullie positieve ondersteuning ben ik een heel vrolijk, enthousiast en gelukkig mens geworden. Ik ben ontzettend blij met de keuzes die ik heb kunnen maken en het enthousiasme dat jullie mij gaven om mijn dromen te verwezenlijken. Vader dank je wel voor je kritische Nederlandse tekstcorrecties. Lieve Bart, dank je wel voor je positieve discussies en (technisch-farmaceutische) adviezen gedurende mijn onderzoek. Lieve Marius en Riny, dank jullie wel voor het nauwkeurig doorlezen van mijn stukken. De overeenkomst tussen het glasvocht en Shell werd pas duidelijk toen het glasvocht door 6M HCl bij 110 °C compleet "gekraakt" was (Hoofdstuk 6). Diego Keijzer, tevens paranimf, je bent al meer dan 25 jaar een trouwe rots in de branding en zodoende een meer dan belangrijk aandeel in mijn leven.

Lieve Rosette, mijn ware, de laatste loodjes van mijn onderzoek waren, met en door onze Marius, oprecht het zwaarst. Jouw onvoorwaardelijke liefde en steun hebben het mogelijk gemaakt het onderzoek ook daadwerkelijk af te ronden. De komst en eerste blikken van onze Marius op deze wereld (5 april 2008) zijn een onbetaalbaar geschenk en ik ben enorm trots op je! De eerstvolgende vakantie neem ik geen artikelen mee.....

Curriculum Vitae

Dirk Ponsioen was born in Leiden on January 12th 1977. Living his entire youth in Oegstgeest, he completed his secondary school education at the Stedelijk Gymnasium in Leiden in 1995. In September of the same year, he started his medical studies at the University of Groningen and joined Vindicat atque Polit, a student union. In 1997, he started rowing at Aegir, where he took place in the executive committee from 1998 to 1999. During his study, he also assisted students in human anatomy, gave first aid lessons on a primary school, and worked for the "domiciliary care Groningen".

In 2000, he started his internships, which were followed mainly in the University Medical Center Groningen (UMCG) and in the "Isala Klinieken" (Zwolle). In order to finish his Master's degree, he did two scientific trainings at the department of Ophthalmology (UMCG) under supervision of dr N.M. Jansonius and dr L.I. Los, in 2001 and 2002, respectively. In April 2002, he obtained his Master's degree in medicine and started his PhD project "Age-related liquefaction and the role of the vitreoretinal border" at the department of Ophthalmology (UMCG) and at the Department of Pathology and Medical Biology of the University of Groningen. His PhD-project started in combination with his final internship, which was done at the ophthalmology department in the "Isala Klinieken". In October 2002, he received his Medical Degree and continued his PhD project on a full-time basis. Since August 2005, he is in training to become an ophthalmologist (head and supervisor Prof. Dr. J.M.M. Hooymans).

In August 2006, Dirk Ponsioen married Rosette Bremmer. They are currently living in Groningen. On April 5th 2008, their son Marius was born.