Age-Related Changes on the Surface of Vitreous Collagen Fibrils

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PURPOSE. To determine whether aging vitreous collagen fibrils undergo ultrastructural changes that might underlie vitreous liquefaction and posterior vitreous detachment.

METHODS. Vitreous collagen fibrils from 21 human subjects (age range, 3–89 years) and from bovine eyes were isolated on electron microscopy grids. Cupromeronic blue labeling in the presence of 0.5 M MgCl2 and immunogold labeling for collagen types II and IX were analyzed by transmission electron microscopy.

RESULTS. Aging was associated with marked changes on the surface of human vitreous collagen fibrils, including an exponential loss of type IX collagen along with its chondroitin sulfate side-chains (half-life, 11 years) and a fourfold increase in the exposure of type II collagen.

CONCLUSIONS. Despite being a minor component of vitreous collagen fibrils, type IX collagen, probably by virtue of its chondroitin sulfate side-chains, shields type II collagen from exposure on the fibril surface. With aging, this shielding diminishes, resulting in the surface exposure of "sticky" type II collagen and thus predisposing the vitreous collagen fibrils to fusion. These changes could underlie vitreous liquefaction and weakening of vitreoretinal adhesion. (Invest Ophthalmol Vis Sci. 2004;45:1041-1046) DOI:10.1167/iovs.03-1017

The vitreous humor is a transparent gel consisting almost entirely of extracellular matrix. The cortical gel is normally adherent to a basement membrane on the inner surface of the retina called the inner limiting lamina (ILL).1 During aging there is progressive gel liquefaction2 that, in combination with weakening of the vitreoretinal adhesion, can eventually result in posterior vitreous detachment (PVD), an event where the vitreous cortex is dissected from the ILL of the posterior retina by fluid vitreous. The overall rate of PVD in a study of autopsy eyes was 23%, but the rate increased to 27% in the seventh decade and reached 63% by the eighth decade.3 While PVD itself does not affect vision, it plays a pivotal role in a number of common blinding eye diseases including rhegmatogenous retinal detachment,3 proliferative diabetic retinopathy,4 and macular hole formation.5

The gel state of the vitreous humor is maintained by a dilute network of long, thin collagen fibrils.1 These collagen fibrils are initially distributed throughout the vitreous gel in bundles that form a contiguous network through individual collagen fibrils traversing from one bundle to another.6 Within and between these bundles the individual collagen fibrils are normally spaced apart. However, evidence suggests that with aging there is a progressive lateral fusion of the individual fibrils.1,7 This process results in aggregates of collagen that are perceived as “floaters” by subjects with these degenerative changes. Furthermore, fibril fusion results in the redistribution of the collagen fibrils with an increased concentration in the residual gel, but a decreased concentration and ultimate absence of fibrils in other areas, resulting in liquefaction.8 Recently an alternative mechanism for vitreous liquefaction has been proposed whereby the collagen fibrils are fragmented,9 and it is conceivable that both of these mechanisms could contribute to vitreous liquefaction.

Vitreous collagen fibrils are heterotypic (mixed) in composition and contain collagen types II, V/XI, and IX, with type II collagen predominating.1 The collagen molecules within these fibrils are cross-linked together to provide the fibrils with mechanical stability. Collagen types II and V/XI are fibrillar collagens that self-assemble into staggered arrays and cross-link to form the rope-like core of the fibrils. Type IX collagen is covalently linked to the surface of the fibrils in a D-periodic distribution.9–11, a repeat of 67 nm that can be discerned at an ultrastructural level using stains such as uranyl acetate. Type IX collagen is composed of three collagenous domains (COL1 to 3) interspersed between four noncollagenous domains (NC1 to 4), and it is covalently bound to type II collagen through the COL2 domain.12 Type IX collagen in vitreous humor is a proteoglycan with a single chondroitin sulfate side-chain covalently linked to the collagenous protein core.13 In other tissues, glycosaminoglycan chains such as chondroitin sulfate may contribute toward the maintenance of spacing between adjacent collagen fibrils.14,15

Fibrillar collagens, such as type II collagen, have a propensity to fuse on contact (as occurs within fibrils during fibrillation). We hypothesize that components on the surface of the vitreous collagen fibrils, such as type IX collagen, shield type II collagen and thus prevent fusion between fibrils. However, during aging, this shielding is lost and type II collagen becomes increasingly exposed on the fibril surface, thus predisposing to fusion of adjacent fibrils on contact. To test this hypothesis, we undertook ultrastructural analyses to determine whether the surface composition of the vitreous collagen fibrils changed with increasing age. Initial studies were performed with bovine vitreous to establish experimental protocols. Analysis of human vitreous collagen fibrils then revealed profound aging changes, including a loss of type IX collagen
and a corresponding increase in type II collagen exposure on the surface of the fibrils.

METHODS

Sample Collection and Preparation

Eyes of 2-year-old steers were obtained from a local abattoir and kept on ice. The vitreous gels were either dissected from the eyes within 2 hours postmortem or the eyes were kept at 4°C for 2 weeks before removal of the gels. Some vitreous gels that had been removed after 2 hours underwent six freeze/thaw cycles, the gels being thawed for 3 hours on each occasion. The vitreous gels were then Dounce homogenized, thus creating a viscous liquid containing a suspension of collagen fibrils.

Human cadaver eyes were obtained from the Manchester Royal Eye Hospital Eye Bank (UK) after removal of the corneoscleral rim for transplantation. Donor eyes were only used when specific permission had been granted for research, and the research adhered to the tenets of the Declaration of Helsinki. The donors ranged in age from 3 to 89 years and had no history of ocular disease. The vitreous gel was dissected from one of the eyecups within 48 hours postmortem and stored at −20°C. On thawing, each vitreous gel was Dounce homogenized.

For electron microscopy, collagen fibrils were mounted on 400 mesh nickel electron microscopy (EM) grids by immersing the grids in the homogenate for at least 30 seconds. The collagen fibrils were thereby attached to the grids at certain points along their length, but between these points were suspended freely in the solutions while being labeled with cupromeronic blue or immunogold. This technique provided the fibrils with uniform access to reagents during labeling procedures and, as a backing of carbon film was added after the labeling (see below), background labeling was virtually absent.

Antibody Preparation and Western Blot Analysis

Intact and pepsinized type IX collagen were both purified from bovine vitreous humor as previously described.15 Intact type IX collagen was used to generate a (rabbit) polyclonal antiserum. Pepsin-resistant fragments of type IX collagen containing the COL1 and COL2 domains were separated by gel filtration chromatography using a Sephacryl 200 HR column (100 × 2.5 cm) equilibrated in 20 mM Tris, pH 7.4, containing 1 M NaCl, before dialysis against distilled water and lyophilization. The COL2-containing fraction was redissolved in 0.1 M NaHCO3 and coupled to cyanoan bromide-activated Sepharose 4B resin (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. The resultant resin was used to affinity purify antibodies to the COL2 domain from the polyclonal antiserum. The antiserum was applied to the column which was then washed with 0.5 M NaCl, 0.05% Tween-20 in 0.1 M Tris/HC1 (pH 8.0) before elution with 0.5 M NaCl, 0.05% Tween-20 in 50 mM glycine-HC1 (pH 2.5). The eluted antibodies (called anti-IX) were then equilibrated to pH 8 with 1 M Tris/HC1 (pH 9).

Samples were subjected to SDS-PAGE using 4 to 12% NuPAGE Bis Tris gels with a MOPS buffer system (Invitrogen, Paisley, UK). The gels were then stained with silver or subjected to Western blot analysis, Tris gels with a MOPS buffer system (Invitrogen, Paisley, UK). The gels underwent six freeze/thaw cycles, the gels being thawed for 3 hours on each occasion. The vitreous gels were then Dounce homogenized, thus creating a viscous liquid containing a suspension of collagen fibrils.

Immunolabeling for Electron Microscopy

The anti-IX antibody was used at a dilution of 1:10 and C-19 (Autogen Bioclear UK Ltd., Wiltshire, UK), a (goat) polyclonal antibody specific for type II collagen (anti-II), was used at a 1:50 dilution. Appropriate nonimmune sera were used for controls. All incubations were performed in 0.5 mL microcentrifuge tubes. Fibrils were mounted on grids and incubated in TBS containing 2% milk protein for 10 minutes. The grids were then incubated in primary antibody diluted in TBS containing 0.05% milk protein (TBS-milk) and protease inhibitors (as above) overnight in the dark at ambient temperature. The grids were then washed with TBS-milk before incubation for 2 hours with the appropriate 5 nm gold particle-conjugated secondary antibody (British BioCell Int. Ltd., Cardiff, UK) diluted 1:25 in TBS-milk. Finally, the grids were washed briefly with TBS-milk, backed by picking up carbon film floating on distilled water, negatively-stained with 2% uranyl acetate (pH 4.2), and air dried.

Transmission Electron Microscopy and Analysis of Data

Specimens were examined in a JEOL 1200 EX transmission electron microscope operated at 60 kV and images were recorded at x30,000 on Emar EM film (Kodak, Hemel Hempstead, UK). Micrographs were collected from areas of grids that contained segments of collagen fibrils that were neither parts of aggregates nor very close to neighboring fibrils. The discrete cupromeronic blue/glycosaminoglycan complexes formed electron-dense filaments. These were counted along long segments of collagen fibril and the number of electron dense filaments per micron of axial length calculated. To measure the lengths of the fibrils segments analyzed, the micrograph images were loaded into the SemMetrics analysis program (Synoptics Ltd., Cambridge, UK). In total for each sample, fibril segments adding up to approximately 135 microns were measured distributed between 50 and 100 fibrils. Immunogold labeling was quantified in the same way, but in addition to the above criteria, gold particles were only counted when they were within 30 nm of the fibrils. This limit of 30 nm was set to encompass the maximum size of the primary and gold-conjugated secondary antibody complex easily, but also to ensure that particles associated with adjacent fibrils were not counted.

RESULTS

Immunoelectron Microscopy and Cupromeronic Blue Labeling

Initial experiments were undertaken with bovine collagen to optimize conditions and to determine whether storage of vitreous gel at 4°C and/or multiple freeze/thaw cycles affected cupromeronic blue labeling or immunogold labeling with the antibodies to type II and IX. When compared with freshly prepared grids, no significant differences could be observed between the different groups (data not shown). Therefore, for
human samples, we used the relatively stringent protocol of a maximum postmortem time of 48 hours (for eyes kept at 4°C) before isolation of the vitreous gel, and one freeze/thaw cycle before homogenization.

Cupromeronic blue labeling took the form of electron-dense filaments on the surfaces of both bovine and young human vitreous collagen fibrils. These electron-dense filaments were often distributed in a regular, D-periodic distribution along the fibrils. The electron-dense filaments were generally aligned parallel to the fibril surfaces unless fibrils were close together in which case orthogonally-orientated filaments were observed that appeared to bridge between the adjacent fibrils as has previously been described (Fig. 1A). These different filament morphologies may not represent in vivo states as the chondroitin sulfate chains are artificially collapsed to form crystals with the cupromeronic blue, whereas in vivo they will be in a highly hydrated state. To establish the specificity of the cupromeronic blue labeling, vitreous homogenate was digested with chondroitin ABC lyase before incubation with cupromeronic blue. This resulted in an almost complete abolition of labeling (Fig. 1B), thus establishing that the cupromeronic blue formed filamentous complexes with chondroitin/dermatan sulfate glycosaminoglycan chains.

Vitreous collagen fibrils from young human eyes were decorated with the electron-dense cupromeronic blue filaments. However, even in the youngest samples, the complexes appeared to be less frequent than on the bovine vitreous collagen fibrils (Figs. 1 and 2). Moreover, the electron dense filaments were virtually absent on collagen fibrils from aged subjects (Fig. 2). When the data from all 21 vitreous humor samples was analyzed and mean cupromeronic blue filaments per micron of axial fibril length plotted against age for each sample, an exponential decay curve fitted the data points well (Fig. 3A). A half-life for the cupromeronic blue filaments of 11.4 years was derived from this exponential fit.

As we surmised that the cupromeronic blue filaments represented the chondroitin sulfate side-chains of type IX collagen, experiments were also undertaken to determine whether there were changes in the concentration of type IX collagen core protein on the surface of the collagen fibrils with age. An antibody (anti-IX) was generated against type IX collagen and tested by Western blot analysis of bovine vitreous collagens (Fig. 4). Anti-IX labeled the COL2 and COL1 domains of the type IX collagen, but showed no cross-reactivity with types II or V/XI collagen. Attempts were also made to test anti-IX against pepsin digests of adult human vitreous collagens. There was no evidence of cross-reactivity with type II or V/XI, but because of the virtual absence of type IX collagen from these samples (see below) it could not be clearly detected by Western blot analysis (data not shown). The immunolabeling exper-

**Figure 1.** Cupromeronic blue labeling of bovine vitreous collagen fibrils. The electron micrographs show labeling with cupromeronic blue in 0.3 M MgCl$_2$ (A) without and (B) with prior chondroitin ABC lyase digestion. The electron dense cupromeronic blue filaments on the collagen fibril surfaces were almost completely removed by the enzyme digestion, demonstrating that they represent chondroitin/dermatan sulfate glycosaminoglycan chains. Bar 100 nm.

**Figure 2.** Exemplar electron micrographs of collagen fibrils from the youngest (age 3 years) and oldest (age 89 years) subjects. The top two panels show labeling with cupromeronic blue in 0.3 M MgCl$_2$, the middle panels immunogold labeling with the antibody to type IX collagen (anti-IX), and the lower panels immunogold labeling with the antibody to type II collagen (anti-II). Both cupromeronic blue filaments and anti-IX immunogold labeling are evident in the young sample, but virtually absent in the sample from the elderly subject. Conversely, there was very little immunogold labeling with the anti-II antibody of the young sample, but extensive labeling of the sample from the elderly subject. Bar 100 nm.
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iments with human vitreous samples revealed scattered immunogold labeling of vitreous collagen fibrils from young eyes, but this labeling was at a very low level on the collagen fibrils from adult subjects (Fig. 2). Incubation of samples with preimmune serum produced essentially no immunogold labeling (data not shown). When mean gold particles per micron of axial fibril length was plotted against age for each sample, a good fit was obtained to an exponential decay curve and a half-life of 11.0 years was derived (Fig. 3B). Further analysis of these data was undertaken by plotting log mean cupromeronic blue filaments per micron against log mean immunogold particles per micron for each sample; this revealed a linear relationship (Fig. 3C). From this analysis we inferred that the loss of the chondroitin sulfate chains was closely related to the loss of type IX collagen. Therefore, the loss of the glycosaminoglycan chains is likely to be secondary to removal or fragmentation of the type IX collagen core proteins, as it is these that are covalently linked into the fibrils.12

To determine whether the loss of type IX collagen (and possibly other molecules) resulted in an increased exposure of type II collagen at the surface of the fibrils, immunolocalization studies were undertaken with anti-II. The collagen fibrils from young eyes revealed very little anti-II immunogold labeling (Fig. 2), implying that there was very little type II collagen exposed on the surface of the collagen fibrils as only surface epitopes would have been available to the antibody.17 However, collagen fibrils from elderly eyes showed extensive immunogold labeling, so in these vitreous samples type II collagen molecules (or at least the epitopes for anti-II) were exposed on the fibril surfaces. When mean anti-II immunogold labeling per micron of axial fibril length was plotted against age for each sample, a fourfold increase in immunogold labeling was observed between the youngest and oldest vitreous collagen fibril samples (Fig. 5A). In a further analysis, log mean anti-II immunogold particles per micron was plotted against log mean anti-IX immunogold particles per micron for each sample, revealing an inverse relationship (Fig. 5B). Therefore, the loss of type IX collagen from the fibril surfaces correlated...
Here we show for the first time that there are major and progressive changes on the surface of vitreous collagen fibrils as a result of aging, and we believe this is the first study to analyze age-related surface changes on collagen fibrils from any tissue comprehensively. The collagen fibrils lost type IX collagen proteoglycan from their surface with aging and there was a concomitant increased surface exposure of type II collagen.9,13,25,26 However, in adult human vitreous, type IX collagen content has been variously estimated to be between 7% and 25% of the total collagen.24

The age-related loss of type IX collagen from the surface of vitreous fibrils is presumed to involve proteolysis as this collagen is covalently cross-linked into the fibrils.12 The exponential fit to the graphs in Figures 3A and 3B suggests that this proteolytic process occurs at a constant rate. The underlying mechanisms remain unclear but one possible explanation is that matrix metalloproteinase activity may play a role; MMP2 has been demonstrated in human vitreous humor25 and this enzyme is capable of fragmenting type IX collagen and causing vitreous liquefaction.26

In young adult bovine vitreous, type IX collagen content has been variously estimated to be between 7% and 25% of the collagen.9,13,25,26 However, in adult human vitreous, type IX collagen content is likely to be much lower as it was virtually undetectable by Western blot analysis of pepsin-derived extracts. Cartilage collagen fibrils also contain collagen types II, IX, and V/IX, and, similarly, in mature human cartilage, type IX collagen is at low levels, representing only 1–2% of the total collagen.12 Mature cartilage contains thin collagen fibrils that resemble vitreous collagen fibrils and thick fibrils that probably represent aggregates of these thin fibrils.27 Interestingly, only the thin fibrils are coated with type IX collagen, so a similar process may be occurring in vitreous and cartilage whereby the thin fibrils aggregate with increasing age because of surface changes including the loss of type IX collagen.27

**DISCUSSION**

The age-related surface changes on the vitreous collagen fibrils may cause decreased vitreoretinal adhesion which, combined with the vitreous liquefaction, predisposes to PVD. The vitreous collagen fibrils at the vitreoretinal interface do not directly insert into the ILL, but are adherent to it while otherwise being oriented parallel to the ILL surface.18,19 Therefore, it is likely that vitreoretinal adhesion is afforded by components on the surface of vitreous collagen fibrils linking (directly or indirectly) to the ILL and if the surfaces of the vitreous collagen fibrils are radically altered, vitreoretinal adhesion is consequently modified.

There was an exponential loss of cupromeronic blue labeled filaments with aging that is likely to reflect a loss of type IX collagen molecules (and their chondroitin sulfate side-chains) for the following reasons. Cupromeronic blue-labeled filaments are absent in chondroitin ABC lyase-treated vitreous (Fig. 1), demonstrating that the filaments contain chondroitin or dermatan sulfate. Vitreous contains two chondroitin sulfate proteoglycans, namely type IX collagen and versican,16 but there is no evidence for the presence of dermatan sulfate.16,20 To date, no small leucine-rich repeat chondroitin sulfate proteoglycans have been identified associated with vitreous collagen fibrils (these often decorate collagen fibrils in other tissues). Instead, vitreous collagen fibrils are decorated with a small leucine-rich repeat glycoprotein called opticin, which possesses sialylated O-linked oligosaccharides but no glycosaminoglycan chains.21,22 The chondroitin sulfate chains on the vitreous collagen fibrils are likely to represent type IX collagen glycosaminoglycan side-chains (as opposed to versican) because they were often observed, like the type IX collagen core protein,9,11 to be regularly aligned along the fibril surfaces in a D-periodic distribution (whereas versican is linked to the hyaluronan network that fills the spaces between the vitreous collagen fibrils).16 Finally, our results here show a strong correlation between the loss of chondroitin sulfate and the loss of immunogold labeling of type IX collagen core protein, with both having a half-life of 11 years, thus substantiating the argument that the filaments revealed by cupromeronic blue represent the glycosaminoglycan side-chains of type IX collagen. It is conceivable that instead of the chondroitin sulfate chains being lost from the fibril surface they are enzymatically desulfated, thus losing their ability to form crystallites with cupromeronic blue under the conditions of these experiments. However, this explanation is unlikely because of the clear correlation between the loss of the cupromeronic blue staining and the loss of type IX collagen immunogold labeling from the fibril surface.
The age-related loss of type IX collagen and the concomitant increase in surface exposure of type II collagen could modulate other vitreoretinal disease processes. Cells proliferate within the vitreous cavity in conditions such as proliferative diabetic retinopathy and proliferative vitreoretinopathy, and the ability of cells to interact with vitreous collagen (e.g., through integrins) is likely to be influenced by the surface composition of the fibrils. Furthermore, the vitreous humor provides an important reservoir of growth factors within the eye, and it is likely that some of these are retained by binding vitreous collagen fibrils. Therefore, growth factor content and availability could change with aging owing to altered interactions with vitreous collagen.

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**References**