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Age-Related Liquefaction of the Human Vitreous Body: LM and TEM Evaluation of the Role of Proteoglycans and Collagen

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Purpose. To evaluate morphologic aspects of age-related liquefaction of the human vitreous body by light and electron microscopy to provide a basis from which future studies directed at the pathogenesis of this phenomenon can be undertaken. The study focuses on changes in fibrillar collagen and proteoglycans (PGs).

Methods. Morphologic aspects of intravitreal liquefied spaces and matrix areas surrounding them were examined in 13 adult human donor eyes (aged 21–80 years) by light (LM) and transmission electron microscopy (TEM). Collagen fibrils were visualized by using standard contrasting methods. PGs were specifically stained by cupromeronic blue (CB).

Results. Eyes from older donors contained larger spaces than eyes from younger ones. Transitions between matrix and spaces were abrupt or gradual. In transition areas of all specimens, a gradual decrease in the number of collagen fibers, and to a lesser extent of PGs was observed. In addition, a fragmentation of collagen fibers and an aggregation of PG-molecules around these fragments were found. Neither cells nor their fragments were observed in these areas.

Conclusions. This is the first study to evaluate vitreous liquefaction at the light and electron microscopic level. A breakdown of collagen fibrils into smaller fragments seems to be crucial to the pathogenesis of age-related liquefaction of the human vitreous body. The mechanism inducing fragmentation of vitreous fibrils has yet to be elucidated. From the absence of cells and cellular remnants in all specimens, it is tentatively concluded that an extracellular process is involved. (*Invest Ophthalmol Vis Sci.* 2003;44:2828–2833) DOI:10.1167/iovs.02-0588

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The human vitreous body is subject to age-related changes. Morphologically, two distinct structural alterations can be observed. On the one hand, there is a progressive increase in the volume of liquefied spaces (synchysis),[1–4] and on the other, there is an increase in optically dense areas (syneresis).[5–7] Clinically, liquefaction is an important phenomenon, because it ultimately results in posterior vitreous detachment (PVD),[1–3,8] which may induce potentially serious pathologic events at the vitreoretinal interface (e.g., retinal tears, intravitreal hemorrhage, or retinal detachment).

These structural changes have been observed by macroscopic techniques, in vivo by slit lamp biomicroscopy,[1] and in vitro with the naked eye, low-power magnification,[8] and dark-field slit lamp microscopy.[6,7]

The pathophysiological mechanisms underlying synchysis and syneresis have not yet been elucidated. Earlier studies have speculated that these two phenomena are pathophysiologically interrelated,[4,7,9] and are the structural manifestation of a destabilization of the vitreous matrix. Vitreous matrix structure supposedly is maintained by interactions between its main macromolecular components: collagens, proteoglycans (PGs), glycoproteins (GPs), and hyaluronan (HA).[9–12] Fibrillar collagen presumably is the most essential molecule in the maintenance of gel structure. Other macromolecules are thought to stabilize the collagen network by various interactions, which ultimately result in the formation of infinite macromolecular networks. Chondroitin sulfate PGs observed in the vitreous include type IX collagen and versican.[13] Recently, opticin, an extracellular matrix leucine-rich-repeat protein, has been identified in the vitreous.[13] It has been suggested that PGs and opticin are essential in maintaining the spacing between collagen fibrils and prevents them from self-aggregating.[13] Generally, liquefaction is supposed to start with changes in the noncollagenous components of the matrix[9–13] and to result in an aggregation of collagens. This would cause a simultaneous collapse of the collagen framework (syneresis) and the formation of collagen-free, liquid-filled spaces (synchysis).

As mentioned, data on macroscopic aspects of vitreous liquefaction are readily available in the literature. Also, biochemical studies have been directed at changes in the composition of the vitreous matrix on aging and differences in macromolecular composition between gel and liquid parts of the vitreous.[4,14–16] However, data on the light (LM) and electron (EM) microscopic appearances of structural changes in the vitreous due to liquefaction are extremely scarce, and to our best knowledge, the subject has never before been systematically evaluated at these levels. In our view, such data are fundamental to any hypothesis on the pathophysiology of vitreous liquefaction. As previously described,[17] we embed large specimens in glycol methacrylate (GMA) medium (Technovit 7100; Heraeus Kulzer, Wehrheim, Germany), which enables us to get an overview of large parts of the vitreous matrix by LM. We then select specific parts to evaluate these in more detail by transmission EM (TEM). The present LM and TEM study concentrates on a possible role for PGs in stabilizing vitreous

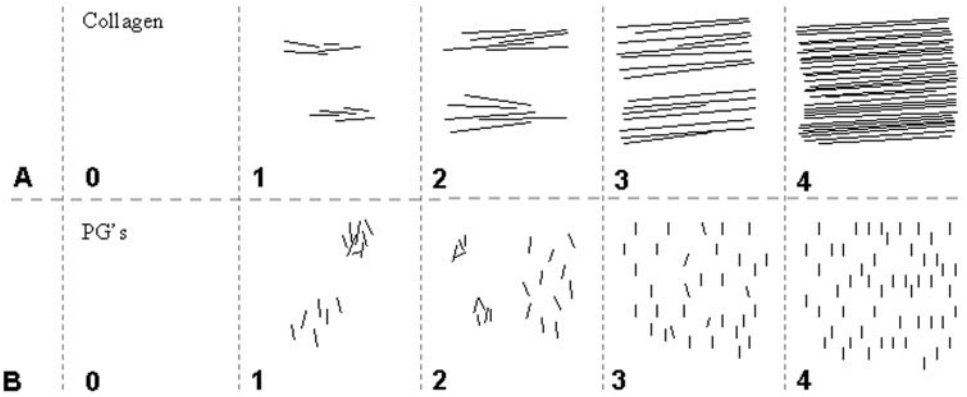

FIGURE 1. Representation of collagen (**A**) and proteoglycan (**B**) densities. Fragmentation of collagen fibrils (i.e., the presence of shorter fragments) is clearly visible in (**A-1**). Clustering of PGs is clearly visible in (**B-1**) in the *top right* corner, and (**B-2**) on the *left*.

collagens in conjunction with their proposed function as "spacers" of fibrillar collagen in the cornea.[12,18] PGs can be specifically demonstrated in extracellular matrices by the critical electrolyte concentration (CEC) method, which was introduced by Scott.[19]

We assumed that structural changes reflecting vitreous liquefaction could best be evaluated at the borders of intravitreal spaces—that is in areas where a transition between normal vitreous gel structure and liquid vitreous takes place. Quite unexpectedly, we never observed any prominent aggregation of collagen fibrils in these areas. On the contrary, a fragmentation of collagen fibrils was consistently found in all specimens. This necessitates a reevaluation of previously forwarded theories on vitreous liquefaction in which collagen fibril aggregation is a central event, and it opens the way to future studies exploring which pathophysiological mechanisms may induce a breakdown of the collagen framework itself.

MATERIALS AND METHODS

We studied 13 human donor eyes (13 donors) with ages varying between 21 and 80 years (median donor age was 63 years). Eyes were obtained from the Cornea Bank (Amsterdam, The Netherlands) after removal of the corneas for transplantation. The provisions of the Declaration of Helsinki for research involving human tissue were observed. Specimens were studied by LM and TEM. Specimens were fixed by immersion within 48 hours after death in 2% glutaraldehyde (GA, TAAB Laboratories, Aldermastron, UK) in 0.1 M phosphate buffer (pH 7.4) for several days. After removal of small parts of the globe at 6 and 12 o'clock, specimens were fixed for an additional 4 hours in the same fixative. Specimens were washed in 6.8% sucrose in phosphate-buffered saline (PBS) for 2 hours and briefly in double-distilled water. After transection of the globes, specimens were immersed for 20 hours in 0.2% aqueous cupromeronic blue (CB; Seikagaku Co., Tokyo, Japan) containing 0.025 M NaAc, 0.3 M $MgCl_2$, and 2.5% GA. Specimens were briefly washed in staining solution minus CB, washed in double-distilled water, and either dehydrated through ethanol-propylene oxide mixtures and embedded in Epon 812 (Serva Feinbiochemica, Heidelberg, Germany) or dehydrated through increasing concentrations of ethanol and embedded in GMA (Technovit 7100; Heraeus Kulzer). The GMA-embedded materials were cut on a microtome (Reichert Jung, Vienna, Austria). Sections 3 to 4 μm thick were stained with toluidine blue (TB) and evaluated by LM. Areas of interest were selected for evaluation by TEM and cut from larger plastic blocks. Small plastic blocks were cut on a second microtome (Sorvall, Newtown, CT). Thin sections approximately 200 nm thick were mounted on polyvinyl formal–coated grids (Formvar; SPI, West Chester, PA), counterstained with uranyl acetate in 25 centipoise methylcellulose and evaluated with a TEM (model 201; Philips, Eindhoven, The Netherlands) operated at 80 kV. For the Epon-embedded materials, the areas of interest were directly selected for evaluation by TEM and cut from the larger plastic blocks. Semithin sections (1–3 μm) were stained with TB and evaluated by LM. Ultrathin sections (80–90 nm) were contrasted with uranyl acetate and lead citrate and examined with the TEM operated at 60 kV.

Chondroitin sulfate proteoglycan (CS-PG) is the predominant PG in the vitreous.[20,21] It can be removed by the action of chondroitinase ABC. Parts of some specimens ($n = 5$) were subjected to chondroitinase ABC treatment before CB staining as follows. Specimens were incubated overnight at 37°C in chondroitinase ABC 0.1 U/mL (C-3667; Sigma-Aldrich, St. Louis, MO) in 25 mM Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$, washed in Tris buffer and in double-distilled water.

Statistical Analysis

Morphologic data were semiquantitatively analyzed as follows: Within each specimen ($n = 13$) transition areas were identified. Micrographs were taken by one of the authors (RJW) from three morphologically distinct areas within each transition area: (1) regular matrix (area I); (2) transition area in the strict sense (area II), and (3) border area (area III). All micrographs were printed at the same magnification and randomly presented to two independent masked observers (LIL, MJAL). In each micrograph, four characteristics were independently scored: collagen density (scales 0–4); proteoglycan density (scales 0–4); collagen fragmentation (+, ±, −); and clustering of PGs (+, ±, −). Scales 0 to 4 were defined graphically (Figs. 1A, 1B), and the scales +, ±, and − were defined as: (+) markedly present, (±) moderately present, and (−) (almost) completely absent. The four characteristics were compared between areas I and II, and areas II and III by a paired *t*-test. Data are presented as the mean ± SEM. Statistical significance was defined as $P < 0.05$. Interobserver variability was tested using the two-factor ANOVA with replication test. Individual scores per photograph were averaged when interobserver differences did not reach statistical significance ($P > 0.05$), otherwise data were presented separately as individual scores for each observer.

RESULTS

Light Microscopy

Liquefied spaces were observed in all specimens. In general, eyes from older donors contained larger spaces than eyes from younger ones. A certain structural variability was present at the borders of intravitreal spaces: At some borders, a gradual and at others a more abrupt transition between formed vitreous and liquefied spaces was observed (Figs. 2, 3A, 4A). The most striking structural change observed in transition areas between regular matrix and liquefied spaces consisted of a decrease in the number of collagen fibrils and the appearance of scattered elements that stained intensely with TB (Fig. 2).

For evaluation by TEM, we mainly selected border areas between regular matrix and liquefied spaces located in the intermediate vitreous (i.e., the area between the vitreous cen-

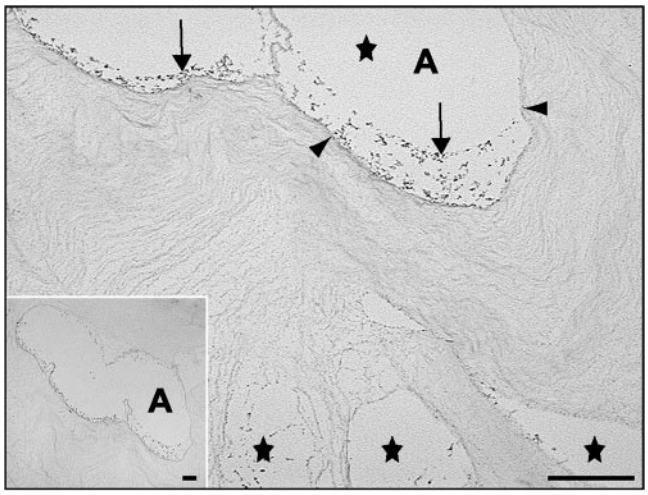

FIGURE 2. LM of section of a 21-year-old donor eye (GMA-embedding). Overview of vitreous matrix containing a number of spaces (★). A rather abrupt transition between formed matrix and intravitreal space (A) is seen (*arrowhead*). *Arrows*: Granular, intensely stained material at the border of (A). *Inset*: overview of superior space. Bars, 50 μm.

ter and the cortex). Spaces were observed in the anterior, equatorial, and posterior vitreous. In most eyes, several samples per bulbus, obtained from different anteroposterior locations, were evaluated.

Transmission Electron Microscopy

Transition areas identified by LM were selected for subsequent TEM-evaluation. They were subdivided in (1) regular matrix, (2) transition area in the strict sense, and (3) border area.

The regular matrix (1) had a regular organization pattern consisting of fibrillar collagen in an essentially parallel orientation, interspersed with CB-positive structures at more or less regular intervals and oriented at various angles to the collagen fibers (Figs. 3B, 4B).

In transition areas (2), a decrease in the number of fibers and a decrease in the length of individual fibers (fragmentation) was observed. In addition, changes were observed in the distribution of PGs. Most strikingly, PGs lost their orderly distribution and formed aggregates alongside the collagen fragments. This phenomenon was most clearly seen (3) at the borders of the spaces (Figs. 3D, 4D). It could be appreciated more easily in the GMA-embedded specimens (Fig. 3) than in the Epon-embedded material (Fig. 4), because ultrathin sections from the former were somewhat thicker than those from the latter and the GMA-material therefore contained more matrix components per section. In addition, a decrease in the number of PG molecules was observed. This was most clearly apparent in the Epon-embedded specimens (Fig. 4C). The intravitreal spaces themselves appeared completely devoid of macromolecular components (Fig. 2). Cupromeronic blue–positive structures were absent from chondroitinase ABC–treated specimens (not shown), which would be consistent with their chondroitin sulfate proteoglycan (CS-PG) nature.[20,21]

Neither by LM nor by TEM did we observe cells or cellular debris in transition areas between regular matrix and liquefied spaces in any of the specimens.

Statistical Analysis

Interobserver variability, as tested by two-factor ANOVA, was observed only for clustering of PGs ($P = 0.0006$; Fig. 5). This was caused by a systematic higher score given by one of the observers (Fig. 5D). On comparison of areas I and II, significant

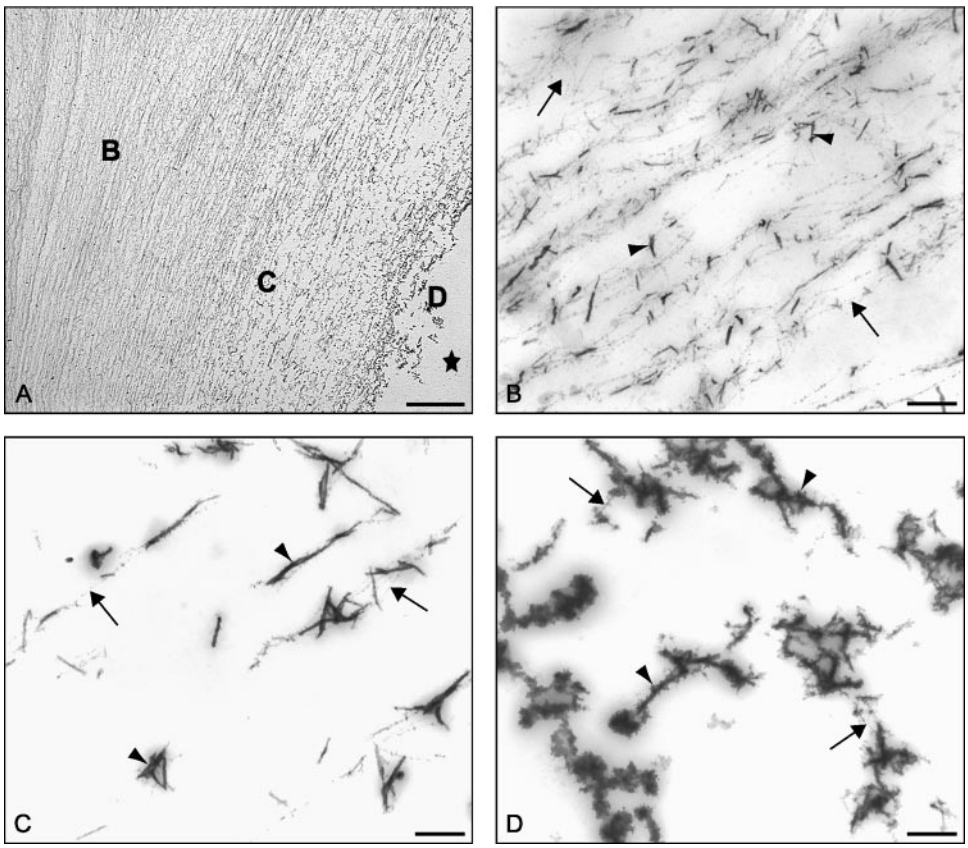

FIGURE 3. Micrographs of sections from a 63-year-old donor eye (GMA-embedding). (**A**) LM: Transition areas indicated by B, C, and D; intravitreal space (★). (**B–D**) TEM: details of areas corresponding to (B, C, D) in (**A**). (**B**) Regular matrix. *Arrows*: collagen fibrils; *arrowheads*, PGs. (**C**) Fragmentation of collagen framework and a decrease in the number of collagen fibrils (*arrows*). In addition, a decrease in the number of PGs and clustering of remaining PGs (*arrowheads*) on collagen fragments. (**D**) Larger proteoglycan aggregates (*arrowheads*) clustered around smaller collagen fragments (*arrows*). Bar: (**A**) 100 μm; (**B–D**) 1 μm.

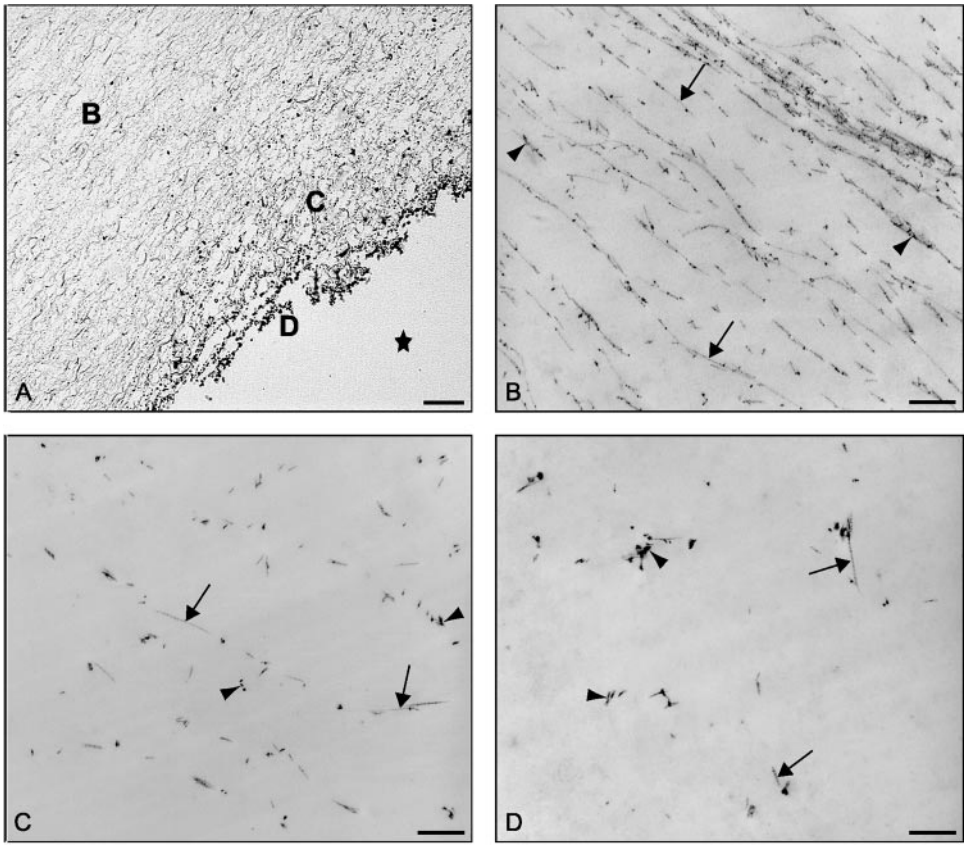

FIGURE 4. Micrographs of sections from a 56-year-old donor (Epon embedding). (**A**) LM: Transition areas indicated by B, C, and D; intravitreal space (★). (**B–D**) TEM: details of areas corresponding to B, C, and D in (**A**). (**B**) Regular matrix. *Arrows*: collagen fibrils; *arrowheads*: PGs. (**C**) Fragmentation of collagen framework, showing a decrease in the number of collagen fibrils (*arrows*) and PGs (*arrowheads*). Aggregation of PGs is much less evident than in Figure 3C. (**D**) Aggregation of PGs (*arrowheads*) around collagen fragments (*arrows*). Bar: (**A**) 30 μm; (**B–D**) 1 μm.

differences were observed in collagen density ($P = 0.000002$), collagen fragmentation ($P = 0.00013$), proteoglycan density ($P = 0.00004$), and clustering of PGs ($P = 0.013$; observer 1 vs. $P = 0.0012$; observer 2). On comparison of areas II and III, significant differences were observed in collagen density ($P = 0.00054$), proteoglycan density ($P = 0.00056$), and clustering of PGs ($P = 0.00029$; observer 1 vs. $P = 0.003$; observer 2). In this case, fragmentation of collagen was of borderline significance ($P = 0.052$).

DISCUSSION

In the present study, performed at LM and TEM levels, we found evidence of collagen breakdown and a loss of PGs in age-related vitreous liquefaction. Statistical analysis of the morphologic data strongly supports the impression obtained by morphologic evaluation, that there was a gradual decrease in collagen and proteoglycan densities from area I (regular matrix) toward area III (border of liquefied space). Also, a gradual increase in clustering of PGs from areas I to III was present. In addition, a statistically significant increase in collagen fragmentation was observed from areas I to II (transition area in the strict sense). A further increase in fragmentation was observed toward area III, but this increase is only of borderline significance ($P = 0.052$).

Collagen breakdown as a mechanism of vitreous matrix destabilization has not been considered in macroscopic morphologic studies. The latter invariably suggests that liquefaction is the result of a change in noncollagenous matrix components, normally acting as "spacers" for the collagen framework. Changes in these components would result in an aggregation and an increase in cross-linking of collagen fibrils (syneresis) on the one hand and liquefaction (synchysis) on the other. Hereby, synchysis and syneresis presumably are induced simultaneously and through the same pathogenetic mechanism.

Whereas our findings do not exclude an additional role for noncollagenous matrix components in vitreous liquefaction, the finding of collagen fragmentation instead of collagen aggregation in transition areas, necessitates a reevaluation of the previously proposed interrelationship between synchysis and syneresis. For, if collagen breakdown, and not its aggregation, is a crucial mechanism underlying vitreous liquefaction, how do synchysis and syneresis then relate to each other? Furthermore, if collagen breakdown takes place with aging, what explains the constant amount of vitreous collagen in various age groups observed in a biochemical study?[4]

We propose that the seeming inconsistency between a constant amount of collagen throughout aging and the finding of collagen breakdown on aging could be resolved by assuming that collagen synthesis may still occur in the adult eye. Newly synthesized collagen could then be incorporated in the matrix at sites not necessarily related to the areas of liquefaction and give rise to an increase in optically dense structures during aging. This implies that synchysis and syneresis may be induced by two pathogenetically unrelated mechanisms. In effect, our study questions the long-held assumption that the vitreous matrix is an almost inert extracellular matrix (ECM) in which collagen turnover would not occur (see also item 3, turnover of matrix components, further on).

Our findings of a fragmentation and overall decrease in the number of collagen fibrils and PGs and an aggregation of PGs at the borders of intravitreal spaces could be consistent with a focal breakdown of the collagenous vitreous meshwork. Because multiple small fragments were found, instead of few large fragments, we consider it more logical to assume an enzymatic, rather than a mechanical type of breakdown, even though traction forces may play a secondary role in enzymati-

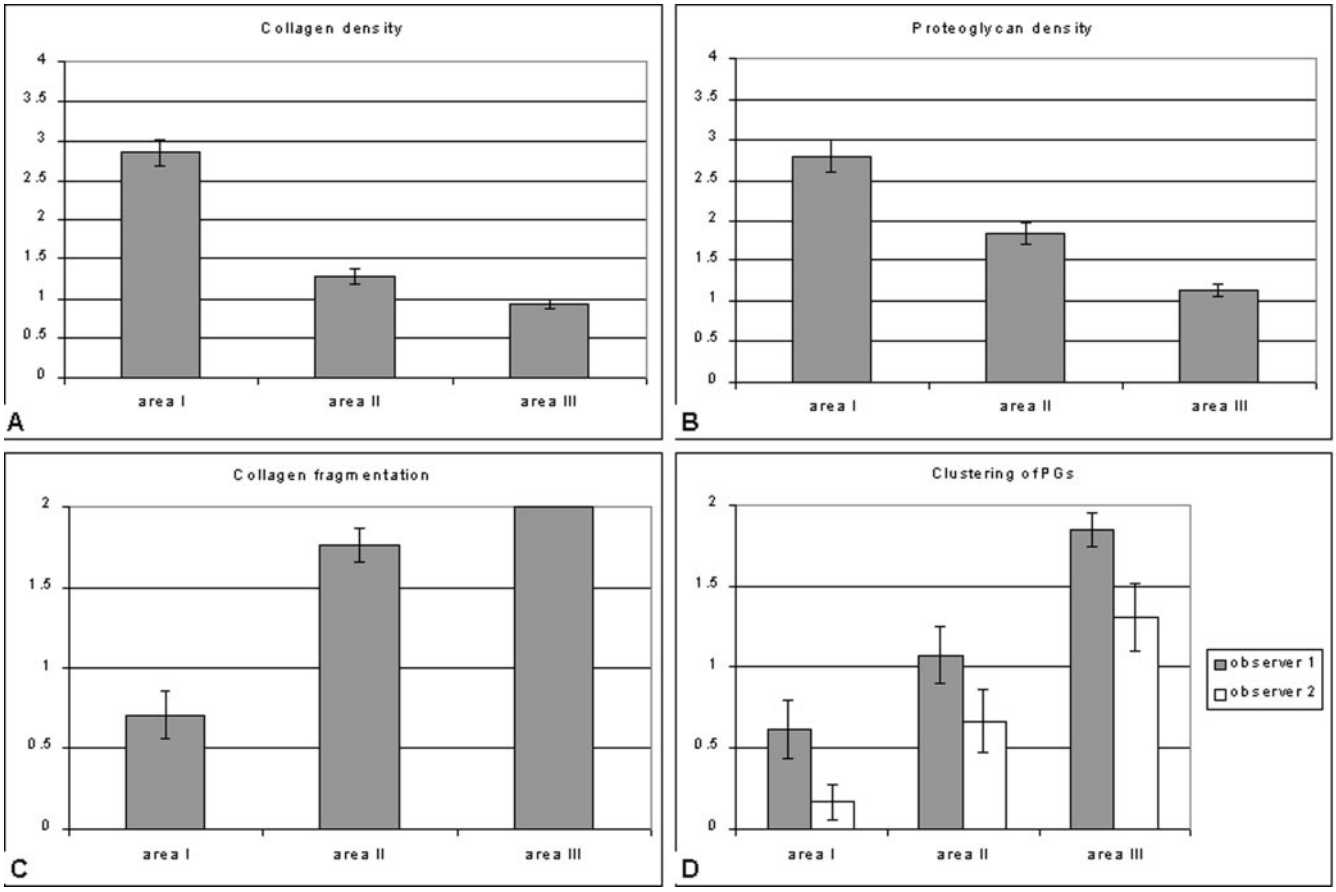

FIGURE 5. Data represent the mean ± the SEM (vertical bars) of results in 13 specimens. (**A**) Collagen density in areas I (regular matrix), II (transition area in a strict sense), and III (border area). Values differ significantly between areas I and II and II and III. (**B**) Proteoglycan density in areas I to III. Values differ significantly between areas I and II and II and III. (**C**) Fragmentation was scored +, 2; ±, 1; or − 0. Data differ significantly between areas I and II but significance is only borderline between areas II and III. (**D**) Clustering of PGs was scored +, 2; ±, 1; or − 0. Interobserver variability was statistically significant; therefore, the results of individual observers are shown. There is a systematic difference in scores between observer 1 and 2, the former giving a consistently higher score than the latter. For both observers, data differ significantly in areas I and II and II and III.

cally weakened matrix areas. Cells do not seem to play a role in the process, because no evidence whatsoever of their presence was observed.

Collagen turnover has been studied in more detail in matrices other than the vitreous. In a review by Everts et al.,[22] both an intracellular and an extracellular pathway of collagen breakdown have been described. The latter seems of primary importance in conditions such as morphogenesis, inflammation, and metastasis. However, its role in physiological steady state conditions is not yet clear. Therefore, the role of such a mechanism in vitreous liquefaction remains to be determined in future studies. Generally, enzymatic breakdown of collagenous matrices may be induced by the action of matrix metalloproteinases (MMPs) and related enzymes.[23,24] The proposed heterotypic constitution of collagen fibrils,[25] which are thought to consist of mixtures of collagens II, V/XI, and IX, provides a variety of potential sites for the action of MMPs. Several MMPs have been demonstrated in vitreous and retina, including MMP-1; (pro) MMP-2, which can cleave vitreous collagens type V/XI and type IX fragments; MMP-3 (stromelysin); and MMP-9.[23,26] Further, an age-related increase in vitreous concentrations of plasmin(ogen), which may activate MMP-2, was demonstrated.[24]

If we assume that a focal enzymatic breakdown of vitreous matrix components, in particular of fibrillar collagen but possibly also of PGs, is an important pathophysiological mechanism of vitreous liquefaction, various topics still must be examined:

1. Matrix (gel) structure. Our results are in accordance with previous studies that indicated that fibrillar collagen is the essential component of vitreous gel structure.[10,11,27] The present study suggests that collagen fibrils may act as carrier molecules for PGs, because PGs are distributed regularly along collagen fibrils in regular matrix and are found to aggregate along fragments of fibrillar collagen in border areas. The role of other macromolecular components in maintaining gel structure remains unresolved. In particular, a possible role of PGs in vitreous liquefaction remains obscure, and the hypothesis that changes in PG distribution may be crucial in the process of liquefaction, as we assumed at the onset of this study, could not be substantiated.
2. Fate of breakdown products. We observed fragmented collagen fibrils and aggregated PG molecules at the borders of the spaces. The spaces themselves, however, seemed free of tissue fragments. This could be explained by assuming that fragmented matrix components remain in the spaces and were washed out during our fixation and embedding procedures, that further digestion of fragments occurs in the ECM, or that these components are transported by bulk flow toward metabolically more active cellular areas bordering the vitreous. The latter is imaginable, since previous macroscopic studies have shown that larger spaces are interconnected and extend

toward the area adjacent to the ciliary body anteriorly and to the prepapillary and premacular areas posteriorly.[28–30] In these areas, fragments could be further digested by an intracellular route, for example.

3. Turnover of matrix components. The vitreous matrix has long been regarded as an almost inert ECM, in which almost no turnover of macromolecular components occurs. Recent studies have modified this view by demonstrating the ongoing turnover of noncollagenous ECM components in adult eyes. For example, turnover of vitreous HA in adult rabbits was demonstrated by Laurent and Fraser.[31] Hyaluronan-synthase (HAS) activity, which is indicative of HA synthesis, was found in the ciliary body region and hyalocytes of adult human and monkey eyes.[32] However, observations in the latter study should be regarded with caution, because Weigel et al.[33] later demonstrated that the HAS-antibody used is not specific. A number of studies demonstrated the production and turnover of glycoproteins in the ciliary body region of adult rabbit eyes.[34–37] A possible turnover of vitreous collagen (production and breakdown) has not been conclusively demonstrated. However, Snowden et al.[14] found a relatively large number of immature crosslinks in adult bovine vitreous, which could be consistent with collagen production in the adult vitreous.

In summary, our results would be consistent with a possible extracellular pathway of vitreous collagen breakdown. This questions the formerly proposed interrelationship between synchysis and syneresis. It now seems that these phenomena need not automatically be interrelated and/or interdependent, but may be induced by separate (patho)physiological mechanisms. In future studies, it will be interesting to explore which pathophysiological mechanisms may be responsible for the breakdown of fibrillar collagen, and whether synthesis of vitreous collagen in the adult eye can really be demonstrated.

Acknowledgments

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