3 Dimensional Meshwork Structure of Vitreous Body

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Abstract

It was found by light microscopic observation, using phosphomolibdenic acid hematoxylin and Alcian blue stains, that vitreous body has a three-dimensional meshwork structure and the beads are located at positions of joints in the fibrils. The HA coats the outer surface of the meshwork fibrils and the beads. The protein beads may reinforce the whole meshwork structure. One of non-collagenous protein beads is identified as αA-crystallin, which is a major component of the lens. It was suggested that vitreous liquefaction is induced by deformation of meshwork fibril structure and detachment of the non-collagenous protein beads, located at the joint of meshwork structure, resulting in the collapse of the structure to release watery liquid trapped within the meshwork.

Introduction

The vitreous body is a transparent matrix that fills the posterior cavity of the eye, occupying more than two thirds of the intraocular volume. The mammalian vitreous body is a hydrophilic gel with a water content higher than 99%. It provides ideal support for the retina and allows light to reach the retina and metabolic solutes to diffuse.

Due to the highly viscous hydrogel, the precise structure of the vitreous body has not yet been clarified, although some fibrous structures have been identified (1,2) within the vitreous body. The model proposed by

Balazs (3) shows that the vitreous body is composed of a sparse, random meshwork of collagen fibrils and that HA is dissolved in the interstices of the collagen fibrils without chemical links to the collagen. It has been thought that polymeric HA may adopt a random coil configuration and that the high viscosity of HA in solution is due to entanglement. But, a vitreous liquefaction phenomenon of phase change from gel to sol cannot be explained by this model. Recently, the vitreous body of most mammals has been shown to contain three polymeric components, namely, collagen, HA, and beaded fibrils (4,5). However, the composition and function of these beads have remained unknown. It was suggested (6) that the beads may serve to stabilize the three-dimensional structure of vitreous body.

With aging, in the human vitreous body there is a localized breakdown of the gel structure, with aggregation of the fibrils (7), which appears to be a frequent precursor to retinal detachment, resulting in liquefaction which means the phase transition from gel to sol. The liquefaction of the vitreous body is the most common degenerative change occurring in senile conditions, which sometimes cause damage to the eyesight. Interleukin 1β (8) and light (9) may be the potent factors to induce this liquefaction.

When the vitreous body of rabbit is melted at the room temperature from the frozen state (-20°C), it is liquefied and separated into two phases, namely, more tight gel phase and aqueous phase. This is considered to be a good model to reveal about the liquefaction. We studied the structure of the vitreous body, by using this model to make clear the mechanism of liquefaction.

Methods

This experiment conformed to the ARVO Resolution on the Use of Animals in Research.

Preparation of specimen for microscopic observation

Rabbit's eyes were dissected usually within 1 hour after sacrifice by excess extracorporeal injection of pentobarbital (100 mg/kg). The specimens were first prefixed with 2 M glutaraldehyde solution for 20 min. and small incisions were made on the outer surface of the eyeball, which reached the vitreous humor near the nerve to allow entry of fixing reagents. Eyes were further fixed with 2 M glutaraldehyde solution for 5 min., and then with 10% formaldehyde in phosphate buffer (pH 7.5) for 1 day. This

method is essentially the same as Kobayashi's method (10).

The samples were stained by the following methods, phosphomolibdenic acid hematoxylin stain (11). They were examined on a Leica Microscope LMD at both visible and dark phase.

Glucosaminoglycan analysis

After digestion of the samples by actinase E and chondroichinase ABC, the aliquots were applied to HPLC with ion exchange column and each glycosaminoglycan (GAG) components were determined. GAG is analyzed by Seikagaku Industry Co.Ltd.

Amino acid analysis of gel and sol after liquefaction

The peptides were hydrolyzed with gas-phase 6 n HCl in vacuo at 108℃ for 24 hrs (PicoTag Work Stations, Waters). The amino acids were derivatized by 6-aminoquinolyl-N-hydroxysuccinimidiyl carbamate and analyzed by RP-HPLC (Schimazu LC 10AD) with an AccQ column (3.9 x 150 mm, Waters), using fluorescence detection (250 nm excitation wavelength and 395 nm emission length).

Amino acid sequence of protein beads

The sample was digested with lysyl-endoprotease (Wako Pure Chemicals) for 5 h at 35C in a 10 mM Tris-HCl buffer, pH 9.0. The resulting peptides were fractionated by reversed-phase (RP)-HPLC (Schimazu LC 10AD), and analyzed by a protein sequencer. Amino acid sequences were determined by Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (Applied Biosystems 477A/120A, Foster City, CA, U.S.A.).

Two-dimensional gel electrophoresis of the sol

The sols of vitreous humors were dissolved in 6M urea, 5%(v/v)TritonX-100,2%(v/v),2mercaptoetanol. 2%(v/v) Ampholyto (pH3-10) and a small quantity of bromo-phenol blue. The immobiline DryStrips pH3-10 were rehydrated with 8M urea, 5%(v/v)TritonX-100, 10mM dithiothreitol, and 1M acetic acid at least 6h at room temperature. After the 1-st dimension run, the gels were incubated with SDS equilibration solution A (0.05M Tris-HCl, 6M urea, 30%(v/v)glycerol, 1%Sodium Dodecylsulfate and 16.2mM dithiothreitol) and SDS equilibration B (0.05M Tris-HCl, 6M urea, 30%(v/v) glycerol, 1%Sodium Dodecylsulfate, 243mM iodoacetamide, and a small quantity of bromophenol blue) for 10min at room temperature, respectively. 1-st dimensional gels were placed at the surface of the Exel Gel SDS (2-nd

dimensional gel, 8-18%gradient) catiodic side.

Results

A typical meshwork structure of a rabbit native vitreous body is shown in Fig. 1. Bead-like substances of 5-8 μm in diameter are present at most joints of the fibril meshwork (Fig.1 arrowheads). This meshwork structure is more clearly observed by dark phase picture, rather than by phase contrast or bright phase.

When the vitreous body is melted from the frozen state (-20°C) (hereinafter this deformed vitreous body is designated as the melted vitreous body), the melted vitreous body is changed into two phases, that is, associated fibrils (arrowhead A, Fig. 2A) and amorphous aggregates (arrowhead B, Fig. 2B). Beads were not detected at the joints of the associated fibrils of the melted vitreous body in Fig. 2A. The amorphous aggregates seem to be released from the associated fibrils (Fig. 2B).

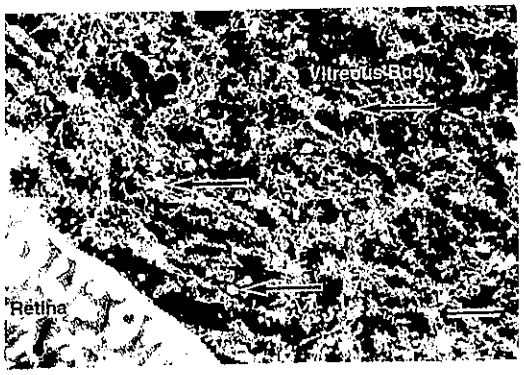

Fig. 1 Cross section of an native eye of rabbit dark phase image (bars 50 μm) by phosphomolibdenic acid hematoxylin stain.

The gel of the melted vitreous body has HA of 91.4 μg/ml, ChS of 12.4 μg/ml. HA is a major component of GAG in vitreous body. And the sol of the melted vitreous body has just HA of 18.5 μg/ml. After liquefaction, most of HA still remain in gel.

The proteins released from the amorphous aggregates in Fig. 2B, which correspond to the proteins, underwent amino acid analysis. Column A of Table 1 shows that these proteins are not collagenous substances. The hydrogel of the melted vitreous body is mainly a collagenous substance, making up approximately 70% of the protein of the hydrogel, as estimated from the hydroxyproline ratio (column B, Table 1). These results indicate that collagen is the main protein of the meshwork of the vitreous body and that the beads are composed of non-collagenous proteins.

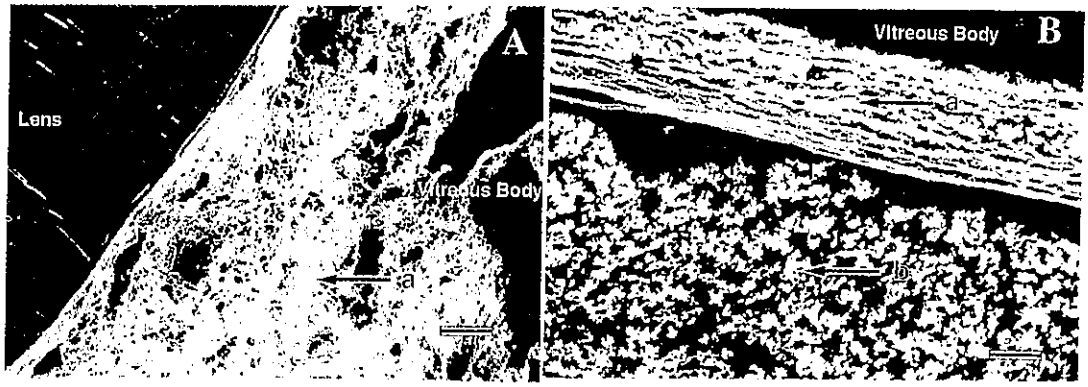

Fig. 2 Cross section of the melted vitreous body of dark phase image (bars 50μ m) by phosphomolibdenic acid hematoxylin stain.
(a) associated fibrous area (arrow A), (b) associate fibrous area (arrow A) and amorphous aggregates area (arrow B)

Amino Acid	A	B
Hy-Pro	0	94
Asp	88	67
Ser	52	39
Glu	182	111
Gly	71	215
His	23	9
Arg	22	33
Thr	29	21
Ala	84	78
Pro	51	76
Cys	9	0
Tyr	13	11
Val	79	48
Met	8	20
Lys	66	27
Ile	45	44
Leu	115	83
Phe	63	54
Total	1000	1000

Tabel 1 Amino Acid Analysis of Vitreous Body. Non collagenous substances (Column A) released from the melted vitreous body and collagenous substances (Column B) from associated fibrils - opaque hydrogels.

The soluble proteins obtained from the melted vitreous body (protein beads) were directly applied to the protein sequencer. However, these proteins could not be analyzed because of N-terminal blocking. After digestion by the protease, the resulting peptides were separated by HPLC. The fractions were collected, and aliquots of the samples were analyzed by a protein sequencer. One of the peaks was determined as VQSGLDAGH and according to the database

(BLSTO), the sequence was consistent with V146QSGLDAGH of the αA-crystallin fragment of lens(12). The two-dimensional gel electrophoresis patterns of the soluble proteins from native and melted vitreous bodies are presented in Fig. 3. There was little protein in the solution of the native vitreous body (Fig. 3A). On the other hand, the pattern of the melted vitreous body (Fig. 3B) clearly shows many kinds of soluble proteins, with molecular weights ranging from 20,000 to 35,000, were observed (Fig. 3B).

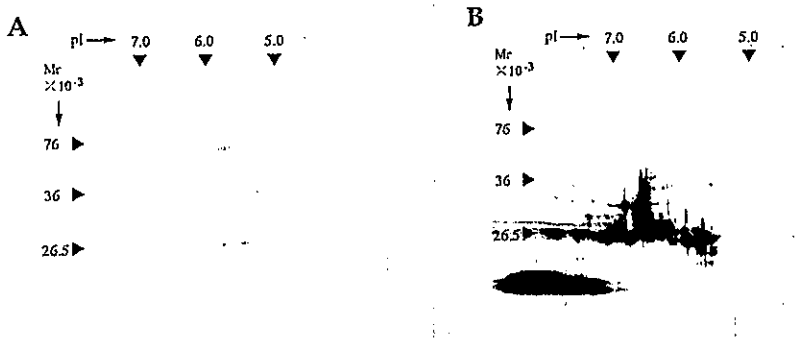

Fig. 3 Two-dimensional gel electrophoresis patterns of soluble proteins obtained from native and melted vitreous bodies. (A) soluble proteins of the native vitreous body, (B) soluble proteins of the melted vitreous body.

Discussion

A typical meshwork structure of a rabbit native vitreous body, of which the fibrils diameter are 2-4 μm, can be observed as shown in Fig. 1. This meshwork structure of vitreous body could not be detected with hematoxylene eosin stain or Azan stain. It may due to the surface chemical structure of hyaluronic acid. Bead-like substances of 4-6 μm in diameter are present at most joints of the fibril meshwork as shown by arrow in Fig. 1. It is suggested from the results of GAG analysis that HA does not exist in the vitreous body as amorphous hydrogel or as dissolved state, but as stable biding state with collagen. HA is a major (about 90%) glucosaminoglycan component of rabbit vitreous (GAG analysis). In this experiment, the role of chondroitin sulfate, which occupies about 10% of vitreous gel, is not clear. The glycosaminoglycans of the vitreous (HA and chS) aggregate with themselves and with each other in solution (13). It was found (14) that depolymerization of all vitreous hyaluronan and

of chondroitin sulfate rested in gel wet weight reduction but not gel destruction.

Many paper (14,15,16) pointed that there are collagen fibers in vitreous not binding with hyaluronic acid. But, Azan or van Gieson stain for connective tissue cannot reveal existence of collagen in native and normal vitreous. It is very clear that the connective tissues in vitreous body do not exist separately or expose to outside of fibrils.

Asakura (17) found that ruthenium red positive glycosaminoglycans exist as amorphous masses on collagen fibrils, as well as filaments interconnecting collagen fibrils and amorphous materials. It is proposed that HA binds with collagen fibrils via chondroitin sulfate.

This meshwork can hold so much watery liquid in vitreous body to form gel-like structure. After liquefaction, the structure is changed into the two phases. One phase is associated fibrils as Fig.2 (A) shows, which do not have the beads. The native meshwork fibrils are easily associated each other to release watery liquid, after detachment of the beads. Another phase is amorphous aggregates as Fig.2(C) shows, which are detached from the meshwork structure. After washing the melted vitreous body with saline, the amorphous aggregates as Fig.2(C) shows were not observed except the associated fibrils. The amorphous aggregates are water-soluble and thought to be the beads assembly.

From the results of two dimensional gel electrophoresis, it is also revealed that these soluble proteins (Fig.3B) were released from the meshwork structure after being melted. Before liquefaction, any proteins were not detected in Fig.3A. These beads proteins are different from collagen (Table 1). This result can be compared with Davidson's result (4), which also show non-collagenous substance of beads.

One of the beads proteins was identified as αA-crystallin by amino acid sequence study. αA-crystallin is one of the main components of the lens (18) and a lens-specific protein, even though it is expressed in non-ocular tissues (19). This is the first observation of the presence of αA-crystallin in the vitreous body, suggesting that there is a relationship between the lens and the vitreous body and the beads have αA-crystallin. Other type crystallins were also found in vitreous (20, 21). It was found that αB-crystallin (22) appear in the subendothelial region of Schlemm's canal and outer corneosceleral in normal eye.

What is the mechanism of liquefaction? Liquefaction is a sort of phase transition from gel phase to sol phase. If water (like aqueous humor) merely is trapped as binding waters of hyaluronic acid in vitreous body, this phase transition is not easily elucidated. If aqueous is trapped with three dimensional meshwork structure of hyaluronic acid-collagen fibrils as shown in this study, this aqueous is easily released from the meshwork after this structure is broke down. This break down trigger may be detachment of beads protein from the joint of meshwork structure. It was suggested (2) that the gel vitreous can be liquefied by removing or destroying the collagen fibrillar network. The former can be achieved by filtration or by centrifugation, the latter by homogenization or by digestion with proteases (collagenases) which destroy the fibrillar collagen. The residue after filtration or the sediment after centrifugation was called "residual protein". In this experiment, the residual protein itself must be beads proteins.

There are so many works (4,6,17) which show very small fibrils and beads by transmission electron micrograph. On the other hands, Worst (5) found a characteristic fine structure in vitreous consisting of a number of radially and concentric running wavy lines, in a fairly complex arrangement. It may be due to the difference of the structural hierarchy, namely, macroscopic revel (100~1000 micron), microscopic revel (10~100 micron), and electron microscopic revel (0.1~10 micron). It seems likely that the structure of microscopic revel is also very important to elucidate the physical changes in liquefaction. It is very clear that the connective tissues in vitreous body do not exist separately or expose to outside of fibrils. It was also revealed by the transmission electron micrographs (23) that some collagen fibrils in human vitreous appeared to be packed in a bundle of parallel fibrils to form big fibrils of diameter 10~20 μm. And the existence of amorphous beads like substance of diameter 10~40 μm were also observed. The three dimensional structure of the vitreous (2) is thought to be formed by water-insoluble collagen fibrils, of which are remarkably uniform in diameter (10 – 20 um).

Our results suggest that vitreous body is not merely support hydrogel to retina, but has functional structure and some relationship with lens and other eye tissues. And furthermore, it is necessary to reveal the native vitreous body at native state.

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