Characterization of Membranes Removed during
Open-sky Vitrectomy

Benigno D. Peczon,* John K. Wolfe, Ilene K. Gipson, Tatsuo Hirose,
Sheldon M. Buzney, and Charles L. Schepens

Membranes removed during open-sky vitrectomy have been characterized by electron microscopy, reaction with anti-human fibrinogen, susceptibility to enzymatic digestion, amino acid analysis, and electrophoresis in sodium dodecyl sulfate. There were significant differences between longstanding and newly formed membranes. Longstanding membranes contained substantial amounts of hydroxyproline, glycine, and hydroxylysine, were capable of digestion by collagenase but not by plasmin, yielded faint positive results with anti-human fibrinogen, and showed fibrils characteristic of collagen by electron microscopy. After digestion with pepsin, electrophoresis revealed bands that migrated the same distance as vitreous collagen chains. This type of membrane is evidently collagenous in nature. A second type of membrane, which developed in the course of vitrectomy, contained no hydroxyproline, only traces of hydroxylysine, and relatively small amounts of glycine, was digested by plasmin, yielded strong positive results with anti-human fibrinogen, and showed fibers that were not characteristic of collagen by electron microscopy. Electrophoresis demonstrated bands similar to authentic fibrin in these newly formed membranes. These data suggest that this second type of membrane is composed largely of fibrin. Prevention of the formation of this second type of membrane during vitrectomy may require the addition of agents that inhibit fibrin formation. Invest Ophthalmol Vis Sci 24:1382–1389, 1983

Open-sky vitrectomy is a surgical procedure that may be indicated for the removal of dense vitreous opacities and the release of vitreoretinal traction. During this operation, transparent membranes occasionally appear in the vitreous cavity or around the anterior segments, particularly on the surface of the iris. The clinical impression is that formation of these membranes within the posterior vitreous segment may precipitate severe and often incurable massive preretinal retraction after existing membranes have been adequately removed. If this hypothesis proves to be true, the prevention of formation or the elimination of these newly formed membranes may make the treatment of severe massive preretinal retraction by open-sky vitrectomy considerably more effective. First, however, the nature of these membranes must be investigated. Although longstanding membranes obtained from the vitreous cavity have been reported as being collagenous, those formed during surgery are more likely to originate from fibrin. The studies presented here—electron microscopy, reaction with anti-human fibrinogen, susceptibility to enzymatic digestion, amino acid analysis, and electrophoresis in sodium dodecyl sulfate—provide more definitive data on the nature of these two types of membranes.

Materials and Methods

Samples

Longstanding membranes attached to the anterior retinal surface were obtained from patients undergoing open-sky vitrectomy. Newly formed membranes, which often became evident during or after vitrectomy, were obtained from the anterior iris surface and from the vitreous cavity. All membranes were collected in TC-199 culture media (GIBCO, Grand Island, NY) or in distilled water; they were either cooled on ice (if analyzed immediately) or frozen at −20°C (if stored prior to analysis).

Electron Microscopy

Membranes collected in culture media were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer,
postfixed in 1% osmium tetroxide in the same buffer, dehydrated in an ethanol series, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips® 200 electron microscope.

Direct Immunofluorescence

Membranes were fixed in absolute ethanol, embedded in paraffin, and sectioned according to the technique described by Sainte-Marie.2 Samples were incubated with FITC conjugated goat anti-human fibrinogen (Meloy, Springfield, VA) diluted 1:1 with phosphate-buffered saline, pH 7.4, at room temperature for 30 minutes. Following application of a glass coverslip using Gelvatol (Monsanto, St. Louis, MO), the samples were examined with a Zeiss® Photomicroscope III. Specificity for fibrin was established by adsorbing the anti-human fibrinogen serum with human fibrinogen powder (Sigma, St. Louis, MO); depletion of antibody was monitored by assaying the reagent against fixed and sectioned fibrin powder.

Enzyme Digestion

Streptokinase and human plasminogen were obtained from Sigma Chemical Co., St. Louis, MO. Purified collagenase (234136) was obtained from Calbiochem (La Jolla, CA). Samples were removed from TC-199, washed once with tris(hydroxymethyl)aminomethane (Tris) buffer, and incubated at room temperature with streptokinase-activated plasmin. Final concentrations were 0.15 M NaCl, 50 mM Tris (pH 7.4), 400 U streptokinase, and 0.05 U human plasminogen in a final volume of 1 ml. Solubilization was observed under a dissecting microscope. Samples that did not dissolve in plasmin within 24 hours were solubilized with solubilizing buffer by 1) dialysis overnight against solubilizing buffer, 2) shaking in solubilizing buffer at 37°C for 16 hours, or 3) boiling for 60 seconds followed by incubation at 70°C for 2 hours. Protein that was not precipitated with 3 M NaCl was recovered by dialysis against water, lyophilized, and resolubilized by shaking in solubilization buffer at 37°C for 20 hours.

Globular protein standards (Pharmacia Fine Chemicals, Piscataway, NJ) consisting of phosphorylase b (MW 94,000), albumin (MW 67,000), catalase (MW 60,000), ovalbumin (MW 43,000), and lactate dehydrogenase (MW 36,000) were included as molecular-weight standards with newly formed membranes. Acid-soluble rat-tail collagens (Type I) and partially purified bovine cartilage collagen (Type II), which were gifts of Dr. P. Davison of the Boston Biomedical Research Institute and Dr. B. Chakrabarti of the Eye Research Institute of Retina Foundation, respectively, were used as molecular-weight markers of pepsin-treated and reduced and alkylated longstanding membranes. Molecular weights of the bands in newly formed membranes were estimated from standard curves constructed by plotting the mobilities of globular proteins against the logarithms of their molecular weights.6 Molecular weights of pepsin-insoluble bands were estimated similarly, using the known collagen chains as standards.

Results

Morphology

Electron microscopy clearly showed two types of vitreous membranes. Longstanding membranes (Figs. 1A–B) were composed chiefly of collagen fibrils. The periodicity of the fibril bands was 600 to 700 Å, which suggests that they are indeed vitreous collagen.7 Ran-
domly embedded in these collagen fibrils was cellular debris (Fig. 1A).

The morphologic features of newly formed membranes (Figs. 2A–B) differed substantially from the membranes shown in Figures 1A–B. No evidence of collagen fibrils with a well-defined periodicity was found. Instead, filament masses similar in appearance to fibrin clots were present. Cells and cellular debris were found randomly in these filament masses (Fig. 2A).

**Immunofluorescence**

Longstanding membranes typically exhibited fluorescence for fibrin within the interstices of the specimen and rarely on the surface (Fig. 3C). In contrast, newly formed membranes demonstrated marked deposition of fibrin on the surfaces and to a much lesser extent within the samples (Fig. 3D). Adsorption of anti-serum with fibrin powder eliminated fluorescence in both membranes and fibrin control.
Enzymatic Digestions

Longstanding membranes incubated overnight with plasmin were not digested to an appreciable degree. Incubation with collagenase for 3 hours, however, did dissolve a large fraction of these membranes. In contrast, digestion with plasmin dissolved a large fraction of newly formed membranes.

Amino Acid Analysis

Amino acid analysis of longstanding membranes showed the presence of substantial amounts of hydroxyproline, hydroxylysine, and glycine (Table 1). The amino acid compositions of these membranes taken from different parts of the eye did not differ substantially from each other. In contrast, newly
formed membranes contained no hydroxyproline, traces of hydroxylysine, and less than 100 residues of glycine per 1,000 amino acid residues. There was relatively little compositional variation among samples of the newly formed membranes that were analyzed.

Electrophoresis

Electrophoresis in SDS of newly formed membranes showed the presence of a doublet (apparent MW, 109,000 and 104,000), faint bands (76,000, 73,000, and 69,000), a major band (62,000), a faint band (56,000), and protein that failed to enter the gel (>550,000) (Fig. 4). Faint bands (arrowheads, Fig. 4) with apparent molecular weights of 208,000, 37,000, 31,000, and 16,000, which were likely derived from cellular debris, also were observed.

Independent of mode of resolubilization, the major band upon electrophoresis in SDS of twice pepsin-treated and reduced and alkylated longstanding membranes migrated the same distance as rat tail \( \alpha_1 \) (I) and cartilage \( \alpha_1 \) (II) collagen. Less intensely stained components consisted of bands with apparent molecular weights in excess of 320,000 (bracket, Fig. 5), bands that migrated the same distance as \( \beta_1 \), \( \beta_1 \), and \( \alpha_2 \) (I) (bands 1, 2, and 6, respectively, Fig. 5), and bands with apparent molecular weights of 170,000 and 130,000 (bands 3 and 4, respectively, Fig. 5).

Discussion

Electron microscopic examination showed that longstanding membranes, which were found to consist primarily of collagen fibrils, differed from newly formed membranes. Immunologic staining with anti-human fibrinogen likewise showed differences between the two membranes. Longstanding membranes displayed evidence for previous deposition of fibrin within interstices of specimens with fluorescence intensities that were generally low. In contrast, newly formed membranes displayed evidence for fibrin within their interstices and marked fluorescence on their surfaces.

Digestion of newly formed membranes with plasmin strongly suggests that they are composed primarily of fibrin. In contrast, the failure of plasmin to dissolve longstanding membranes and their subsequent solubilization by collagenase are consistent with their being collagenous in nature.

Amino acid analysis confirms these assignments. Vitreous collagen contains at least 103 hydroxyproline,
Table 1. Amino acid compositions of longstanding and newly formed membranes (residues/1,000)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Longstanding</th>
<th>Newly formed</th>
<th>Fibrinogen</th>
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<tr>
<td></td>
<td>1 2 3</td>
<td>1 2 3 4</td>
<td>Ref 21</td>
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<tr>
<td>Hyp</td>
<td>80.1 98.9 91.3</td>
<td>0 0 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Asp</td>
<td>47.6 43.6 46.9</td>
<td>99.5 75.5 88.4 102.4</td>
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<td>Thr</td>
<td>27.5 24.9 27.2</td>
<td>62.8 55.3 64.4 64.7</td>
<td>65.1 64</td>
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<tr>
<td>Ser</td>
<td>38.3 34.7 38.6</td>
<td>84.4 67.7 79.5 89.5</td>
<td>86.8 78</td>
</tr>
<tr>
<td>Gln</td>
<td>96.7 109.7 104.4</td>
<td>116.7 134.8 146.8 121.1</td>
<td>112.4 115</td>
</tr>
<tr>
<td>Pro</td>
<td>105.4 94.3 94.8</td>
<td>71.3 61.8 70.5 51.3</td>
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</tr>
<tr>
<td>Gly</td>
<td>268.5 283.8 270.4</td>
<td>74.3 40.0 80.7 99.0</td>
<td>99.6 97</td>
</tr>
<tr>
<td>Ala</td>
<td>92.8 85.8 87.3</td>
<td>60.6 92.7 56.0 55.4</td>
<td>51.1 44</td>
</tr>
<tr>
<td>Val</td>
<td>30.5 27.4 28.4</td>
<td>55.6 85.5 48.8 55.2</td>
<td>46.0 45</td>
</tr>
<tr>
<td>Met</td>
<td>10.4 9.5 9.0</td>
<td>19.9 5.2 18.9 22.4</td>
<td>24.3 21</td>
</tr>
<tr>
<td>Ile</td>
<td>20.6 17.1 19.0</td>
<td>39.0 17.0 32.0 34.4</td>
<td>40.9 43</td>
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<tr>
<td>Leu</td>
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<td>78.7 109.2 74.3 63.0</td>
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<tr>
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<td>37.0 34</td>
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<tr>
<td>Phe</td>
<td>18.0 17.7 18.6</td>
<td>37.2 45.8 34.0 32.9</td>
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<td>His</td>
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<td>23.4 27.4 20.6 22.6</td>
<td>23.0 22</td>
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<tr>
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<td>0 0</td>
</tr>
<tr>
<td>Arg</td>
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<td>53.0 38.8 57.0 55.0</td>
<td>53.6 52</td>
</tr>
<tr>
<td>Cys*</td>
<td>6.4 2.7 7.0</td>
<td>22.4 39.5 20.9 16.3</td>
<td>8.9 23</td>
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</table>

* Denotes sum of cysteic acid and cystine in the membranes.

306 glycine, and 19 hydroxylysine residues per 1,000 amino acid residues.9 It is apparent from an examination of the compositions of longstanding membranes (Table 1) that they are composed primarily of collagen. If collagen is defined as protein in which one-third of the residues are glycine, and assuming that noncollagenous proteins in the samples contain 100 residues of glycine per 1,000 amino acid residues, then the longstanding membranes analyzed are 72 to 79% collagen. The absence of hydroxyproline and the presence of only trace amounts of hydroxylysine and of less than 100 glycine residues per 1,000 residues in newly formed membranes (Table 1) indicate that they are not derived from collagen. The trace amounts of hydroxylysine found in these membranes most likely originate from the cells trapped within them. Cultures of cells present in membranes adhering to the vitreous obtained from patients with retinal detachments have shown the presence of epithelial-like cells and other cell types; the epithelial-like cells were found to contain basement membrane-like material and extracellular collagen.10 The general similarity of the amino acid compositions of newly formed membranes and purified fibrinogen strongly suggests the identification of the major component of these membranes as fibrin. Comparison of newly formed membranes with fibrinogen rather than fibrin is valid because peptides released upon formation of fibrin from fibrinogen account for only 3 to 4% of the protein.11,12

Further evidence that newly formed vitreous membranes are mainly fibrin is provided by their electrophoretic pattern in SDS. McKee et al13 have reported that electrophoresis of insoluble human fibrin in SDS yielded bands with molecular weights of 105,000 (γ-
Fig. 5. Gel electrophoresis in sodium dodecyl sulfate. Lane 1, rat tail (Type I) collagen standards. \( \beta_{11}, \beta_{12}, \alpha_1, \) and \( \alpha_2 \) denote known collagen chains. Lanes 2 to 5, twice pepsin-treated and reduced and alkylated longstanding membranes; lane 2, solubilized by dialysis vs. solubilizing buffer at 4°C overnight; lane 3, shaken in solubilizing buffer at 37°C for 16 hours; lane 4, boiled in solubilizing buffer for 60 seconds and shaken at 70°C for 2 hours; lane 5, supernatant fraction from salt precipitation dialyzed vs. water and redissolved in solubilizing buffer at 37°C for 20 hours. Arrowhead denotes elution position of Type II bovine cartilage collagen. Double arrowhead denotes pepsin. DF, tracking dye front. Gel electrophoresis was performed as described in Figure 4.

dimers), 73,000 (\( \alpha \)-monomer), 60,000 (\( \beta \)-monomer), 53,000 (\( \gamma \)-monomer), and >400,000 (\( \alpha \)-polymer). The molecular weights of the bands in newly formed vitreous membranes that entered the polyacrylamide gel are very similar: 104,000, 73,000, 62,000, and 56,000. The difference in estimates of molecular weights of protein that did not enter the gels (>400,000 vs. >550,000) is a function of the concentrations of the polyacrylamide gels used. More detailed work will likely show that the molecular weights of these high-molecular-weight polymers do not differ. The electrophoretic patterns are similar in that the low-molecular-weight \( \gamma \)-chain bands are faint. This is expected since upon conversion of fibrinogen to fibrin, \( \gamma \)-chains are converted to dimers. The multiplicity of \( \alpha \)-chains that we found (MW 76,000, 73,000, and 69,000) has been observed previously by McKee et al, who found two to three such bands ranging in molecular weight from 68,000 to 76,000.13 The superior resolution in our polyacrylamide gels show a band with an apparent molecular weight of 109,000, distinct from the band with a molecular weight of 104,000. It is possible that this band constitutes \( \alpha \)-\( \gamma \) dimers.14,15

Digestion of longstanding membranes with pepsin followed by reduction and alkylation and a second treatment with pepsin was performed to remove non-collagenous components. When Swann and Sotman16 treated bovine vitreous humor by such a technique, they found that the major band migrated the same distance as \( \alpha_1 \) (I) collagen on electrophoresis in SDS. Less intensely stained bands larger than and the same size as Type I \( \beta_{11} \) chains also were found. More detailed characterization showed the alpha-sized molecules to be a special \( \alpha_1 \) (II) chain and the larger molecules to be multiples of the vitreous \( \alpha_1 \) (II) chains. Other investigators have presented evidence for the presence of chains other than \( \alpha_1 \) (II) chains in the vitreous. Chromatography on carboxymethylcellulose of denatured lathyritic vitreous collagen has shown the elution of 10% of the alpha-chains in the \( \alpha_2 \) region.17 Ten percent of the biosynthetic product of chicken retinal cells, which are known to synthesize the collagen deposited in the vitreous body,18,19 differ from \( \alpha_1 \) (II) collagen.20 On the basis of their mobilities upon electrophoresis in SDS, the bracketed bands and bands 1 and 5 in Figure 5 are likely to be \( \alpha_1 \) (II) and its aggregated forms, whereas bands 2, 3, and 4 may constitute forms of collagen other than \( \alpha_1 \) (II). Positive identification of these bands, however, requires additional characterization that is not possible at this time because of the limited amounts of longstanding membrane available.

In summary, morphologic analysis, reaction with anti-human fibrinogen, susceptibility to proteolytic enzymes, comparisons of amino acid compositions, and electrophoretic patterns show the presence of two distinct types of membranes obtained during vitrectomy, one composed chiefly of collagen and the other chiefly of newly formed fibrin. Identification of the constituent collagen(s) of longstanding membranes requires additional studies. Identification of the newly formed membranes as fibrin suggests that the prevention of the formation of this membrane during vitrectomy may require the addition of agents that inhibit its formation.

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**Key words:** membranes, open-sky vitrectomy, fibrin, vitreous collagen

**References**