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,

† Human informed consents were received prior to undertaking this study.
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. 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, 1 mM CaCl₂, 50 mM Tris (pH 7.4), 400 U streptokinase, and 0.05 U human plasminogen (Sigma, St. Louis, MO); depletion of antibody was monitored by assaying the reagent against fixed and sectioned fibrin powder.

**Amino Acid Analysis**

Samples collected in distilled water were lyophilized. After evacuation and repeated flushing with nitrogen, dried samples were hydrolyzed in constant-boiling HCl (Pierce Chemical Co., Rockford, IL) at 105°C for 24 hours. Amino acid analyses of the hydrolyzates were performed as described previously.

**Electrophoresis**

Newly formed membranes collected in distilled water were lyophilized and dissolved in 0.0134 M H₂SO₄—0.027 M Tris-24 mM dithiothreitol-20 g/l sodium dodecyl sulfate (SDS) buffer, pH 6.1 (solubilization buffer). Proteolytic enzyme inhibitors (5 mM ε-aminocaproic acid, 5 mM p-aminobenzamidine, 10 mM ethylene-diaminetetraacetic acid, and 1.5 mM phenylmethysulfonylfluoride) were added to the solubilization buffer. The samples were dissolved by placing them in boiling water for 60 seconds followed by shaking at 70°C for 2 hours. To prevent reoxidation of sulfhydryl groups, samples were alkylated with 144 mM iodoacetamide at 50°C for 15 minutes in the dark. SDS polyacrylamide gel electrophoresis was performed as described previously.

Prior to electrophoresis, non-collagenous components of 10 pooled longstanding membranes were removed essentially according to a three-step procedure involving limited proteolysis by pepsin, reduction, and alkylation of disulfide bonds under nonreducing conditions, followed by a second digestion with pepsin. Collagens were precipitated with 3 M NaCl and resolubilized 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.

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.
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 hydroxyllysine, 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 α1(I) and cartilage α1(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 β11, β12, and α2(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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Hyp</td>
<td>80.1</td>
<td>98.9</td>
<td>91.3</td>
</tr>
<tr>
<td>Asp</td>
<td>47.6</td>
<td>43.6</td>
<td>46.9</td>
</tr>
<tr>
<td>Thr</td>
<td>27.5</td>
<td>24.9</td>
<td>27.2</td>
</tr>
<tr>
<td>Ser</td>
<td>38.5</td>
<td>34.7</td>
<td>38.6</td>
</tr>
<tr>
<td>Glx</td>
<td>96.7</td>
<td>109.7</td>
<td>104.4</td>
</tr>
<tr>
<td>Pro</td>
<td>105.4</td>
<td>94.3</td>
<td>94.8</td>
</tr>
<tr>
<td>Gly</td>
<td>268.5</td>
<td>283.8</td>
<td>270.4</td>
</tr>
<tr>
<td>Ala</td>
<td>92.8</td>
<td>85.8</td>
<td>87.3</td>
</tr>
<tr>
<td>Val</td>
<td>30.5</td>
<td>27.4</td>
<td>28.4</td>
</tr>
<tr>
<td>Meth</td>
<td>10.4</td>
<td>9.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Ileu</td>
<td>20.6</td>
<td>17.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Leu</td>
<td>44.9</td>
<td>44.2</td>
<td>48.4</td>
</tr>
<tr>
<td>Tyr</td>
<td>9.5</td>
<td>7.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Phe</td>
<td>18.0</td>
<td>17.7</td>
<td>18.6</td>
</tr>
<tr>
<td>His</td>
<td>9.6</td>
<td>7.6</td>
<td>11.1</td>
</tr>
<tr>
<td>Hyl</td>
<td>16.5</td>
<td>20.8</td>
<td>19.6</td>
</tr>
<tr>
<td>Lys</td>
<td>26.1</td>
<td>22.9</td>
<td>26.0</td>
</tr>
<tr>
<td>Arg</td>
<td>50.5</td>
<td>47.0</td>
<td>44.7</td>
</tr>
<tr>
<td>$\varepsilon_{1/2}$ Cys*</td>
<td>6.4</td>
<td>2.7</td>
<td>7.0</td>
</tr>
</tbody>
</table>

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

306 glycine, and 19 hydroxylysine residues per 1,000 amino acid residues. 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. 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.

Further evidence that newly formed vitreous membranes are mainly fibrin is provided by their electrophoretic pattern in SDS. McKee et al. 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. α1, α2, α3, and α4 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.

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.

Acknowledgments

We thank Nancy Hetu and Prudencia Bucay for technical assistance, Leona W. Greenhill for editorial assistance, and...
Drs. Peter Davison and Bireswar Chakrabarti for providing collagen standards.

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

References