Morphologic, Biomechanical, and Compositional Features of the Internal Limiting Membrane in Pathologic Myopic Foveoschisis

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PURPOSE. To investigate alterations in the morphologic, compositional, and biomechanical properties of the internal limiting membrane (ILM) in pathologic myopic foveoschisis (MF) eyes.

METHODS. ILM specimens were peeled from 61 eyes with MF and 56 eyes with stage III/IV idiopathic macular hole (IMH) as a control. Samples were analyzed for transmission electron microscopy (TEM), scanning electron microscopy, immunofluorescence, Western blotting, and atomic force microscopy. ILM characteristics were compared between the two groups.

RESULTS. TEM findings revealed that thickness of the MF ILMs significantly decreased compared with that of IMH ILMs (0.753 ± 0.215 vs. 1.894 ± 0.247 μm; P < 0.0001). The vitreal side stiffness of the MF ILMs was markedly higher than that of the IMH ILMs (3.520 ± 0.803 vs. 0.879 ± 0.230 MPa, P < 0.0001). Comparing with the IMH group, collagen IV exhibited decreased concentration and different immunofluorescence distribution in ILMs of MF eyes, so also protein α3 (IV), α4 (IV), and α5 (IV). The immunofluorescence staining results showed that astrocytes were observed in none of the IMH eyes and in 12 of 16 MF eyes (75%, P < 0.0001).

CONCLUSIONS. These alterations in the MF ILMs appear to be associated with Müller cell and astrocyte reactive gliosis. The present findings contribute to a more in-depth understanding of the pathogenesis of MF.

Keywords: myopic foveoschisis, internal limiting membrane, collagen IV, Müller cell, astrocyte

Myopic foveoschisis (MF), characterized by splitting of retinal layers, is a deteriorative complication of pathologic myopia.1,2 It was first demonstrated and described in detail by Takano and Kishi in 1999 using optical coherence tomography (OCT). In pathologic myopic eyes with posterior staphyloma, the prevalence of MF was 9% to 20%. Once MF develops, macular function is thought to be severely impaired.

According to the current knowledge, vitreoretinal adhesion and traction at the site of the internal limiting membrane (ILM) might be the fundamental factors in the development of MF. For this reason, surgical peeling of the ILM has been introduced as a potentially useful method and has greatly improved the visual and anatomic prognosis of patients with MF.3-5 It was suggested that the ILM plays a key role in the course of MF.

Due to the importance of ILM in clinical practice, histopathologic studies on surgically peeled ILM tissues, which help us to better understand the pathogenesis of MF, had been studied previously. For example, Yokota et al.5 carried out ultrastructure research and found cells on the vitreal side of the ILM in MF eyes. The result was in accordance with morphologic findings of Bando et al., who revealed fibrous glial cells and collagen in transmission electron microscopy (TEM) micrographs of MF ILMs. However, due to the difficulty of obtaining and handling the very delicate human native ILMs, most data on the characteristics of MF ILMs were based on ultrastructural analysis of small case series using TEM. Furthermore, there is less detail on the collagen composition and intrinsic biomechanical properties of ILM tissues in MF eyes, which are also important in the development of MF.

Therefore, the aim of this study was to quantitatively investigate ultrastructural, compositional, and nanomechanical profiles of ILMs from patients with MF, which in turn could explain a detailed pathogenesis of the disease.

METHODS

This was a consecutive interventional case series of surgically peeled ILM specimens removed from 117 eyes of 117 patients with pathologic MF (61 eyes) and stage III/IV idiopathic macular hole (IMH, 56 eyes) who underwent vitrectomy at Sun Yat-sen University, Zhongshan Ophthalmic Center, between February 2016 and May 2017. OCT (Optovue RTVue XR; Optovue, Inc., Fremont, CA, USA) was used to confirm the diagnoses of IMH (stage III to stage IV) and MF and to exclude the presence of an epiretinal membrane. Patients were
excluded for systemic diseases, such as arterial hypertension, diabetes mellitus, rheumatoid arthritis or malignant tumors, previous ocular surgery, or trauma and ocular diseases, such as glaucoma, diabetic retinopathy, and uveitis. Patients’ clinical records were reviewed for sex, age, measurement of best-corrected visual acuity, tonometry, axial length, refractive error, fundus examination, and slit-lamp biomicroscopy of the anterior and posterior segments of the eyes. This study was approved by the Institutional Review Board of Zhongshan Ophthalmic Center affiliated with Sun Yat-sen University (Guangzhou, China) and was conducted in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from each study participant.

**Surgical Procedure**

All the patients in the study underwent 25-gauge three-port pars plana vitrectomy performed by one experienced surgeon (S.Z.). Indocyanine green (ICG) was applied to visualize the ILM at a concentration of 0.25% for 10 seconds. The ILM specimen was peeled with ILM forceps (Grieshaber Revolution DNP 25Gauge ILM forceps; Alcon Laboratories, Inc., Fort Worth, TX, USA). Next, the ILM tissues were harvested from the central macula, with a diameter of 3 mm, and immediately placed into specific solutions for different types of measurements. After fluid-air exchange, the vitreous body was filled with filtered air, and the patient was required to keep a face-down position for seven days.

**TEM and Scanning Electron Microscopy (SEM)**

Six samples from each group were measured by SEM. Fourteen excised ILM specimens from IMH eyes and 19 from MF eyes were measured by TEM. Among the ILMs prepared for TEM testing, seven specimens from seven patients were cut into halves. Half of the specimens were processed for TEM, and half were prepared for atomic force microscopy (AFM) imaging to compare the thickness between hydrated and dehydrated ILMs.

Immediately after removal, ILM specimens were fixed overnight (2.5% glutaraldehyde and 2.0% paraformaldehyde), osmicated, and dehydrated through an ascending graded ethanol series.

For TEM, ILM specimens were embedded in Epon overnight and polymerized at 60°C for 48 hours. Ultrathin sections of 60 nm were obtained and stained with uranyl acetate and lead citrate. Using a transmission electron microscope (Philips CM 10; Eindhoven, Holland), 20 measurements of thickness per ILM sample were taken at ×13,500 magnification at random. Müller cell debris quantification was assessed in four randomly selected fields at ×13,500 magnification per ILM sample. The size of Müller cell debris was analyzed and graded: The debris size was regarded as small if less than 0.5 μm, as large if greater than 0.5 μm, and as medium if between 0.5 and 0.1 μm.

For SEM, ILM specimens were dried in a Hitachi HCP-2 (Tokyo, Japan) critical-point drying apparatus coated with platinum in an ion-sputtering machine (Hitachi E-102). Afterward, the specimens were examined under a scanning electron microscope (Hitachi S-2500).

**Atomic Force Microscopy**

AFM was used to detect the thickness and stiffness properties of seven ILMs in both groups of IMH and MFL flat mounts were immobilized on the glass slides and immersed in PBS at 4°C overnight until AFM imaging. All the experiments were conducted with an atomic force microscope (Dimension FastScan, Bruker, Germany).

Three different modes, height sensor, peak force error, and DMTModulus, were selected to present the characteristics of ILMs. The height sensor, namely, surface shape appearance, demonstrates the collagen morphology and measures the thickness of ILMs by detecting the height difference between the underlying glass substrate and the edge of the ILM specimen. The peak force error reflects the rolling of the collagen surface. DMTModulus measures the property of Young’s modulus, which refers to the relationship between stress (force per unit area) and strain (proportional deformation) in a material. Young’s modulus represents the material property of stiffness, which is defined as a material’s ability to resist elastic deformation against external stress. A stiffer material has a higher Young’s modulus.

The stiffness values of both the vitreal surface and the retinal side of the ILMs were analyzed for comparison. Young’s modulus was taken as the average of measurements from 100 random locations. For thickness imaging, 25 measurements were taken at four randomly selected fields.

**Immunofluorescence (IF)**

Sixteen ILM specimens from each group were investigated using IF. Each specimen was cut into halves. Half of the specimens were processed for cross sections, and half were prepared for flat-mount staining.

For IF cross sections, samples were fixed (4% paraformaldehyde), dehydrated, embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA), and frozen at −20°C. Afterward, tissue sections were obtained by cryosectioning at a thickness of 6 μm using a Leica CM1900 cryostat (Rankin Biomedical Corp., Holly, MI, USA). For ILM flat-mount staining, the ILM specimens were immobilized on glass slides and fixed with 4% paraformaldehyde for 30 minutes.

Performing IF, the samples were incubated with laminin (LS-C25105, 1:500; LifeSpan), Seattle, WA, USA), collagen IV (ab6586, 1:1000; Abcam, Cambridge, UK), z1 (IV) (ab189408, 1:1000; Abcam), z2 (IV) (LS-C119455 1:500; LifeSpan), z3 (IV) (LS-C119456 1:500; LifeSpan), z4 (IV) (LS-C119457 1:500; LifeSpan), z5 (IV) (LS-C119458; 1:500; LifeSpan), z6 (IV) (LS-C119459; 1:500; LifeSpan), glial fibrillary acidic protein (GFAP) (ab7260, 1:1000; Abcam), or vimentin (ab8978, 1:1000; Abcam) primary antibodies at 4°C overnight. As secondary antibody, we used either donkey anti-mouse IgG (H+L) (Alexa Fluor 488) or donkey anti-rabbit IgG (H+L) (Alexa Fluor 594) (Thermo Fisher Scientific, Rockford, IL, USA). 4′,6-Diamidino-2-phenylindole (DAPD) was used to stain cell nuclei. Images were obtained with a Zeiss LSM 710 confocal microscope and Zen software (Carl Zeiss AG, Oberkochen, Germany). Cell counting was acquired by measuring DAPI-stained nuclei in 5 to 12 nonoverlapping fields on the same ILM. Each field was photographed using the confocal microscope at ×200 magnification to count DAPI-stained nuclei.

**Western Blotting (WB)**

Twenty peeled ILM tissues from each group were further tested for antibody specificity using WB. For WB analysis, human ILMs were immersed in lysis buffer, phacofragmented, pelleted by centrifugation, and dissolved in 5X sodium dodecyl sulfate (SDS) sample buffer. The samples were boiled for 10 minutes and loaded onto 8% to 12% SDS gradient gels. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, and blots were labeled with antibodies to laminin (LS-C25105, 1:500; LifeSpan), collagen IV (ab6586, 1:1000; Abcam), z1 (IV) (ab189408, 1:1000; Abcam), z2 (IV) (LS-C119455, 1:500; LifeSpan), z3 (IV) (MAB7546, 1:250; RD, Minneapolis, MN, USA), z4 (IV) (LS-C374350, 1:400; LifeSpan), and z5 (IV) (LS-C119456, 1:500; LifeSpan), z6 (IV) (LS-C119457, 1:500; LifeSpan), z7 (IV) (LS-C119458; 1:500; LifeSpan).
Characters of ILM in Myopic Foveoschisis

RESULTS

The demographic information for ILM specimens in the IMH and MF groups is listed in Table 1. The total number of eyes prepared for TEM, SEM, AFM, IF, and WB is listed as well.

Ultrastructural Analysis

The morphology of representative ILMs from each group is shown in Figure 1. Through the use of TEM, cavities of various shapes and sizes (Fig. 1F; arrows) were observed in the ILM in 17 MF eyes (89.5%) and 1 IMH eye (7.14%, \( P < 0.0001 \)). Compared to the IMH group, the retinal side of the MF ILMs had more frequent and highly irregular long extensions deep into the retina (17 vs. 0, \( P < 0.0001 \) (Fig. 1E). Collagen fibers were found attached to the vitreous side of the ILMs in 1 of 14 IMH eyes (7.14%) and in 1 of 19 MF eyes (5.26%), but no significant difference was noted (\( P > 0.05 \)). The microscopic findings for ILMs are shown in Table 2.

ILM thickness could be measured by TEM and AFM. By TEM observations, the average ILM thickness was significantly thinner in the MF group compared with that in the IMH group (0.753 ± 0.215 vs. 1.894 ± 0.247 \( \mu \text{m}; P < 0.0001 \)). Consistent with this difference, the minimum ILM thickness was markedly lower in the MF eyes than in the IMH eyes (0.457 ± 0.187 vs. 1.194 ± 0.252 \( \mu \text{m}; P < 0.0001 \)), and so was the maximum ILM thickness (1.207 ± 0.306 vs. 2.890 ± 0.540 \( \mu \text{m}; P < 0.0001 \)) (Fig. 2A). AFM measurements of the vitreal side of ILMs revealed similar results (1.220 ± 0.434 vs. 2.877 ± 0.234 \( \mu \text{m}, P < 0.0001 \)) (Fig. 2A). Comparing thickness measurement by TEM and AFM showed that hydrated ILMs prepared for AFM were thicker than dehydrated ILMs measured by TEM (Fig. 2B).

Mean ILM thickness was significantly correlated with age in the IMH group (Table 3; Fig. 2C), whereas this relation was not observed in the MF group. Axial length and refractive error were not correlated with the average ILM thickness in the MF or IMH group (Table 3, all \( P > 0.05 \)).

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, y</th>
<th>Sex, Male/Female</th>
<th>Refractive Error, D’</th>
<th>Axial Length, mm’</th>
<th>TEM, n</th>
<th>SEM, n</th>
<th>AFM, n</th>
<th>IF, n</th>
<th>WB, n</th>
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<tbody>
<tr>
<td>IMH, n = 56</td>
<td>55.41 ± 4.86</td>
<td>24/32</td>
<td>0.11 ± 0.64</td>
<td>22.87 ± 0.76</td>
<td>14</td>
<td>6</td>
<td>7</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>MF, n = 61</td>
<td>53.57 ± 7.02</td>
<td>27/34</td>
<td>−15.78 ± 6.63</td>
<td>29.75 ± 2.46</td>
<td>19</td>
<td>6</td>
<td>7</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) (Student’s \( t \)-test).

\( \sigma 5 \) (IV) (LS-C314274, 1:400; LifeSpan), \( \sigma 6 \) (IV) (LS-C382816, 1:1000; LifeSpan), GFAP (ab7260, 1:10,000; Abcam), or vimentin (ab8978, 1:1000; Abcam). WB data were obtained from four pairs each of IMH and MF eyes. The results for each ILM sample pair were reproduced at least three times.

Statistical Analysis

All data analyses were performed using SPSS (SPSS, Inc., Chicago, IL, USA, version 24.0). All the data were expressed as the mean ± standard deviation (SD). The statistical analyses were performed using Student’s \( t \)-tests and Pearson correlations. Differences with \( P \) values less than 0.05 were considered statistically significant.

Table 1. Overview of Clinical Characteristics of Patients in the IMH and the MF Groups; Also Includes the Number of Eyes Used for TEM, SEM, AFM, IF, and WB Experiments

Figure 1. Ultrastructure of the ILM in two groups. The upper row shows ILM micrographs of a 54-year-old female with IMH. The lower row shows ILM micrographs of a 51-year-old female with MF. The vitreal surface (V) of the ILM is smooth, and the retinal surface (R) is irregular. (A) Representative transmission electron micrograph illustrates that the ILM resembles a typical basement membrane with networks of polymerized laminins and cross-linked collagen IV fibers. (B) Higher magnification of the ILM shows a homogeneous meshwork of extracellular matrix. (C) The scanning electron micrograph shows that the retinal side of the ILM displays a scaffold ultrastructure with multiple layers. (D) Higher magnification of the ILM clearly confirms the same detail as in (C). (E) The ILM dramatically decreases in thickness, becomes highly irregular at its retinal surface, and has long extensions into the deep retina. (F) Cavities (arrows) emerge in the ILM. (G) The scanning electron micrograph indicates that the ultrastructure of the ILM on the retinal surface (R) was disorganized. (H) A high-magnification SEM scan illustrates irregular meshwork and numerous cellular fragments on the retinal side of the ILM. Original magnification: (A, E) \( \times 13,500 \), (B, F) \( \times 46,000 \), (C) \( \times 5000 \), (G) \( \times 6000 \), (D, H) \( \times 20,000 \).
### Biomechanical Characteristics Analysis

The parameter of Young’s modulus was determined by AFM to evaluate the stiffness of ILMs. Young’s modulus of ILM segments on the vitreal side was significantly higher (3.520 ± 0.805 MPa) in the MF group compared with that in the IMH group (2.37 ± 0.230 MPa); similar results were obtained for Young’s modulus on the retinal side (6.255 ± 1.08 MPa vs. 1.629 ± 0.417 MPa; P < 0.0001) (Fig. 3A). Furthermore, in the MF group, Young’s modulus on the vitreal side was positively correlated with axial length (Table 4; r = 0.934, P = 0.002) (Fig. 3B), but not related to age, ILM thickness, or refractive error (Table 4, all P > 0.05). In the IMH group, there was no correlation between Young’s modulus on the vitreal side and axial length, age, ILM thickness, or refractive error (Table 4, all P > 0.05). In addition to alterations in stiffness value, the distribution of ILM stiffness on the vitreal side also differed between the two groups. Representative distributions of ILM stiffness on the vitreal side from each group are shown in Figures 3D and 3E.

### Protein Composition Analysis

To understand the detailed mechanisms of ILM ultrastructural and stiffness alterations, IF and WB analyses were carried out to examine the concentration and distribution patterns of collagen IV and its isoforms (Figs. 5A, 5B). Similarly, AFM probing demonstrated that the density of collagen fibers was much lower in the MF group than in the IMH group (Fig. 5C).

### Cellular Components Analysis

In the TEM observations, Müller cell fragments were found on the retinal surface of all ILM specimens from both groups (Fig. 6A, arrows). The amount of Müller cell fragments was abundant on the retinal surface of the ILMs in the MF group compared with that in the IMH group (42.25 ± 3.19 vs. 10.16 ± 0.92; P < 0.0001). Large Müller cell debris was identified on the retinal surface of ILMs in 12 (63.2%) of the MF eyes, significantly more than in the IMH subjects (0%, P = 0.0002).

To further confirm the cellular involvement on the ILMs, the total cell count and cell type were analyzed using IF. Specimens in the MF eyes showed a significantly higher cell count than those in the IMH group (28.69 ± 2.37 vs. 3.67 ± 0.52, P < 0.0001) (Fig. 6C). Fluorescent images staining positive for GFAP and vimentin revealed that in the MF group, cells exhibited a star-shaped appearance, indicating the presence of astrocytes (Fig. 6D). The astrocytes were observed in none of the IMH eyes and in 12 of 16 MF eyes (75%, P < 0.0001). GFAP is the principal intermediate filament in astrocytes. In the present study, significantly more GFAP debris was observed in the flat-mount preparations of ILMs in the MF group than in the IMH group (Fig. 6F). WB data showed upregulated GFAP expression in the MF group compared with that in the IMH group (Fig. 6E).

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**Table 2.** The Microscopic Findings of ILMs in the IMH and the MF Groups. Statistically Significant P Values Are Shown in Bold

<table>
<thead>
<tr>
<th>Findings</th>
<th>Method</th>
<th>IMH Group</th>
<th>MF Group</th>
<th>P Value</th>
</tr>
</thead>
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<tr>
<td>Cavities, No. (%)</td>
<td>TEM</td>
<td>1 (7.14%)</td>
<td>17 (89.5%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Long irregular indentations, No. (%)</td>
<td>TEM</td>
<td>0 (0%)</td>
<td>17 (89.5%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collagen attached to ILMs, No. (%)</td>
<td>TEM</td>
<td>1 (7.14%)</td>
<td>1 (5.26%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Müller cell debris counts, No./field</td>
<td>TEM</td>
<td>10.16 ± 0.92</td>
<td>42.25 ± 3.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Large Müller cell debris, No. (%)</td>
<td>TEM</td>
<td>0 (0%)</td>
<td>12 (63.2%)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Total cell counts, No./field</td>
<td>IF</td>
<td>3.67 ± 0.52</td>
<td>28.69 ± 2.47</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Astrocytes, No. (%)</td>
<td>IF</td>
<td>0 (0%)</td>
<td>12 (75%)</td>
<td>&lt;0.0001</td>
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</tbody>
</table>

* Student’s t-test.
† Fisher’s exact test.
DISCUSSION

To the best of our knowledge, this study constitutes the first use of multiple advanced modalities to elucidate the morphology, composition, and biomechanical contribution of the ILM to the pathogenesis of MF. However, there is a debate on the terminology of MF. The term “schisis” refers to “absolute breaking up of adhesions or attachments,” whereas in pathologic myopia, the retinal tissues are not separated but rather stretched with “bridges” of neural retinal elements spanning between the retinal layers. Therefore, the term “myopic ectatic retinopathy” was proposed by some researchers to accurately describe the stretching of retinal layers that occurred in pathologic myopic patients.11

TABLE 3. Correlations Between Mean ILM Thickness and Age, Axial Length, Refractive Error

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age</th>
<th></th>
<th>Axial Length</th>
<th></th>
<th>Refractive Error</th>
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<tr>
<td>IMH group</td>
<td>0.788</td>
<td>0.001</td>
<td>0.086</td>
<td>0.769</td>
<td>0.05</td>
<td>0.864</td>
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<tr>
<td>MF group</td>
<td>-0.319</td>
<td>0.183</td>
<td>0.119</td>
<td>0.627</td>
<td>-0.091</td>
<td>0.711</td>
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</table>

Statistically significant P values are reported in bold. Statistical significance was tested using Pearson correlation.

FIGURE 3. AFM probing of Young’s modulus. (A) Young’s modulus of the ILM is much higher in the MF group compared with that in the IMH group (P < 0.0001). (B) The diagrams illustrate correlations of Young’s modulus with axial lengths. (C) A typical extension-retraction cycle of AFM obtained in this study. (D) The summary of all stiffness measurements from one typical IMH ILM confirms an appearance of normal distribution, whereas the distribution of Young’s modulus is disorganized and inhomogeneous in one typical MF ILM. (E) In the IMH group, the distribution pattern of Young’s modulus shows an appearance of concentric circles. However, the distribution of Young’s modulus is irregular in the MF group.
The ILM is a specialized basement membrane (BM) located at the border between the vitreous body and retinal neuroepithelium. During embryogenesis, ILM plays a vital role in eye development and stability. In the adult eye, ILM has been demonstrated to be involved in the aging process and pathogenesis of vitreoretinal diseases. As a principal property, BM thickness is important in the physical homeostasis and maintenance of the mechanical stability of tissue walls. Histopathologic studies showed that the thickness of ILM was 1 to 2 μm in healthy cadaver eyes and 1.8 ± 0.6 μm in

<table>
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<tr>
<th>Group</th>
<th>TEM Thickness</th>
<th>AFM Thickness</th>
<th>Age</th>
<th>Axial Length</th>
<th>Refractive Error</th>
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<tr>
<td></td>
<td>r</td>
<td>P</td>
<td>r</td>
<td>P</td>
<td>r</td>
</tr>
<tr>
<td>IMH group</td>
<td>0.194</td>
<td>0.677</td>
<td>0.1</td>
<td>0.831</td>
<td>0.284</td>
</tr>
<tr>
<td>MF group</td>
<td>0.603</td>
<td>0.152</td>
<td>0.347</td>
<td>0.445</td>
<td>−0.542</td>
</tr>
</tbody>
</table>

Statistically significant P values are reported in bold. Statistical significance was tested using Pearson correlation.

**TABLE 4.** Correlations Between Young’s Modulus on the Vitreal Side and ILM Thickness, Axial Length, Refractive Error

**FIGURE 4.** Immunoreactivity distribution of collagen IV and its isoforms in cross sections of the ILM. Collagen IV stain is prominent in the vitreal side of ILM in the IMH group, whereas it exhibits bipolar-like expression in the MF group. Collagen α5 (IV) colocalizes with α4 (IV)/α5 (IV) and contributes to an abundant staining on the vitreal surface of the ILMs in the IMH group. By contrast, in the MF group, collagen α3 (IV) and α4 (IV) are predominantly expressed on the retinal surface, and α5 (IV) is evenly distributed throughout the ILM. The assessment of collagen α1 (IV) protein shows a similar distribution pattern between the MF and IMH groups, as well as α2 (IV) and α6 (IV). Scale bar denotes 20 μm.
patients with IMH. The current data closely match earlier results with the average thickness of ILMs in the IMH group (1.89 ± 0.24 μm). More importantly, the present study provides the first analysis of the thickness of freshly peeled ILM specimens from patients with MF. TEM micrographs showed that the MF group exhibited a markedly reduced ILM thickness (0.75 ± 0.21 μm). The result is supported by earlier publications showing that several layers of the eyeball became severely thinner in pathologic myopic patients. As shown by OCT previously, the subfoveal choroidal thickness of highly myopic eyes was significantly thinner than that of normal eyes (118 ± 68 vs. 287 ± 76 μm), and so was the scleral thickness in the posterior pole (602 ± 190 vs. 752 ± 148 μm). Hypothetically, the reason for ILM thinning was similar to high myopia–induced thinning of the choroid and sclera, which were the results of biomechanical stretching induced by excessive axial elongation of the eyeball.

Alterations of the ILM thickness in MF eyes were accompanied by morphologic changes. In the present study, all the ILM samples were folded and showed two surfaces, which was consistent with observations in previous publications. Our findings showed that all the ILM specimens in the MF group had lost their homogeneous spongy meshwork. Abnormal ultrastructural features were confirmed, including highly irregular long indentations and vastly larger amounts of cellular debris. As demonstrated by Sebag, the contours of these indentations were attributable to insertions of Müller cells into the retinal side of the ILM. Thus, the highly irregular long indentations in the ILMs indicated abnormally long insertions of Müller cells into the ILMs. Cell debris in the ILM was defined as fragments of Müller cell end-feet. In the present study, TEM and SEM imaging detected a vastly larger amount of cellular debris on the retinal sides of the ILMs in the MF group, which was in accordance with previous studies. Increased cellular debris might be caused by long irregular insertions of Müller cells into the ILMs, which reflect the response of Müller cells to anterior–posterior stretching of the eye.

Proper ILM stiffness helps maintain mechanical balance and stability between the vitreoretinal interface and is critical for intrinsic homeostasis of the eye. Young's modulus is used to evaluate a material's intrinsic biomechanical stiffness and was measured between 0.75 and 1.5 MPa in healthy ILMs. The current data showed that Young's modulus of ILM segments on the vitreal side in the IMH group was 0.87 ± 0.23 MPa, which was in agreement with previous data. Thus far, no data have been reported regarding the quantitative analysis of ILM stiffness in patients with MF. Our findings showed that Young's modulus of the MF ILMs was significantly higher than that in the IMH group. Additionally, the distribution pattern of ILM stiffness in MF group was altered as well. A summary of ILM stiffness measurements from MF eyes showed that the distribution pattern of Young's modulus was rather irregular and did not exhibit distinct features. The disorganized topographic distribution of Young's modulus might lead to a loss of surface tension stability. Potentially, ILMs with significantly increased stiffness generate a centripetal traction and cannot follow the progressive scleral elongation and

Figure 5. The concentrations of collagen IV and its isoforms in the ILMs between the IMH and the MF groups. (A) WBs were probed for collagen IV, α1 (IV), α2 (IV), α3 (IV), α4 (IV), α5 (IV), α6 (IV) and GAPDH (as loading control). Representative images are shown. (B) Densitometry analysis of collagen IV and its isoforms from ILM tissues. The graphs show decreased expression levels of collagen IV, α3 (IV), α4 (IV), and α5 (IV) in ILMs of MF eyes compared with those in the IMH group. There were no significant changes in collagen α2 (IV) concentrations between the two groups, as well as α2 (IV) and α6 (IV). (C) Air-mode measurements of AFM demonstrate that collagen fibers are sparse in the MF group compared with the IMH group. Asterisk denotes P < 0.05.
stretching, contributing to the cause of retinal splitting in the pathogenesis of pathologic myopia.

Since the basic framework of the ILM is composed of networks of collagen IV and polymerized laminins, it was assumed that ILM stiffness disparity might be influenced by altered protein composition and the density of collagen cross-links. Collagen IV is the dominant protein and contributes to the elastic qualities of human ILM tissues. It is composed of six α chains, α1(IV) to α6(IV), which assemble into three distinct collagen IV trimers—α1α1α2, α3α4α5, and α5α5α6. Different heterotrimeric structures have distinct side-selective distributions and physical characteristics, which might reflect a tissue’s altering demands for biomechanical stability. For example, a histopathologic study suggested that a large number of molecular cross-links were present in α3/α4/α5 heterotrimers. As a result, α3/α4/α5 heterotrimers were supposed to be more resistant to biomechanical stress. In the current study, IF staining revealed that α3 (IV), α4 (IV), and α5 (IV) were abundant on the vitreal side of the ILMs in the IMH group, which was in accordance with previous results. However, in the MF eyes, collagen α3/α4 (IV) was prominently localized on the retinal side of the ILMs, whereas α5 (IV) was evenly distributed. This might contribute to the increased stiffness on the retinal side of ILM in MF eyes. Taken together, altered expression levels and distributions of α3 (IV) to α5 (IV) chains might influence the biomechanical properties of the ILMs and reflect their compensation for perpendicular traction during the elongation of axial length in the MF group.

To further explore the underlying cellular mechanism of ultrastructural and biomechanical changes in ILMs, particular attention was paid to investigate the cell types that might be responsible for the pathogenesis. Müller cells are the dominant macroglial cells of the retina, spanning the entire depth of the retina. In addition to Müller cells, astrocytes are another type of neuron-supporting macroglial cell in the mammalian retina. It has been reported that Müller cells and astrocytes can undergo reactive gliosis in response to mechanical...
stiffness and certain disease features. The process was characterized by hypertrophy of the cell body, process extension, and increased GFAP expression, which might alter both the tissue-wide and cellular biomechanics of the retina to maintain mechanical integrity. A study exploring Müller cell remodeling in a cat model of retinal detachment revealed the growth of Müller cells into the inner retina, increasing the visibility of cell processes on the vitreal surface of the ILM. Therefore, abnormal ultrastructure including long irregular indentations and increased cell debris, which represented Müller cell process extension, might result from Müller cell reactive gliosis responding to mechanical stress during the elongation of axial length.

Ultrastructural analyses of ILMs excised from highly myopic eyes with myopic traction maculopathy demonstrated that fibrous astrocytes were the major cell population. In the present study, the number of astrocytes in the MF group was higher than that in the IMH group. The presence of a higher number of astrocytes might be caused by the migration of retinal glial cells to the inner surface of the ILM, which might facilitate the development of tangential traction at the vitreoretinal interface.

Müller cell reactive gliosis and astrogliosis are characterized by rapid synthesis of GFAP, which is an indicator of stress in the central nervous system (CNS) and helps maintain mechanical strength of cells. As an extension of the brain, the retina also responds to stress in a similar fashion, inducing GFAP upregulation in both reactive Müller cells and astrocytes. Thus, the biomechanics of the retina would be modified and play a potential role in maintaining homeostasis. Our results showed that the concentration of GFAP in ILMs, secreted by Müller cells and astrocytes, was higher in the MF group. Therefore, upregulation of GFAP in ILMs provided more evidence for reactive Müller cell gliosis and astrogliosis, which might increase mechanical support for the cells and surrounding ECM.

The present study had some limitations. First, the present study did not uncover the cell signal pathway involved in the pathogenesis. Future studies should focus on the underlying mechanism of ILM alterations because it might be crucial to the development of a new potential therapeutic target in MF progression. Second, ICG has been reported to exert adverse effects on ILM morphology, stiffness, and the amount of debris on the retinal side of the ILM. However, out of consideration of patients' safety, ICG was injected intravitreally to study the ILM in all MF patients in the present study. To reduce the effect of ICG on the ILM, the concentration and the duration of ICG staining were strictly monitored. Moreover, the IMH group served as control in this study, in which the staining time and concentration of ICG were exactly the same as the MF group. As a result, the toxic effect of ICG was minimized and the result was reliable.

In summary, the present study demonstrated MF-related changes in the ILM, including the peculiar stiffness, thickness, long irregular indentations, and a large amount of cell debris on the retinal side along with decreased concentration and altered distribution of collagen IV. These alterations were caused by mechanical stress resulting from elongation of the axial length, the formation of staphylomas in pathologic myopia, and consequent Müller cell and astrocyte gliosis. It is conceivable that increased stiffness of ILM has a major role in the formation of MF. However, the present study did not uncover the cell signaling pathways involved in MF pathogenesis. Future investigations are needed to elucidate these underlying molecular mechanisms, which have direct relevance for the development of novel therapeutic strategies for preventing MF progression.

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