TIMP-3 in Bruch’s Membrane: Changes during Aging and in Age-Related Macular Degeneration

Motobiro Kamei and Joe G. Hollyfield

Purpose. To assess the distribution, content, and function of tissue inhibitor of metalloproteinases (TIMP)-3 during aging in normal eyes for comparison with the levels observed in eyes with age-related macular degeneration (AMD).

Methods. Donor tissues analyzed included 36 normal eyes (14–96 years old) and 15 AMD eyes (74–98 years old). A tissue strip including the fovea was used for immunohistochemistry. Western blot analysis was performed on extracts of the retinal pigment epithelium (RPE)–choroid complex from the posterior part of each eye. Immunoreactivity of TIMP-3 bands in each western blot was densitometrically quantitated. The inhibitory function of TIMP-3 was evaluated with reverse zymography.

Results. TIMP-3 was present uniformly across Bruch’s membrane in the normal samples. In samples from donors more than 50 years of age, immunostaining was intense. TIMP-3 content ranged from 92 to 1061 ng/cm² and increased with age (r = 0.66). In AMD eyes, TIMP-3 distribution in Bruch’s membrane was abundant in areas of continuous soft drusen but absent in areas below RPE atrophy. TIMP-3 levels in AMD eyes were significantly higher than in age-matched normal eyes (577 versus 877 ng/cm²; P = 0.009). Inhibitory activity correlated well with TIMP-3 content (r = 0.82) and was also significantly higher in AMD eyes than in age-matched normal eyes (P < 0.001).

Conclusions. During normal aging, TIMP-3 content in Bruch’s membrane of the macula shows a significant increase. TIMP-3 content in AMD eyes was elevated relative to that of age-matched normal eyes. Higher levels of TIMP-3 may contribute to the thickening of Bruch’s membrane observed in AMD. (Invest Ophthalmol Vis Sci. 1999;40:2367–2375)

Matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs) play important roles in regulating the turnover of the extracellular matrix (ECM). MMPs constitute a family of secreted enzymes, currently with more than 20 members, that are involved in degrading components of the ECM in the normal course of matrix turnover and renewal. ¹ MMPs are also implicated during the initial stages of neovascularization, in which they are thought to be required, along with other proteases, for degradation of components of the capillary basement membrane as a prerequisite for new vessel outgrowth.² The TIMPs, which are represented by four distinct gene products, are thought to suppress excessive degradation of ECM and may play an important functional role in limiting neovascularization.³⁻¹⁰ TIMP-3 is unique in that once secreted, it binds to component(s) of the ECM, whereas other TIMPs do not.¹¹ In the outer eye wall, immunohistochemical studies indicate that TIMP-3 is present in normal Bruch’s membrane,¹²⁻¹⁵ and in situ hybridization studies indicate that the RPE is a major site of TIMP-3 gene expression.¹⁴⁻¹⁵ One role of TIMP-3 in Bruch’s membrane may be as a potent local inhibitor of MMP activity, regulating the rate of Bruch’s membrane turnover, as well as limiting choroidal neovascularization.

Sorsby’s fundus dystrophy is an early-onset, inherited form of macular degeneration, characterized by thickening of Bruch’s membrane and submacular neovascularization, which are also features of AMD. Mutations in the gene coding for TIMP-3 have been found in families with Sorsby’s fundus dystrophy.¹⁶ Immunohistochemical studies of a donor eye from a Sorsby’s patient showed extensive TIMP-3 accumulation in the thickened Bruch’s membrane.¹⁷ These observations led us to evaluate TIMP-3 content and distribution in Bruch’s membrane of AMD donor eyes, which are known to accumulate drusen and exhibit abnormal thickening of this layer. Although no mutations in the coding region or the regulatory elements of the TIMP-3 gene have been discovered in patients with AMD to date,¹⁸⁻¹⁹ excess TIMP-3 within the ECM could prevent normal matrix remodeling and could be causally involved in the increased thickening of Bruch’s membrane that occurs in AMD.

Because of the importance of Bruch’s membrane permeability in the trafficking of metabolites between choroid and RPE²⁰ and the known early alterations in this lamina in AMD,²¹ it is important to understand the relationship of TIMP-3 in normal aging and AMD. In this study we follow the age-related changes in TIMP-3 distribution, content, and inhibitory activity during normal aging and compare these levels with those present in age-matched AMD donor eyes.
**METHODS**

**Donor Tissues**

Thirty-two eyes from normal human donors (Table 1) and 15 eyes from AMD donors (Table 2) were used. Normal eyes were obtained through the Cleveland Eye Bank, Ohio, and the National Disease Research Interchange (Philadelphia, PA). They were enucleated between 1.5 and 7 hours after death and preserved at 4°C for 1 to 14 hours. Immediately after arrival at our laboratory or National Disease Research Interchange, they were frozen in liquid nitrogen and stored at −80°C until sample preparation. AMD donor eyes were obtained through the Eye Donor Program of the Foundation Fighting Blindness (Hunt Valley, MD). They were enucleated between 1.5 and 8 hours after death and retained at 4°C for 0.5 to 11.5 hours before freezing in liquid nitrogen before storage at −80°C.

**Tissue Preparation**

Each tissue sample consisted of a 10 × 12 mm rectangle, cut from the posterior pole of the frozen globe, temporal to the optic nerve head. The exact area to be removed was first defined with vernier calipers using the optic nerve, the long posterior ciliary artery and the insertion of the inferior oblique muscle as external markers to localize the position of the fovea internally. An incision through the sclera into the vitreous was made on each side of the rectangle to free the sample. A 2-mm-wide strip of the retina–RPE-choroid–sclera complex, centered on the fovea, was separated for immunohistochemistry.

For biochemical samples, the retina, any adhering vitreous, and sclera were removed from the remaining 10 × 10-mm aerial expanse of tissue and the RPE–Bruch’s membrane–choroid complex was retained for extraction. Each sample was weighed and then homogenized in 350 μl of extraction buffer, consisting of 500 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1% Triton X-100, and protease inhibitors (400 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 100 μg/ml phenylmethylsulfonyl fluoride). After incubation for 15 minutes on ice followed by centrifugation at 13,000 rpm for 30 minutes at 4°C, the supernatant was collected and stored at −70°C. Protein concentration of each sample was measured using bicin-

**Table 1. Normal Donor Eyes Used for TIMP-3 Analysis**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Postmortem Time* (h)</th>
<th>Cause of Death</th>
<th>Weight† (mg)</th>
<th>Protein Concentration (mg/ml)</th>
<th>TIMP-3 Content (ng/cm²)</th>
<th>Relative Inhibitory Activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOR-1</td>
<td>14</td>
<td>F</td>
<td>4/6</td>
<td>Auto accident</td>
<td>139</td>
<td>1.31</td>
<td>91.5</td>
<td>0.10</td>
</tr>
<tr>
<td>NOR-2</td>
<td>14</td>
<td>F</td>
<td>2/4</td>
<td>Asthma</td>
<td>138</td>
<td>1.55</td>
<td>161.6</td>
<td>0.15</td>
</tr>
<tr>
<td>NOR-3</td>
<td>16</td>
<td>F</td>
<td>1.5/4.5</td>
<td>Auto accident</td>
<td>198</td>
<td>1.90</td>
<td>177.7</td>
<td>0.27</td>
</tr>
<tr>
<td>NOR-4</td>
<td>18</td>
<td>F</td>
<td>2/2.5</td>
<td>Auto accident</td>
<td>156</td>
<td>1.94</td>
<td>215.4</td>
<td>0.21</td>
</tr>
<tr>
<td>NOR-5</td>
<td>29</td>
<td>M</td>
<td>6/5</td>
<td>Adenocarcinoma</td>
<td>178</td>
<td>1.58</td>
<td>113.1</td>
<td>0.21</td>
</tr>
<tr>
<td>NOR-6</td>
<td>39</td>
<td>M</td>
<td>2/10</td>
<td>Acute cardiac events</td>
<td>273</td>
<td>2.48</td>
<td>280.0</td>
<td>0.27</td>
</tr>
<tr>
<td>NOR-7</td>
<td>60</td>
<td>M</td>
<td>4/1</td>
<td>Acute cardiac events</td>
<td>176</td>
<td>1.35</td>
<td>295.1</td>
<td>0.64</td>
</tr>
<tr>
<td>NOR-8</td>
<td>61</td>
<td>M</td>
<td>2/NA</td>
<td>Acute myoccardial infarction</td>
<td>169</td>
<td>1.73</td>
<td>317.7</td>
<td>0.58</td>
</tr>
<tr>
<td>NOR-9</td>
<td>61</td>
<td>M</td>
<td>4/1</td>
<td>Rupture of aortic aneurysm</td>
<td>202</td>
<td>2.07</td>
<td>210.0</td>
<td>0.47</td>
</tr>
<tr>
<td>NOR-10</td>
<td>62</td>
<td>F</td>
<td>4/2</td>
<td>Cardiomyopathy</td>
<td>202</td>
<td>2.00</td>
<td>486.3</td>
<td>0.83</td>
</tr>
<tr>
<td>NOR-11</td>
<td>65</td>
<td>F</td>
<td>4/4</td>
<td>Rupture of aortic aneurysm</td>
<td>279</td>
<td>2.53</td>
<td>323.3</td>
<td>0.58</td>
</tr>
<tr>
<td>NOR-12</td>
<td>65</td>
<td>M</td>
<td>4.5/4.5</td>
<td>Acute cardiac events</td>
<td>175</td>
<td>1.20</td>
<td>590.7</td>
<td>0.90</td>
</tr>
<tr>
<td>NOR-13</td>
<td>66</td>
<td>M</td>
<td>4/2</td>
<td>Acute cardiac events</td>
<td>224</td>
<td>1.78</td>
<td>405.0</td>
<td>0.59</td>
</tr>
<tr>
<td>NOR-14</td>
<td>69</td>
<td>F</td>
<td>3.5/10</td>
<td>Adenocarcinoma</td>
<td>182</td>
<td>1.18</td>
<td>388.8</td>
<td>0.71</td>
</tr>
<tr>
<td>NOR-15</td>
<td>70</td>
<td>F</td>
<td>4/5</td>
<td>Acute cardiac events</td>
<td>122</td>
<td>1.28</td>
<td>507.3</td>
<td>0.51</td>
</tr>
<tr>
<td>NOR-16</td>
<td>71</td>
<td>M</td>
<td>4/1</td>
<td>Acute cardiac events</td>
<td>193</td>
<td>1.49</td>
<td>390.4</td>
<td>0.72</td>
</tr>
<tr>
<td>NOR-17</td>
<td>71</td>
<td>F</td>
<td>5.5/13</td>
<td>Acute cardiac events</td>
<td>289</td>
<td>1.37</td>
<td>329.0</td>
<td>0.54</td>
</tr>
<tr>
<td>NOR-18</td>
<td>73</td>
<td>F</td>
<td>3/8</td>
<td>Acute cardiac events</td>
<td>211</td>
<td>1.70</td>
<td>743.1</td>
<td>0.59</td>
</tr>
<tr>
<td>NOR-19</td>
<td>81</td>
<td>M</td>
<td>2.5/4.5</td>
<td>Acute myoccardial infarction</td>
<td>205</td>
<td>1.56</td>
<td>700.1</td>
<td>0.77</td>
</tr>
<tr>
<td>NOR-20</td>
<td>81</td>
<td>F</td>
<td>6.5/14</td>
<td>Pneumonia</td>
<td>193</td>
<td>1.28</td>
<td>942.4</td>
<td>0.82</td>
</tr>
<tr>
<td>NOR-21</td>
<td>83</td>
<td>F</td>
<td>3/4.5</td>
<td>Pneumonia</td>
<td>164</td>
<td>1.73</td>
<td>463.1</td>
<td>0.58</td>
</tr>
<tr>
<td>NOR-22</td>
<td>85</td>
<td>M</td>
<td>2.5/6.5</td>
<td>Congestive heart failure</td>
<td>181</td>
<td>2.03</td>
<td>554.7</td>
<td>0.69</td>
</tr>
<tr>
<td>NOR-23</td>
<td>85</td>
<td>F</td>
<td>6.5/5</td>
<td>Gastrointestinal bleeding</td>
<td>156</td>
<td>1.22</td>
<td>533.1</td>
<td>0.58</td>
</tr>
<tr>
<td>NOR-24</td>
<td>85</td>
<td>M</td>
<td>5.5/5</td>
<td>Sepsis</td>
<td>226</td>
<td>1.13</td>
<td>576.2</td>
<td>0.55</td>
</tr>
<tr>
<td>NOR-25</td>
<td>89</td>
<td>F</td>
<td>2.5/14</td>
<td>Pneumonia</td>
<td>176</td>
<td>1.64</td>
<td>1060.8</td>
<td>0.83</td>
</tr>
<tr>
<td>NOR-26</td>
<td>90</td>
<td>F</td>
<td>5/6.5</td>
<td>Acute myoccardial infarction</td>
<td>130</td>
<td>1.18</td>
<td>263.9</td>
<td>0.66</td>
</tr>
<tr>
<td>NOR-27</td>
<td>92</td>
<td>M</td>
<td>2/NA</td>
<td>Acute myoccardial infarction</td>
<td>142</td>
<td>1.57</td>
<td>1010.0</td>
<td>0.63</td>
</tr>
<tr>
<td>NOR-28</td>
<td>95</td>
<td>F</td>
<td>5/11</td>
<td>Acute myoccardial infarction</td>
<td>134</td>
<td>1.31</td>
<td>263.0</td>
<td>0.35</td>
</tr>
<tr>
<td>NOR-29</td>
<td>95</td>
<td>F</td>
<td>7/6</td>
<td>Congestive heart failure</td>
<td>148</td>
<td>1.46</td>
<td>424.0</td>
<td>0.39</td>
</tr>
<tr>
<td>NOR-30</td>
<td>94</td>
<td>M</td>
<td>5/9</td>
<td>Squamous cell carcinoma</td>
<td>152</td>
<td>1.42</td>
<td>524.0</td>
<td>0.41</td>
</tr>
<tr>
<td>NOR-31</td>
<td>94</td>
<td>F</td>
<td>6/1</td>
<td>Respiratory failure</td>
<td>147</td>
<td>1.07</td>
<td>710.8</td>
<td>0.90</td>
</tr>
</tbody>
</table>

NA, data not available.

* Postmortem time represented as death–enucleation/enucleation–freezing intervals.
† Weight refers to the isolated 10 × 10-mm sample used for TIMP-3 analysis.
§ Ratio (sample pixel density)/(standard pixel density).
cholinic acid (BCA; Pierce, Rockford, IL) and bovine serum albumin (BSA) as a reference standard.

TIMP-3 content was evaluated as the amount per area (10 × 10 mm²). Wet tissue weight and protein content of each sample are presented in Tables 1 and 2.

### Immunohistochemistry

The 2 × 10-mm tissue strip including the fovea was embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen for immunocytochemistry in the absence of fixation. Indirect immunohistochemistry was performed on 8-μm-thick cryosections. After quenching endogenous peroxidase activity with 0.3% hydrogen peroxide and blocking nonspecific antibody binding with 5% BSA in PBS containing 0.3% Triton X-100, the sections were incubated overnight at 4°C with a mouse monoclonal anti-human TIMP-3 antibody (either Clone 136-13H4, provided by Sunee Apte or 136-17B12, provided by Kazushi Iwata, [Fuji Chemical Industries, Toyama, Japan]). The primary antibody was used at 1:1000 dilution. Biotinylated horse anti-mouse IgG (1:200 dilution; Vector, Burlingame, CA) was used as a secondary antibody followed by the avidin–biotin complex method (Elite ABC, Vector). Immunoreactivity was resolved with horseradish peroxidase–aminoethylcarba-

<table>
<thead>
<tr>
<th>Table 2. AMD Donor Eyes Used for TIMP-3 Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. (FFB accession no.)</strong></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>AMD-1 (359)</td>
</tr>
<tr>
<td>AMD-2 (511)</td>
</tr>
<tr>
<td>AMD-3 (503)</td>
</tr>
<tr>
<td>AMD-4 (321)</td>
</tr>
<tr>
<td>AMD-5 (429)</td>
</tr>
<tr>
<td>AMD-6 (446)</td>
</tr>
<tr>
<td>AMD-7 (444)</td>
</tr>
<tr>
<td>AMD-8 (334)</td>
</tr>
<tr>
<td>AMD-9 (344)</td>
</tr>
<tr>
<td>AMD-10 (552)</td>
</tr>
<tr>
<td>AMD-11 (447)</td>
</tr>
<tr>
<td>AMD-12 (335)</td>
</tr>
<tr>
<td>AMD-13 (450)</td>
</tr>
<tr>
<td>AMD-14 (355)</td>
</tr>
<tr>
<td>AMD-15 (EB)</td>
</tr>
</tbody>
</table>

CNV, choroidal neovascularization; NA, data not available; EB, donation obtained directly from a local eyebank.

* Postmortem time represented as death–enucleation/enucleation–freezing intervals.

† Weight refers to the isolated 10 × 10-mm sample used for TIMP-3 analysis.

‡ Total interval from death to freezing.

§ Ratio (sample pixel density)/(standard pixel density).

Microscopic Evaluation

Because no detailed clinical history was available on any of the donor eyes classified as AMD, it was necessary to evaluate the status of the disease in the AMD donor tissues based on the histopathology of the tissue samples. For classification of the AMD disease status, we assessed the following criteria, also used in the recently published ARMD Grading System²²: hard drusen status: (−) no hard drusen, (+) 1 to 3 hard drusen in the 10-mm section, (++) 4 or more hard drusen in the 10-mm section; soft drusen status: (−) no soft drusen, (+) patchy soft drusen present but not continuous, (++) continuous soft drusen extending more than 0.5 mm; the presence and length of choroidal neovascular membranes; and RPE atrophy measured in the full 10-mm length of the section. We could not evaluate basal laminar or linear deposits, because these features cannot be distinguished within drusen with light microscopy on unfixed tissue samples. The listed features are presented for each AMD donor eye in Table 2.
Western Blot Analysis
Immediately before electrophoresis, 3.5 μl of sample buffer (4× NuPage sample buffer, Novex, San Diego, CA) containing dithiothreitol (final concentration, 50 mM) was added to 6.5 μl of the protein extract. The 4× sample buffer (pH 8.5) consisted of 1.17 M sucrose, 563 mM Tris base, 423 mM Tris-HCl, 278 mM sodium dodecyl sulfate (SDS), 2.05 mM EDTA, 0.88 mM Coomassie Blue R250 and 0.70 mM phenol red. After heating for 10 minutes at 70°C, 10-μl volumes of each sample were loaded into gel slots for electrophoresis on 10% gels (bis-Tris; Novex) with 2-(N-morpholino) ethane sulfonic acid SDS running buffer (NuPage MES-SDS; Novex). Recombinant human TIMP-3 protein (10 ng) was included in a separate lane as a positive control. Proteins were transferred from the gel to polyvinylidene difluoride membranes using blotting apparatus (XCell II; Novex). After transfer, membranes were incubated with blocking solution (2% BSA in Tris-buffered saline) for 1 hour. The monoclonal anti-human TIMP-3 antibody (Clone 136-17B12) conjugated to horseradish peroxidase was applied at 1:1000 dilution and incubated overnight at 4°C. After rinsing, immunoreactivity was displayed with the chemiluminescent method (ECL, Amersham, Arlington Heights, IL) and captured on radiographic film during a 10- to 15-second exposure. The immunoreactivity signal was digitized on a Scanwizard (Microtek, Redondo Beach, CA) flatbed scanner. The intensity of immunoreactivity was quantitated from the digitized images obtained were compared with the intensity of Coomassie blue staining of the gelatin in lanes with known concentrations of TIMP-3 standards.

Protein Isolation and N-Terminal Sequence Analysis
In addition to recognizing the TIMP-3 bands at 24 and 27 kDa, the monoclonal antibody also interacted with two additional bands at 37 and 40 kDa. To determine whether these were aggregates of TIMP-3 or unrelated proteins, we excised the higher molecular weight bands from polyvinylidene difluoride membrane after electrotransfer from a separate gel and analyzed the bands by Edman microsequencing using a (Precise 492; PE Biosystems, Foster City, CA) protein sequencer in the Molecular Biology Core Laboratory, Case Western Reserve University (Cleveland, OH).

Reverse Zymography
TIMP-3 functional activity in each sample was established with reverse zymography using protease-substrate gel electrophoresis, as described previously.11 In brief, the extracted protein from the RPE-choroid complex was diluted to 75 times and activated by adding calcium chloride at a final concentration of 10 mM. After mixing with 4× loading buffer (40 mM Tris, 8% SDS, 40% glycerol, and 0.01% bromphenol blue), 20 μl of the sample (without heating or reducing agents) was separated by electrophoresis in a matrix consisting of 0.1% SDS, 12% polyacrylamide gel containing MMP-2, MMP-9, and 0.1% gelatin (from a reverse zymography kit which also includes TIMP-3 standards provided by Dylan R. Edwards, The University of Calgary, Alberta, Canada). To remove SDS, the gel was rinsed overnight in the following buffer (50 mM Tris [pH 7.5], 5 mM CaCl₂, and 25 mg/ml Triton X-100). The gel was then placed in an incubation buffer (50 mM Tris [pH 7.5] and 5 mM CaCl₂) to allow MMPs to degrade gelatin for 24 hours at 37°C. Coomassie blue stains only the protein (gelatin) in the region where MMP activity has been inhibited. Intensity of the Coomassie blue-stained gelatin was quantitated as described earlier. The values obtained were compared with the intensity of Coomassie blue staining of the gelatin in lanes with known concentrations of TIMP-3 standards.

Statistical Analysis
Western blot analysis and reverse zymography were repeated three times on each sample. The results are expressed as the mean ± 1 SD.

Diversity of tissue weight and protein concentration of samples were analyzed with unpaired Student’s t-test. Correlations between TIMP-3 content and age or inhibitory activity were determined with Pearson’s correlation coefficient. Differences in TIMP-3 content and inhibitory activity between AMD and age-matched normal tissue were analyzed with the Mann-Whitney test.

RESULTS
TIMP-3 Distribution and Content in Normal Eyes
Table 1 presents a summary of all the data and vital information from each normal eye used in this analysis.

Distribution. TIMP-3 immunoreactivity was present in Bruch’s membrane in each normal donor tissue used and was distributed across the full thickness of this membrane. Although immunoreactivity was uniform in each donor sample, in general, eyes from younger donors showed less intense immunostaining than eyes from older donors. In addition to TIMP-3 immunolocalization in Bruch’s membrane, samples from 80- and 90-year-old donors commonly exhibited intense staining in the capillary bed matrix. TIMP-3 immunoreactivity was not evident in the neurosensory retina, the choroid, or the sclera. In the control tissues where nonimmune mouse IgG was substituted for the monoclonal IgG, Bruch’s membrane showed no immunoreactivity. Examples of TIMP-3 immunolocalization from donors at a variety of representative ages are presented in Figure 1.

Content. Western blot analysis with the TIMP-3 antibody revealed a major 24-kDa band and a minor 27-kDa band in each of the RPE-choroid tissues samples (Fig. 2). The 27-kDa band was identical in location to the recombinant glycosylated form of TIMP-3, used as a positive control. The 24-kDa band was reported to correspond to the unglycosylated form of TIMP-3.5 Two additional minor bands at 37 and 40 kDa were also detected by the antibody. These bands were separately isolated from electroblotted polyvinylidene difluoride membrane and analyzed by microsequencing. The 37-kDa band yielded an N-terminal sequence of GVKVKVGNGFSRQIQQLVTARA, which corresponds 100% to the N terminus of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, accession no. X01677). Authentic GAPDH (Boehringer Mannheim, Indianapolis, IN) was used in western blot analyses to evaluate cross-reactivity with the anti-TIMP-3 antibody. Cross-reactivity was evident when at least 125 ng GAPDH was loaded, but was not detected when lower loading levels were applied (data not shown). The 40-kDa immunoreactive band yielded the N-terminal
sequence of LAIPALQAEPQGQ, which shows less than 40% homology to any known protein. Although the identity of this 40-kDa protein was not established, the sequence obtained did not identify this protein as an aggregate of TIMP-3.

The immunoreactivity present in the 24- and 27-kDa western blot bands was variable among different samples. Notably, the relative amounts of immunoreactivity were found to be age dependent, with less intense signals present in samples from young donors than from older donors. Densitometric measurements of specific TIMP-3 immunoreactivity in the 24- and 27-kDa bands suggest that the TIMP-3 content ranged from a low of 92 ng/cm² in a sample from a 14-year-old donor of normal eyes to 1061 ng/cm² in a sample from a 90-year-old donor of normal eyes (Table 1). A direct relationship between TIMP-3 content and age (Fig. 3) appears to be significant in all the normal tissue analyzed (correlation coefficient, \( r = 0.66; n = 32 \)).

To evaluate the influence of postmortem intervals on protein degradation, we performed semiquantitative western

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{TIMP-3 immunolocalization in the normal and AMD donor eyes at the indicated ages. TIMP-3 immunoreactivity in Bruch’s membrane is uniform in each normal sample. The younger donor eyes, especially those from the second or third decade, showed less intense immunostaining than the older donor eyes. In the samples from 80- and 90-year-old normal donor eyes, intense staining was also commonly observed extending into the capillary bed matrix. In AMD eyes, note the areas with continuous soft drusen, which show intense immunoreactivity with the TIMP-3 antibody. Original magnification, \( \times 500 \); bar, 20 \( \mu \)m.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Western blot of the samples from normal (A) and AMD (B) donors. The 27-kDa band in lanes 1 through 17 is identical in location with the recombinant glycosylated form of TIMP-3 used as a standard in lane 18. The 24-kDa band, which shows the most intense signal, particularly from the older donor eyes, corresponds to the unglycosylated form of TIMP-3. The intensity of the bands appears to increase with age in the samples from normal donors. All the samples from AMD donor eyes show intense immunoreactivity.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The relationship between TIMP-3 content and age of normal donor eyes. Densitometric measurements of the specific TIMP-3 immunoreactivity in the 24- and 27-kDa bands, when converted to protein amounts, show an age-dependent increase in concentration with a significant correlation coefficient (\( r = 0.66 \)).}
\end{figure}
a fibroblastic scar, TIMP-3 immunoreactivity was observed surrounding a choroidal neovascular membrane or of choroidal neovascularization (Fig. 4). In areas where the RPE present, as described earlier, was always noted below the areas RPE atrophy, where little or no TIMP-3 immunoreactivity was neovascularization was present in 8 of the 15 AMD donor eyes. In regions of transition between areas of RPE atrophy and a normal RPE, TIMP-3 immunoreactivity decreases but is still present in soft drusen and below the central elastin layer of Bruch’s membrane (arrowheads; B). TIMP-3 immunoreactivity is not present in the area of choroidal neovascularization with RPE atrophy but is present surrounding the hyperplastic RPE (open arrowhead, C). Original magnification, ×125 (top); ×500 (bottom); bars, 20 μm.

In general, AMD samples showed more intense immunoreactivity in western blot analysis when compared with age-matched normal eyes. Average densitometric analysis suggests that TIMP-3 content in AMD eyes was 877 ± 325 ng/cm² (n = 15) and 577 ± 241 ng/cm² in the age-matched normal eyes (n = 18). The measurements demonstrating this apparent difference in TIMP-3 content in the macular region between AMD and normal eyes were statistically significant (P = 0.009; Fig. 5).

**TIMP-3 Function in Normal and AMD Eyes**

The inhibitory activity of TIMP-3 was evaluated with reverse zymography. In each sample analyzed, a densely staining gelatin band remained in the zymograms at 24 kDa and a minor stained band remained at 27 kDa, identical with the results of recombinant TIMP-3 (Fig. 6). Inhibitory activity was not present in the areas of the zymograms above 27 kDa. Some samples also showed gelatin remaining at 21 and 22 kDa. The 21-kDa band was identical with the band of recombinant TIMP-2. No inhibitory activity was observed in the location corresponding to TIMP-1 (at 28.5 kDa). The identity of the inhibitor present at the 22-kDa location has not been established; this could represent a partially degraded form of TIMP-3 or an as yet undescribed member of the TIMP family. The intensity of staining of the remaining gelatin in the 24- and 27-kDa locations varied with samples from 0.10 to 1.69 arbitrary units (AU). Therefore, it is reasonable to use samples with various post-mortem intervals as long as the samples are stored less than 12 hours at room temperature. The donor eyes used in this study were enucleated within 8 hours and kept at 4°C until frozen, which was within 12 to 14 hours after enucleation.

** TIMP-3 Distribution and Content in AMD Eyes**

Table 2 presents the summary of all the data and vital information from each AMD eye used in this analysis.

**Distribution.** Representative examples of several features characteristic of AMD eyes are shown in Figure 4. Each AMD eye used contained soft drusen, with 7 of the 15 samples having a continuous expanse of soft drusen for the full 10-mm length of the sample section examined. Whenever soft drusen were observed, whether isolated or continuous, each was intensely immunoreactive with the TIMP-3 antibody (Figs. 1, 4A). Hard drusen, when present, were also strongly immunoreactive. Most of the AMD donor tissues (13 of 15) contained areas of RPE atrophy, which involved from 0.5 to 8.5 mm of the 10-mm section length examined. In Bruch’s membrane below the expanses of RPE atrophy, TIMP-3 immunoreactivity was either not evident or barely detectable (Fig. 4C). Choroidal neovascularization was present in 8 of the 15 AMD donor eyes. RPE atrophy, where little or no TIMP-3 immunoreactivity was present, as described earlier, was always noted below the areas of choroidal neovascularization (Fig. 4). In areas where the RPE had proliferated around a choroidal neovascular membrane or a fibroblastic scar, TIMP-3 immunoreactivity was observed surrounding the hyperplastic RPE (Fig. 4C). The immunoreactivity is subtle, however, and shows irregular distribution, possibly because of a loss of function and polarity of the proliferated RPE. In regions of transition between areas of RPE atrophy and a normal RPE, TIMP-3 immunoreactivity decreases but is still present in soft drusen and below the central elastin layer of Bruch’s membrane (Fig. 4B).

**Content.** In general, AMD samples showed more intense immunoreactivity in western blot analysis after incubating tissue buttons punched out from one eye for various intervals at room temperature. The results showed no apparent change for 12 hours but a decrease after 24 hours (data not shown). This indicates that TIMP-3 can be a considerably stable molecule with six pairs of disulfide bonds. Therefore, it is reasonable to use samples with various post-mortem intervals as long as the samples are stored less than 12 hours at room temperature. The donor eyes used in this study were enucleated within 8 hours and kept at 4°C until frozen, which was within 12 to 14 hours after enucleation.

** TIMP-3 Distribution and Content in AMD Eyes**

Table 2 presents the summary of all the data and vital information from each AMD eye used in this analysis.

**Distribution.** Representative examples of several features characteristic of AMD eyes are shown in Figure 4. Each AMD eye used contained soft drusen, with 7 of the 15 samples having a continuous expanse of soft drusen for the full 10-mm length of the sample section examined. Whenever soft drusen were observed, whether isolated or continuous, each was intensely immunoreactive with the TIMP-3 antibody (Figs. 1, 4A). Hard drusen, when present, were also strongly immunoreactive. Most of the AMD donor tissues (13 of 15) contained areas of RPE atrophy, which involved from 0.5 to 8.5 mm of the 10-mm section length examined. In Bruch’s membrane below the expanses of RPE atrophy, TIMP-3 immunoreactivity was either not evident or barely detectable (Fig. 4C). Choroidal neovascularization was present in 8 of the 15 AMD donor eyes. RPE atrophy, where little or no TIMP-3 immunoreactivity was present, as described earlier, was always noted below the areas of choroidal neovascularization (Fig. 4). In areas where the RPE had proliferated around a choroidal neovascular membrane or a fibroblastic scar, TIMP-3 immunoreactivity was observed surrounding the hyperplastic RPE (Fig. 4C). The immunoreactivity is subtle, however, and shows irregular distribution, possibly because of a loss of function and polarity of the proliferated RPE. In regions of transition between areas of RPE atrophy and a normal RPE, TIMP-3 immunoreactivity decreases but is still present in soft drusen and below the central elastin layer of Bruch’s membrane (Fig. 4B).

**Content.** In general, AMD samples showed more intense immunoreactivity in western blot analysis after incubating tissue buttons punched out from one eye for various intervals at room temperature. The results showed no apparent change for 12 hours but a decrease after 24 hours (data not shown). This indicates that TIMP-3 can be a considerably stable molecule with six pairs of disulfide bonds. Therefore, it is reasonable to use samples with various post-mortem intervals as long as the samples are stored less than 12 hours at room temperature. The donor eyes used in this study were enucleated within 8 hours and kept at 4°C until frozen, which was within 12 to 14 hours after enucleation.
TIMP-3 function showed a significant correlation with age in the normal eyes ($r = 0.67; n = 32$) and was highly correlated with TIMP-3 content in all macular samples ($r = 0.82; n = 47$; Fig. 7). Notably, TIMP-3 inhibitory activity detected by reverse zymography was elevated approximately twofold in the AMD eyes compared with the age-matched normal eyes ($P < 0.001$; Fig. 8).

**DISCUSSION**

The amount of functional TIMP-3 present in Bruch’s membrane below the macula of the normal human retina appears to be age dependent. Sections from the fovea of the donors from the second and third decade of life were weakly immunoreactive with anti-TIMP-3 antibody. This correlated well with lower amounts of immunoreactivity observed in western blot analysis. Additionally, TIMP-3 distribution changed in the 9th and 10th decade samples, with extension of immunoreactivity from Bruch’s membrane into the matrix surrounding the choriocapillaris. Quantitative analysis showed that TIMP-3 content and function in the macula increased with age with a significant correlation coefficient ($r = 0.66$ and 0.67, respectively).

Proteins that increase or decrease with age can be referred to as senescence-related proteins and are candidate molecules for age-related diseases.23,24 Accordingly, we propose that TIMP-3 is a senescence-related protein.

Immunostaining of TIMP-3 was conspicuous in extensive accumulation of drusen, which is characteristic of AMD eyes. Quantitative analysis showed that TIMP-3 levels were significantly elevated in the macula of AMD eyes compared with normal eyes. AMD eyes, however, showed a nonuniform distribution, with virtually no TIMP-3 immunoreactivity below the area of RPE atrophy and abundant immunoreactivity in the area outside the atrophic areas where the RPE was present, which is consistent with the observation in Sorsby’s fundus dystrophy and retinitis pigmentosa.17 This indicates that TIMP-3 distribution in AMD eyes with RPE atrophy is nonuniform. To determine the TIMP-3 content below areas where the RPE remains, we assumed that the area of RPE atrophy was circular and that
The diameter was represented by the length of atrophy measured in the tissue section. We then calculated the area of RPE atrophy and subtracted that area from $10 \times 10$ mm$^2$. With this adjusted denominator, it was apparent that the TIMP-3 content below areas where RPE is present is approximately two times higher in AMD eyes than in age-matched normal eyes (Fig. 5).

The monoclonal antibody used in this study recognized recombinant TIMP-3 in western blot analysis exhibiting characteristic 27- and 24-kDa bands. Although two higher molecular weight bands (approximately 37 and 40 kDa) appeared in the blots of most tissue samples, N-terminal amino acid sequence analysis revealed that these proteins were unrelated to TIMP-3. Reverse zymography also showed that these higher molecular weight components have no inhibitory activity. The 37-kDa nonspecific band was found to be GAPDH, a ubiquitous intracellular enzyme, and the purified protein showed cross-reactivity with the TIMP-3 antibody but with low affinity. In immunohistochemistry, however, no intracellular staining was observed. The presence of cross-reactivity in the western blot analysis and the absence of intracellular staining in immunohistochemistry probably resulted from differences in protein content. Coomassie staining of the transferred membrane showed a large amount of 37-kDa protein in the samples. The total amount of the protein in the tissue extract was large, but the concentration in each cell in the tissue sections was not sufficient to show nonspecific immunoreactivity.

Although neither structural mutations nor mutations in regulatory regions of the TIMP-3 gene are thought to be a cause of AMD, it is possible that a mutation in a gene regulating TIMP-3 translation causes TIMP-3 elevation or that TIMP-3 change occurs downstream of other disorders—for example, changes in the ECM-binding partner. Our immunohistochemical results show that soft drusen were markedly stained with anti-TIMP-3 antibody in AMD eyes. This may imply that an elevated TIMP-3 level in the macula of AMD resulted from TIMP-3 accumulation in soft drusen. Drusen are thought to be composed of incompletely degraded debris that is exocytosed by RPE cells. Various hereditary or nonhereditary factors such as protein alterations, oxidative stress, or disorders of hydrolytic enzymes may participate, along with aging, in accelerating the accumulation of this debris. Those insufficiently digested materials may possess a domain with an affinity for TIMP-3 binding. Because TIMP-3 broadly inhibits MMPs, drusen with excess TIMP-3 may retard Bruch’s membrane renewal. This may result in the thickening of Bruch’s membrane, reducing Bruch’s membrane permeability in the trafficking of metabolites and nutrients between the choroid and RPE, ultimately resulting in RPE and photoreceptor atrophy.

In areas where choroidal neovascularization was observed, the RPE was absent, and virtually no TIMP-3 immunoreactivity was evident in the subjacent Bruch’s membrane. There are two possible sequences to these changes: atrophy of the RPE leads to a decrease in TIMP-3, which is permissive to neovascularization; or choroidal neovascularization causes RPE atrophy, which is followed by TIMP-3 loss. Although current results do not indicate which occurs first, the antiangiogenic activity of TIMP-3 makes the former a more likely scenario. We speculate that, in some AMD eyes, Bruch’s membrane thickening causes an atrophy of RPE, and TIMP-3 levels decrease in the area of RPE atrophy, which is permissive for choroidal neovascularization.

We conclude that the TIMP-3 content in Bruch’s membrane in the macula increases during normal aging and that TIMP-3 content is elevated beyond normal levels in the macular region of AMD eyes. This suggests that TIMP-3 may be one of the key molecules causally involved in Bruch’s membrane thickening during normal aging and in AMD. Further studies are needed to identify the TIMP-3 binding partner(s) in Bruch’s membrane and drusen, to determine whether elevated TIMP-3 levels are caused by increased accumulation or increased synthesis of this inhibitor, and to establish whether TIMP-3 elevation is causally involved in the thickening of Bruch’s membrane, which is associated with age-related macular degeneration.

**Acknowledgments**

The authors thank Joan Fisher of The Foundation Fighting Blindness for her help in securing the AMD donor eyes used in the analysis; Jill Spitzer of the Cleveland Eye Bank for help in securing the normal eyes less than 70 years of age; and the National Disease Research Interchange for obtaining the normal eyes more than 70 years of age; Suneel Apte, Bela Anand-Apte, John W. Crabb, and Dylan R. Edwards for...
valuable discussions and technical advice during the course of this study; and Mary E. Rayborn for help in proofreading the manuscript.

References