Expression of Leukocyte Adhesion Molecules in Human Subfoveal Choroidal Neovascular Membranes Treated with and without Photodynamic Therapy

Deborah C. Yeh, Delsy V. Bula, Joan W. Miller, Evangelos S. Gragoudas, and Jorge G. Arroyo

**PURPOSE.** The purposes of this study were to investigate the immunostaining of the leukocyte adhesion molecules intercellular adhesion molecule (ICAM)-1 and E-selectin in subfoveal choroidal neovascular membranes (CNVMs) surgically excised from patients with age-related macular degeneration (AMD) and to determine whether prior photodynamic therapy (PDT) alters their immunostaining.

**METHODS.** The localization of ICAM-1 and E-selectin in 10 subfoveal CNVMs was determined by immunohistochemistry. Membranes were also immunostained for CD31 to assess vascularity.

**RESULTS.** Significantly higher numbers of CD31-staining vessels per unit membrane area were found in the peripheral regions of the membranes compared with the central regions (P = 0.05). ICAM-1 immunoreactivity in the CNVMs was found predominantly on RPE cells, but also on small vessels in the periphery. ICAM-1 staining was significantly more intense in the peripheral, more cellular areas of the membranes than in the central, more fibrotic regions (P = 0.04). ICAM-1 staining in the periphery of the CNVMs was greater than that in choroidal vessels and the RPE of the normal control eye. ICAM-1 immunostaining grade in peripheral regions of the CNVMs decreased with the increasing number of PDT treatments (P = 0.05). Some of the CNVMs also stained for E-selectin in RPE cells and small vessels in the periphery.

**CONCLUSIONS.** In subfoveal CNVMs from patients with AMD, there is increased immunostaining for leukocyte adhesion molecules, particularly in the peripheral, more cellular regions where angiogenesis may be ongoing. Increasing numbers of PDT treatments may be associated with decreased ICAM-1 immunostaining in the proliferating edges of the CNVMs. Indeed, inflammatory etiologies have been proposed for both nonneovascular and neovascular AMD. Inflammatory responses may be involved in the formation of drusen in nonneovascular AMD, just as chronic inflammatory processes have been implicated in the formation of extracellular plaques in other age-related diseases. An inflammatory etiology of AMD may unify the nonneovascular and neovascular stages of AMD as drusen may serve as nidi of local inflammation that trigger expression of angiogenic factors.

Most vision loss associated with age-related macular degeneration (AMD) results from choroidal neovascularization (CNV), which is the abnormal growth of fragile, leaky vessels from the choroid through Bruch’s membrane into the subretinal space, the subretinal pigment epithelial (RPE) space, or both. Although the triggers for CNV in AMD remain poorly understood, the early phases of angiogenesis in other types of neovascularization are often associated with upregulation of inflammatory mediators and leukocyte adhesion molecules. Examples of inflammation-mediated angiogenesis outside the eye include wound healing and rheumatoid arthritis. Within the eye, growing data suggest a role for inflammation and leukocyte recruitment in the retinal neovascularization of proliferative diabetic retinopathy. Within the choroid, inflammation has been demonstrated histologically in certain types of CNV, such as presumed ocular histoplasmosis syndrome (POHS). These examples raise the possibility that AMD-related CNV may also be mediated by inflammatory processes.

Despite an incomplete understanding of the pathogenesis of CNV in AMD, several new therapies have been developed in recent years, including photodynamic therapy (PDT). PDT involves laser activation and excitation of a photosensitizer, verteporfin, to generate reactive oxygen species that damage the endothelium locally in the choroidal neovascularization, leading to focal thrombosis. The TAP (Treatment of Age-Related Macular Degeneration with Photodynamic Therapy) study has shown significant decreases in vision loss in patients with predominantly classic subfoveal CNVMs when treated with PDT. However, vessel regrowth and recanalization are frequently observed, and multiple PDT treatments (an average of 5.6 treatments in a 2-year period) are often necessary to stop the progression of CNV. It is unclear whether vessel regrowth is due to the underlying pathophysiology of AMD or whether PDT may cause and/or exacerbate local processes that activate angiogenic factors.

Although inflammation and associated leukocyte adhesion molecules have been implicated in the pathogenesis of CNV by animal models of laser-induced CNV and non-AMD causes of clinical CNV, such as POHS, the expression of leukocyte adhesion molecules in CNVMs from patients with AMD has not been investigated to date. Furthermore, there have been no comparisons of leukocyte adhesion molecule expression without and after PDT. Therefore, the purpose of this study was to investigate the immunostaining of leukocyte adhesion molecules ICAM-1 and E-selectin in subfoveal CNVMs surgically excised from patients with AMD. Second, the effects of PDT on vascularity and leukocyte adhesion molecule immunostaining were explored.

From the Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts.

JGA is a recipient of NIH K-23 Mentored Patient-Oriented Research Career Development Award.

Submitted for publication September 6, 2003; revised December 20, 2003 and February 5, 2004; accepted March 1, 2004.

Disclosure: D.C. Yeh, None; D.V. Bula, None; J.W. Miller, None; E.S. Gragoudas, None; J.G. Arroyo, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jorge G. Arroyo, Assistant Professor of Ophthalmology, Harvard Medical School, Retina Service, Division of Ophthalmology, Beth Israel Deaconess Medical Center, 350 Brookline Avenue, Shapiro 5th Floor, Boston, MA 02215; jarroyo@bidmc.harvard.edu.
MATERIALS AND METHODS

CNVMs

After institutional review board approval and informed consent were obtained, and in compliance with the tenets of the Declaration of Helsinki, submacular CNVMs were surgically excised from one eye of 10 different patients with AMD by one of the authors (JGA), as previously described. Some eyes were treated with one or two sessions of verteporfin PDT before excision, as previously detailed. The mean age of the patients was 82 years. In patients who received PDT before CNVM excision, the average time to excision after the last PDT treatment was 2.8 months (range: 0.5–4 months).

Immediately after excision, membranes were fixed in 4% paraformaldehyde for paraffin embedding. Serial 4-μm sections were cut and mounted on slides (Superfrost Plus; VWR, West Chester, PA). One to three sections from each of the CNVMs were processed for immunohistochemistry with each antibody. (These tissue blocks were also used for a separate immunohistochemical study; Bula DV, et al., manuscript submitted.)

Inflamed human tonsil was used as a positive control for immunostaining of ICAM-1, E-selectin, and CD31. Normal whole human eye was also used as a control for ICAM-1 and E-selectin staining.

Immunohistochemistry

Sections were deparaffinized through progressive xylene, 100% ethanol, 95% ethanol, and water baths. Antigen retrieval was performed (Target Retrieval Solution; Dako, Carpinteria, CA) for ICAM-1, CD31, mouse IgG1 isotype control and a separate solution (Basic Antigen Retrieval Solution; R&D Systems, Minneapolis, MN) for E-selectin and the steamer method. Slides were then washed in either Tris-buffered saline plus 0.1% Tween 20 (TBST; Dako) for ICAM-1 or phosphate-buffered saline (PBS; Chemicon, Temecula, CA) for E-selectin and CD31.

For ICAM-1 immunostaining, sections were blocked with 4% normal goat serum for 30 minutes at 25°C in a humidified chamber. Subsequently, sections were incubated with 20 μg/mL anti-ICAM-1 monoclonal IgG1 antibody (MAB2146; Chemicon) for 30 minutes at 25°C, washed in TBST, and incubated with goat anti-mouse antibody alkaline phosphatase labeled polymer and then fast red chromogen provided in a kit (EnVision System AP kit; Dako), according to the manufacturer’s directions.

For E-selectin immunostaining, sections were blocked in blocking reagent (Chemicon) for 30 minutes at 25°C in a humidified chamber, then incubated for 2 hours with a 1:25 dilution of anti-E-selectin monoclonal IgG1 antibody (NCL-CD62E-S82; Vector Laboratories, Burlingame, CA). Sections were then washed in PBS, incubated with biotinylated goat anti-mouse antibody for 30 minutes, washed in TBST, incubated with streptavidin–alkaline phosphatase for 30 minutes, washed in TBST, and incubated with fast red chromogen (Chemicon). CD31 immunostaining was performed using a blood vessel staining kit, alkaline phosphatase system (Chemicon), according to the manufacturer’s instructions, with 20 μg/mL anti-CD31 monoclonal antibody.

Analysis

Slides were analyzed by light microscopy. Immunostaining was graded as 0 (none), 1 (low), 2 (moderate), or 3 (intense) in high power (×400) fields of each section multiple times by two independent observers, and the median score was obtained. Standard grading photographs are in Figure 1. To define the “center” and “periphery” of the membranes, each membrane section was visually divided into four equal lengths along the longitudinal axis. The outer two segments were defined as the periphery and the inner two segments as the center, as demonstrated in Figure 2. Well-defined ringlike clusters or linear patterns of endothelial cells were counted as vessels. The number of vessels per unit membrane area (abbreviated to “vessel number” throughout) was calculated as the sum of the number of CD31-staining vessels per high-power (×400) field in adjacent, nonoverlapping fields covering the entire center or periphery divided by half the total membrane area. Vessel cross-sectional area per unit membrane area (abbreviated to “vessel area” throughout) was calculated as the sum of the luminal cross-sectional areas within CD31-staining vessels noted in adjacent, nonoverlapping high-power (×400) fields covering the entire center or periphery of the CNVM, divided by half the total membrane area. Vessel and membrane areas were measured on computer (OpenLab 2.2.5; ImproVision, Lexington, MA).

Intra- and interobserver agreements on immunostaining grades, as determined by calculating weighted κ values, were good (κw = 0.60–0.65). Spearman rank correlation coefficients were computed to determine the association between CD31 immunostaining grade and vessel number or vessel area. To evaluate the difference between immunostaining grades (or vessel number or vessel area) in the center versus the periphery of the CNVMs, Wilcoxon signed-ranks tests (or two-tailed paired t-tests) were used. To analyze the difference in immunostaining grades (or vessel number or vessel area) in the periphery of the membranes between groups with 0, 1, or 2 PDT treatments, the Kruskal-Wallis test (or one-way ANOVA) was used. Statistical analysis was performed on computer (Analyse-It Software, Leeds, UK).

Leukocyte Adhesion Molecules in CNV Membranes

In at least one set of sections, nonimmune mouse IgG (20 μg/mL, CBL 600; Chemicon) was used in place of the primary antibody, and sections were processed otherwise in parallel. Another set of sections was stained with hematoxylin and eosin for histologic analysis. All sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO), washed in distilled water, and mounted using aqueous mounting medium (Faramount; Dako).

Figure 1. Standard grading photographs for ICAM-1, E-selectin, and CD31 immunostaining. Grading was performed on a high-power field in the designated region (“center” or “periphery”) on the scale: 0 (none), 1 (low), 2 (moderate), and 3 (intense). Magnification, ×400.

Figure 2. Center versus periphery of membranes. To define the center and periphery of each membrane, each membrane was visually divided into four equal lengths along the longitudinal axis; the outer two segments were defined as the periphery, and the inner two segments were defined as the center. Top: CD31 immunostaining of a section from CNVM from patient 5. Bottom: ICAM-1 immunostaining of serial sections from CNVM from the same patient.
Table 1. Immunostaining of CNVMs, Normal Eye, and Tonsil for Leukocyte Adhesion Molecules and Quantification of Vessels

<table>
<thead>
<tr>
<th>Patient</th>
<th>PDT (n)</th>
<th>ICAM-1 Center†</th>
<th>ICAM-1 Periphery‡</th>
<th>E-selectin Periphery‡</th>
<th>CD31 Periphery‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>2.0</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2.5</td>
<td>3.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.5</td>
<td>1.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.5</td>
<td>3.0</td>
<td>0.0</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.5</td>
<td>3.0</td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>0.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>0.0</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0.0</td>
<td>1.0</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>3.0</td>
<td>1.5</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Median or avg.§</td>
<td>0.5</td>
<td>2.5</td>
<td>0.5</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Median or avg.§</td>
<td>1</td>
<td>0.5</td>
<td>2.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Median or avg.§</td>
<td>2</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Median or avg.¶</td>
<td>over all</td>
<td>0.0</td>
<td>2.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Normal eye</td>
<td>0</td>
<td>0.5</td>
<td>0.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Tonsil</td>
<td>—</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient</th>
<th>Vessel Number§</th>
<th>Vessel Area∥</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>218</td>
<td>25.4</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>88</td>
<td>18.2</td>
</tr>
<tr>
<td>7</td>
<td>161</td>
<td>36.6</td>
</tr>
<tr>
<td>8</td>
<td>97</td>
<td>24.6</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>16.1</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>5.3</td>
</tr>
<tr>
<td>Median or avg.§</td>
<td>84</td>
<td>20.5</td>
</tr>
<tr>
<td>Median or avg.¶</td>
<td>71</td>
<td>10.2</td>
</tr>
<tr>
<td>Median or avg.¶</td>
<td>84</td>
<td>20.6</td>
</tr>
<tr>
<td>Median or avg.¶</td>
<td>81</td>
<td>13.8</td>
</tr>
<tr>
<td>Normal eye</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tonsil</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Immunostaining of sections was graded 0 (none), 1 (low), 2 (moderate), or 3 (intense). See Figure 1 for standard grading photographs.
† Grade was done on a high-power field (400×) within the center of the membrane, with the “center” being defined as in Figure 2.
‡ Grade was done on a high-power field (400×) within the periphery of the membrane, with the “periphery” being defined as in Figure 2.
§ Vessel number, number of vessels per unit membrane area (mm²), calculated as the sum of the number of CD31-staining vessels with well-defined ringlike or linear shapes noted in adjacent, non-overlapping high-power (400×) fields covering the entire center or periphery of the membrane, divided by half the total membrane area.
∥ Vessel area, vessel cross-sectional area per unit membrane area (%), calculated as the sum of the luminal areas within CD31-staining vessels noted in adjacent, non-overlapping high-power (400×) fields covering the entire center or periphery of the CNVM, divided by half the total membrane area.
¶ Medians were calculated for immunohistochemical staining grades; averages were calculated for vessel quantification variables.

RESULTS

Histopathology

Consistent with previous histopathologic studies of CNVMs surgically excised from patients with AMD with and without prior PDT,1,2,19–21 light microscopy analysis demonstrated that all 10 subfoveal CNVMs contained endothelium-lined vascular channels, RPE cells with clumped pigment, fibrocytes, and acellular fibrous connective tissue. Some specimens also contained portions of rod outer segments (patients 1, 5, 6) and photoreceptors (patient 6). A few sections contained extravasated blood, most notably those from patients 2 and 10. The central portions of the CNVMs generally appeared more fibrotic, whereas the peripheral edges were more cellular (Fig. 2).

CD31

In the subfoveal CNVMs, CD31 immunostaining was observed discretely on all noted vascular endothelia, consonant with its use by others as a sensitive and specific marker of vessels.22,25 The anti-CD31 antibody stained large patent vessels and small patent and nonpatent vessels. When the CD31 immunostaining grade was compared with the vessel number, there was a significant positive correlation (r = 0.76, P = 0.01). CD31 immunostaining grade also correlated roughly with vessel area (r = 0.60, P = 0.06).

The majority of specimens contained predominantly small vessels (patients 1, 2, 4, 5, 7, 9, 10), whereas one third contained a mix of small and larger patent vessels (patients 3, 6, 8). When larger vessels were present, they consisted of endothelially lined channels occasionally surrounded by pericytes. Larger vessels tended to be fewer in number and localized to the central, more fibrotic portions of the CNVMs (Fig. 2, top, center). Smaller vessels appeared more numerous and clustered in the peripheral regions of the CNVMs (Fig. 2, top, periphery). The general histologic pattern of numerous small vessels in the periphery and few large vessels in the center is reflected in the significantly greater number of vessels in the periphery than in the center (116/mm² vs. 81/mm², P = 0.05) without a significant difference in vessel area between the periphery and the center (12.3% vs. 13.8%; Table 1).

With increasing numbers of PDT treatments, there was a suggestion of decreasing average vessel number (141, 109, and 95/mm²), average vessel area (20.5%, 5.8%, and 7.3%), and median CD31 immunostaining grade (3, 2, and 2) in the periphery of the membranes, although these tendencies did not reach statistical significance (Table 1).

ICAM-1

In the normal eye, ICAM-1 staining was detected at low levels on vascular endothelium and very minimally in and around the RPE and the external limiting membrane (Fig. 3A). In the CNVMs, ICAM-1 immunostaining was localized predominantly within and around RPE cells (Fig. 3B), but was also found on a small subset of linear and ring-shaped structures corresponding to the vascular endothelia of small vessels (Fig. 3C) that also stained for CD31 in serial sections (data not shown). In 9 of the 10 CNVMs, ICAM-1 staining of RPE cells and vessels was more intense and its distribution more extensive than in RPE cells and vessels of the normal control eye (Table 1, Fig. 3). In almost all the CNVMs, ICAM-1 staining of RPE cells and vessels was more intense in the periphery of the membrane than in the center (Fig. 2, bottom panel). Indeed, the median ICAM-1 immunostaining score in the peripheral, more cellular regions was significantly greater than that in the central, more
fibrotic regions of the membranes (periphery = 2 vs. center = 0, \( P = 0.04 \); Table 1).

With regard to the effect of PDT on ICAM-1 immunostaining, there was no significant relationship between number of PDT treatments and ICAM-1 immunostaining grade in the center of the membranes, but there was a significant decrease in the median ICAM-1 immunostaining grade with increasing number of PDT treatments in the periphery of the membranes (\( P = 0.05 \)). The median ICAM-1 immunostaining grade in the periphery was 2.5, 2.5, and 1 for 0, 1, and 2 PDT treatments, respectively (Table 1).

In the two most fibrotic CNVMs (patients 2 and 10), there was also intense ICAM-1 immunoreactivity on RPE cells in the central fibrotic regions, unlike in the other eight CNVMs where ICAM-1 staining was localized primarily to the periphery (Table 1). The CNVMs from patients 2 and 10 were similar in that they were the only two CNVMs with major extravasation of erythrocytes in the membrane (data not shown). The CNVM from patient 2 did not receive PDT, whereas the CNVM from patient 10 received two PDT treatments before excision.

**E-selectin**

E-selectin immunostaining was not detectable in the normal eye (data not shown). In the CNVMs, E-selectin staining was found predominantly on vascular endothelium, with corresponding CD31 staining in serial sections (Figs. 4E–4G). E-selectin staining was noted primarily on very small vessels in the peripheral regions of the CNVMs (Fig. 4G). In no case was
E-selectin staining seen on larger patent vessels in the central, fibrotic regions of the CNVs (Fig. 4D). In general, CD31-staining vessels in the thicker, central, fibrotic regions of the membrane did not stain for either ICAM-1 or E-selectin in serial sections (Figs. 4B–4D), whereas CD31-staining vessels in the thinner, peripheral, more cellular regions stained specifically for ICAM-1 and E-selectin (Fig. 4E–4G), but not with mouse IgG negative control (data not shown). E-selectin staining was also present on some clumps of RPE cells in the periphery of the membranes that also stained with ICAM-1 in serial sections, although E-selectin staining was seen only on a small subset of ICAM-1-staining RPE cells (data not shown).

The E-selectin immunostaining grade in the periphery of the membranes showed no correlation with vessel number, vessel area, CD31 or ICAM-1 immunostaining grade, or the number of PDT treatments.

**Discussion**

Many lines of evidence point toward leukocyte adhesion molecules—such as ICAM-1—as important molecules in the pathogenesis of AMD-related CNV. First, ICAM-1 appears to be an important mediator of VEGF-induced angiogenesis. In addition, ICAM-1 may have a role in mediating oxidative damage, which may be involved in the pathogenesis of AMD and AMD-related CNV. Moreover, in experimental models of CNV induced by laser photocoagulation, upregulation of E-selectin and ICAM-1 expression precedes macrophage recruitment, which, in turn, precedes CNV and fluorescein leakage. The essential role of ICAM-1 and its ligand LFA-1 (CD18) in the formation of CNVs is bolstered by data in ICAM-1- and leukocyte function-associated antigen LFA-1-deficient mice which form significantly less volume of CNV in response to laser photocoagulation than wild-type mice. Given the growing data that implicate leukocyte adhesion molecules in the process of CNV, the purpose of this study was to examine the vascularity and leukocyte adhesion molecule immunostaining pattern in subfoveal CNVMs from patients with AMD, with and without prior PDT treatment.

To investigate the relationship between leukocyte adhesion molecule immunostaining and vascularity, an anti-CD31 antibody was used. CD31 immunostaining allows for more precise quantification of microvessels than by histology alone and has been used for sensitive and specific detection of endothelial cells in CNVs in several studies. Moreover, the positive correlation between CD31 immunostaining grade and histology-based measurements of vessel number \( (r_c = 0.76, P = 0.01) \) lends support to the semiquantitative nature of the immunostaining grading system used.

When the vascularity of the membranes was assessed, there was a significantly greater number of CD31-staining vessels in the periphery compared with the center \( (P = 0.05) \). Indeed, the general histologic pattern of the CNVMs—fibrotic central regions with fewer, larger, more mature vessels and more cellular peripheral regions with numerous small vessels—corresponds with angiographic data which shows that the centers of CNVMs tend to stain late with fluorescein (suggestive of scarring), whereas the peripheral edges tend to hyperfluoresce early and leak late (suggestive of newly formed, highly permeable vessels). With an increasing number of PDT treatments, there was a tendency toward decreasing vessel number, vessel area, and CD31 immunostaining grade in the periphery of the membranes. These decreases in measures of vascularity in the periphery of the membranes with increasing numbers of PDT treatments did not reach statistical significance probably because of the small sample size, but may be consistent with the hypothesis that PDT may retard—rather than promote—angiogenesis at the periphery of the CNVMs.

With respect to leukocyte adhesion molecules, there was increased ICAM-1 immunoreactivity in the vessels and RPE cells of CNVMs of patients with AMD compared with the choroidal vessels and RPE of normal eyes. Moreover, the significantly higher median ICAM-1 immunostaining grade in the peripheral regions of the membranes over the central regions \( (P = 0.04) \) mirrors the significantly greater number of vessels in the periphery than in the center and suggests that ICAM-1 immunoreactivity may be preferentially localized to the leading edges of new vessel growth in CNVMs. Whereas an immunohistochemical study cannot determine a causal role for ICAM-1 in AMD-related CNV, our study provides important data about the localization of immunoreactive leukocyte adhesion molecules in CNVMs of patients with AMD.

In the normal human eye, ICAM-1 is expressed constitutively at low levels on choroidal and retinal vascular endothelium, the RPE, Bruch’s membrane, and the external limiting membrane. In the CNVMs, we found that ICAM-1 immunostaining was predominantly localized to RPE cells. Several in vitro and in vivo studies in cultured human RPE cells have demonstrated that ICAM-1 expression is upregulated in response to stimulation with inflammatory cytokines (i.e., IFNγ, IL-1β, and TNFα), which may be consistent with the significant higher median ICAM-1 immunostaining grade in the periphery compared with the center \( (P = 0.05) \). Indeed, some evidence also suggests that downregulation of ICAM-1 expression on RPE cells may be the mechanism by which intravitreal triamcinolone retards neovascularization. Our study is the first to demonstrate elevated levels of ICAM-1 immunoreactivity on RPE cells in situ in subfoveal CNVMs from patients with AMD.

With regard to ICAM-1 staining of vascular endothelial cells in the CNVMs, it was less extensive than ICAM-1 staining of RPE cells and limited to small vessels localized to the periphery of the CNVMs. E-selectin was also localized to the periphery of the membranes. The ICAM-1 and E-selectin immunostaining patterns may reflect data from animal models that suggest a temporal evolution of leukocyte adhesion molecule expression on different cell types during the progression of CNV. In one rodent study, photocoagulation (i.e., angiogenic stimulus) triggered upregulation of ICAM-1 expression in RPE cells and choroidal vascular endothelial cells which preceded formation of CNVMs. After development of fluorescein-leaking CNVMs, RPE cells continued to express ICAM-1, but choroidal vascular endothelial cells did not. In the same model, E-selectin was transiently expressed on RPE cells and choroidal endothelial cells after photocoagulation and disappeared once fluorescein-leaking CNVMs were detected. Further study is necessary to explore the hypothesis that there may be a leading front of inflammation that precedes or initiates angiogenesis at the edges of membranes as they grow.

In membranes treated with multiple PDT sessions, the median ICAM-1 immunostaining grade in the periphery was significantly lower \( (P = 0.05) \). The possible mechanisms for less ICAM-1 immunoreactivity in the periphery of PDT-treated CNVMs remain unclear; some studies suggest that PDT-related cell death occurs primarily through activation of caspases and apoptosis, whereas other data suggest that PDT upregulates expression of proinflammatory factors and may promote necrotic cell death. Determination of any causal relationship between PDT treatment, vascularity, and ICAM-1 expression in the periphery of the CNVMs awaits further functional studies. Meanwhile, it remains possible that eyes requiring multiple PDT sessions represent more aggressively growing CNVMs.

In conclusion, our immunohistochemistry study demonstrates for the first time that subfoveal CNVMs surgically excised from patients AMD have greater ICAM-1 and E-selectin immunostaining compared with the normal eye and that the increase in ICAM-1 and E-selectin immunoreactivity occurs...
primarily in the periphery of the CNVMs where there are larger numbers of vessels. Treatment of CNVMs with PDT may be associated with both decreased vascularity and ICAM-1 expression in the periphery of the membranes. Our data provide impetus for continued study of the role of leukocyte adhesion molecules in the pathogenesis of CNV in AMD.

References


