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Choroidal neovascularization (CNV), or choroidal angiogenesis, is the hallmark of age-related macular degeneration and a leading cause of visual loss after age 55. The pathogenesis of new choroidal vessel formation is poorly understood. Although inflammation has been implicated in the development of CNV, the role of complement in CNV has not been explored experimentally. A reliable way to produce CNV in animals is to rupture Bruch’s membrane with laser photocoagulation. A murine model of laser-induced CNV in C57BL/6 mice revealed the deposition of C3 and membrane attack complex (MAC) in the neovascular complex. CNV was inhibited by complement depletion using cobra venom factor and did not develop in C3−/− mice. Anti-murine C6 Abs in C57BL/6 mice inhibited MAC formation and also resulted in the inhibition of CNV. Vascular endothelial growth factor, TGF-β2, and β-fibroblast growth factor were elevated in C57BL/6 mice after laser-induced CNV; complement depletion resulted in a marked reduction in the level of these angiogenic factors. Thus, activation of complement, specifically the formation of MAC, is essential for the development of laser-induced choroidal angiogenesis in mice. It is possible that a similar mechanism may be involved in the pathophysiology of other angiogenesis essential diseases.

Materials and Methods

Animals

Male C57BL/6 mice (6–8 wk old), C3-deficient mice (C3−/−), and their wild-type control ((129 X C57BL6)F1) were purchased from The Jackson Laboratory. This study was approved by the Institutional Animal Care and Use Committee of University of Louisville.

Induction of CNV in mice

Animals were divided into four groups. CNV was induced by laser photocoagulation in C57BL/6 mouse (group 1; n = 10; complement sufficient) with the krypton red laser (50-µm spot size; 0.05-s duration; 250 mW) as previously described by us (29, 30). Three laser spots were placed in each eye close to the optic nerve. In group 2, C57BL/6 mice (n = 10) were treated i.p. with 4 U of cobra venom factor (CVF; Quidel) 2 days before laser photocoagulation and every day after laser treatment. We refer to these animals as “complement depleted” throughout this paper. Group 3 had C3−/− mice (n = 10), and group 4 consisted of 10 wild-type control ((129 X C57BL6)F1) for the C3-deficient mice. Laser photocoagulation in all four groups was performed as described above. These experiments were repeated five times.

Measurement of CNV and CNV lesions

Seven days after laser treatment, all animals were perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran (FITC-dextran; average molecular mass, 2 x 10^6; Sigma-Aldrich) and sacrificed. The eyes were harvested and fixed in 10% phosphate-buffered formalin, and retinal pigment epithelium (RPE)-choroid-scleral flat mounts were prepared as previously described (29, 30). RPE-choroid-scleral flat mounts were stained for elastin using a mAb specific for elastin (1.0 mg/ml; 1/200 dilution; Sigma-Aldrich) followed by a Cy3-labeled secondary Ab (1.0 mg/ml; 1/200 dilution; Sigma-Aldrich). The incidence and the size of CNV...
was determined by confocal microscopy. The size of the CNV complex was graded by morphometric analysis of the images (MetaMorph Image Analysis software package; Universal Imaging) obtained from confocal microscopy (29, 30).

**Abs and treatment**

Polyclonal Ab to mouse C6 (purified rabbit IgG) was obtained from Cell Sciences. C57BL/6 mice were divided into two groups. Animals in group 1 (n = 13) received 40 μg of anti-murine C6 i.p. daily for a total of 8 days. Control animals (group 2; n = 11) received a similar treatment with purified polyclonal normal rabbit IgG (BD Pharmingen). On day 8, animals in both groups were treated with laser to induce CNV as described above. The animals were sacrificed on day 7 post-laser treatment; measurement of CNV and CNV lesions was also performed as described above. Animals (n = 3 each group) were sacrificed at 72 h post-laser treatment, and flat mounts prepared from both Ab-treated and control animals were stained for MAC as described below. Both anti-C6-treated and control (normal rabbit IgG-injected) mice were bled on day 8 of Ab treatment for determination of serum complement hemolytic activity. Hemolytic assay was performed using EZ Complement CH50 Test (Diamedix) according to the manufacturer’s instruction with some modifications. Briefly, 150 μl of Ab-sensitized sheep erythrocytes was incubated with rat serum sequentially diluted to give a total volume of 200 μl at 37°C for 60 min. Serum obtained from normal C57BL/6 mice was used to determine the 100% value for complement-dependent serum hemolytic activity.

**Immunohistochemical studies**

Flat mounts were stained for C3 and MAC. A polyclonal Ab (raised in rabbit; 1.0 mg/ml) reactive with rat/mouse C9 was used to stain for mouse MAC, and the IgG fraction of goat anti-mouse C3 (6.0 mg/ml; ICN) was used for C3 staining. These Abs were used at 1/200 dilution. Cy3-conjugated anti-rabbit IgG and anti-goat IgG obtained from Sigma-Aldrich were used for C3 staining. These Abs were used at 1/200 dilution. The samples were assayed (in triplicate) for β-FGF and VEGF proteins using human β-FGF and mouse VEGF ELISA kits (R&D Systems). These experiments were repeated three times with similar results, and the data are represented as mean ± SD.

**ELISA**

Twenty laser spots were placed in each eye of complement-sufficient and complement-depleted C57BL/6 mice as described above. Animals from each group (n = 10 each time point) were sacrificed at days 1, 3, 5, and 7 post-laser treatment, and RPE-choroid-scleral tissues were harvested from the enucleated eyes. RPE-choroid-scleral tissues for different time points were pooled separately. Hemolytic assay was performed using EZ Complement CH50 Test (Diamedix) according to the manufacturer’s instructions with some modifications. Briefly, 150 μl of Ab-sensitized sheep erythrocytes was incubated with rat serum sequentially diluted to give a total volume of 200 μl at 37°C for 60 min. Serum obtained from normal C57BL/6 mice was used to determine the 100% value for complement-dependent serum hemolytic activity.

**RT-PCR analysis**

Twenty laser spots were placed in each eye of complement-sufficient and complement-depleted (CVF-treated) C57BL/6 mice as described above.

**Results**

**Role of complement in the development of laser-induced CNV**

The role of complement in the development of CNV was investigated by using C57BL/6 mice depleted of systemic complement by
CVF. Our results are summarized in Fig. 1, A and B, and Table I. Flat-mount analysis revealed that the incidence of CNV in complement-sufficient C57BL/6 mice (Fig. 1A) was 98%. However, the in vivo depletion of systemic complement with CVF markedly reduced (p < 0.001) the development of CNV (Fig. 1B) to 3%. A total complement hemolytic activity (CH50) assay (33) confirmed the absence of functionally active complement in CVF-injected mice. On days 1, 3, 5, and 7, CH50 levels in CVF-treated mice were 2, 3, 3, and 2%, respectively, compared with control (non-CVF-treated) mice. CH50 levels in control animals were 100% at these time points. No changes were observed on light-microscopic examination of the heart, kidney, and liver of these animals (data not shown). Each experiment was repeated five times with similar results.

Role of C3 in the development of laser-induced CNV

Using C3−/− mice, we next explored whether the development of CNV required C3. Laser photocoagulation induced CNV in the wild-type ((129 × C57BL/6)F1) control (incidence, 98%; Table I; Fig. 1C), but not in the C3−/− mouse (incidence, 5%; D). No changes were again observed on histologic examination of the heart, kidney, and liver of these animals (data not shown). Each experiment was repeated five times with similar results.

Deposition of C3 in CNV complex

Flat mounts of the CNV complex were stained for C3. The neovascular complex stained for C3 in C57BL/6 (Fig. 2A) and (129 × C57BL/6)F1 (C) mice on day 1 post-laser treatment. Similar results were observed on days 3 and 5 postlaser with weak staining on day 7 (data not shown). In contrast, no C3 staining was observed in the laser spots of CVF-treated mice (Fig. 2B), as well as C3−/− mice on day 1 (D) through day 7 (data not shown). No staining was observed in the control sections stained without the primary Ab (data not shown).

Deposition of MAC in CNV complex

Flat mounts of the CNV complex were also stained for MAC. A pattern similar to C3 deposition was observed in laser spots stained for MAC in C57BL/6 (Fig. 3, A, C, and E) and (129 × C57BL/6)F1 (data not shown) mice on days 1, 3, and 5 post-laser treatment. Very weak staining for MAC was noted on day 7 (Fig. 3E). In contrast, no MAC staining was observed in the laser spots of complement-depleted mice (CVF-treated; Fig. 3, B, D, and F) and C3−/− (data not shown) mice from day 1 through day 7. No staining was observed in the control sections stained without the primary Ab (data not shown).

### Table I. Effect of complement on laser-induced CNV in mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of Mice</th>
<th>Treatment</th>
<th>Laser Spots/Eye</th>
<th>Total Spots</th>
<th>CNV-Positive Spots (No. (%))</th>
<th>CNV-Negative Spots (No. (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>50</td>
<td>None</td>
<td>3</td>
<td>300</td>
<td>289 (96)</td>
<td>11 (4)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>50</td>
<td>CVF</td>
<td>3</td>
<td>300</td>
<td>10 (3)</td>
<td>290 (97)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>8</td>
<td>Anti-C6</td>
<td>3</td>
<td>48</td>
<td>13 (27)</td>
<td>35 (73)</td>
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<tr>
<td>C3−/−</td>
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<td>None</td>
<td>3</td>
<td>300</td>
<td>16 (5)</td>
<td>284 (95)</td>
</tr>
<tr>
<td>WT control</td>
<td>50</td>
<td>None</td>
<td>3</td>
<td>300</td>
<td>292 (97)</td>
<td>8 (3)</td>
</tr>
</tbody>
</table>

*Animals were sacrificed on day 7 post-laser treatment. NR-IgG, Normal rabbit IgG; C3−/−, C3-deficient mice; WT, wild type. Student’s t test of the data showed p < 0.001.
Effect of anti-C6 on MAC formation and the induction of laser-induced CNV

To further examine the role of MAC (C5b-9), we used polyclonal Abs that inhibit the complement cascade at C6, thus blocking the generation of C5b-9. Our results showed that systemic administration of the anti-C6 polyclonal Abs dramatically reduced the deposition of MAC in the laser spots (Fig. 4A) on day 3 post-laser treatment, whereas the laser spots stained very strongly for MAC in control (normal rabbit IgG-treated) mice (Fig. 4B). Our results also showed that C6 inhibition had a dramatic effect on the induction and development of laser-induced CNV (Table I). CNV was significantly (*, p < 0.0001) reduced (65–70%) in anti-C6-treated animals compared with naive animals and IgG-injected controls.

Effect of complement on the ocular production of VEGF, TGF-β2, and β-FGF

Laser-induced CNV in the mouse is associated with the increased production of several angiogenic factors within the retina and RPE. Thus, we studied the relationship between activation of the complement cascade and the intraocular expression of VEGF, TGF-β2, and β-FGF in this model. Using RT-PCR, we detected low levels of VEGF (716, 644, and 512 bp), TGF-β2 (684 bp), and β-FGF (298 bp) mRNA at 24 h in laser-treated C57BL/6 mice (Fig. 5A). We previously noted that complement activation occurred within 24 h of laser treatment with the marked deposition of MAC at the site of laser injury at 24 h (Fig. 3A). VEGF, TGF-β2, and β-FGF transcripts increased on days 3 and 5, and returned to basal levels on day 7 (Fig. 5A). This was similar to the pattern we observed for MAC deposition. In contrast, the mRNA of these growth factors did not change and remained at low basal levels through day 7 in CVF-treated mice (Fig. 5A). A strong band at 983 bp for GAPDH...
indicated equal amounts of RNA in each lane (Fig. 5A). No band was seen in the controls without RNA or reverse transcriptase (data not shown).

Using ELISA, low levels of VEGF protein were observed on days 1 and 3 postlaser with a significant increase on day 5 ($p < 0.001$) in C57BL/6 mice (Fig. 5B). $eta$-FGF protein remained at constitutive levels on day 1 but increased significantly ($p < 0.001$) on days 3 and 5 (Fig. 5C). In contrast, the levels of both proteins remained at basal levels throughout day 7 in CVF-treated (complement-deficient) C57BL/6 mice (Fig. 5, B and C). These findings suggest that complement activation and MAC (C5b-9) deposition are important for the production and release of angiogenic growth factors in laser-induced CNV. Our results of RT-PCR and ELISA demonstrated that the production of angiogenic factors—VEGF, TGF-$eta_2$, and $eta$-FGF—is temporarily regulated and dependent on the presence and activation of the complement system.

**Discussion**

Angiogenesis is a fundamental process occurring during embryonic development but is a characteristic of various pathological conditions in the adult (34, 35). Choroidal angiogenesis (i.e., CNV) associated with AMD is the leading cause of visual loss in individuals over age 55 (1). In the present study, we have used a murine model of laser-induced CNV (3, 27–30) to understand the role of complement in choroidal angiogenesis.

First, we demonstrated that complement was essential for the development of CNV in C57BL/6 mice after laser photocoagulation. C57BL/6 mice depleted of complement with CVF did not develop CNV. Because C3 is a key component of complement, and has been demonstrated to play an important role in various immune responses (20–22, 31), we investigated whether it was required for the development of CNV. We report here for the first time that C3$^{-/-}$ mice were unable to develop CNV after laser photocoagulation.

We then focused our attention on the formation and deposition of the C5b-9 MAC in CNV lesions. MAC has previously been reported to be spontaneously and continuously deposited on self-tissue in small amounts under normal conditions and in larger quantities under various pathological conditions (32, 36–38). Using the mouse model of laser-induced CNV, we observed that the neovascular complex stained very strongly for C3 and MAC in complement-sufficient C57BL/6 mice. In contrast, laser spots in C3$^{-/-}$ mice, as well as
complement-depleted (CVF-treated) C57BL/6 mice, did not stain for C3 or MAC. These results showed a correlation between the presence of complement, MAC deposition, and choroidal new vessel formation following laser photocoagulation.

The importance of MAC in the development of CNV was demonstrated by the in vivo inhibition of complement C6 with anti-murine C6 polyclonal Abs. The systemic administration of anti-C6 Abs inhibited the in vivo formation of MAC and markedly reduced both the incidence and development of CNV in C57BL/6 mice after laser treatment. Although anti-C6 Ab inhibits the sequential assembly of the C5b-9 (MAC) complex, the generation of C3a and C5a is not affected (20–22). Thus, our results suggest that, without the formation of MAC at the site of injury, laser-induced choroidal angiogenesis will not occur. Using anti-C6 or anti-C5 Abs, similar observations were made in experimental autoimmune myasthenia gravis (39), collagen-induced arthritis (40), and lupus-like autoimmune disease in NZB/WF1 mice (41).

Because we established a critical role for MAC deposition in the development of laser-induced CNV, we asked whether the generation of the growth factors observed to be important in the development of CNV (42–47) was affected by MAC. We confirmed that within 3 days of laser photocoagulation, there was an increased production of VEGF, TGF-β2, and β-FGF in CNV lesions. However, when the animals were complement depleted with CVF, so that no MAC deposition occurred, the levels of these growth factors remained low. Interestingly, in complement-sufficient animals, the levels of these growth factors returned to baseline level at day 7 postlaser, because at this time point, very little MAC deposition was observed in the CNV complex of these animals. Thus, the development of choroidal angiogenesis following laser photocoagulation is dependent on the deposition of MAC and the subsequent generation and secretion of angiogenic factors. The release of several growth factors, such as β-FGF and platelet-derived growth factor, from epithelial and endothelial cells has been observed following the deposition of MAC (23–26).

Only recently has it become apparent that complement is important in AMD (48–51). Using the in vitro techniques, some investigators have suggested that impaired macrophage mobilization and recruitment allow the accumulation of C5a (and IgG), which induced VEGF production (19). Our results reported here clearly demonstrate that MAC formation and deposition are critical for the increased production of growth factors—VEGF, β-FGF, and TGF-β2—which eventually leads to the development of laser-induced CNV.

In conclusion, our studies describe the first direct role of complement activation and MAC formation in the laser-induced choroidal angiogenesis. On the basis of the results presented here, we propose the following potential mechanism defining the role of complement in CNV: Complement activation (via the classical or alternative pathway) in the posterior segment of the eye leads to increased formation and deposition of MAC on RPE and/or choroid. This results in transient changes in the membrane permeability followed by induction and release of growth factors. Released growth factors cause abnormal proliferation of choroidal endothelial cells leading to the development of CNV. Thus, in the absence of complement, CNV will not occur and complement inhibition may be used as a suitable therapeutic tool in the treatment of CNV. It is possible that a similar mechanism may be involved in the pathogenesis of other neovascular diseases such as diabetic retinopathy, arteriosclerosis, arthritis, and cancer.

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References


