Early Effects of Intravitreal Triamcinolone Acetonide on Inflammation and Proliferation in Human Choroidal Neovascularization

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Objective: To evaluate the early effects of triamcinolone acetonide (TA) on inflammation, proliferation, and vascular endothelial growth factor (VEGF) in human choroidal neovascularization (CNV).

Methods: Retrospective review of an interventional case series of 29 patients who underwent macular translocation. Fourteen CNV membranes without previous therapy (control CNV group) and 4 CNV membranes excised 3 days after photodynamic therapy (PDT CNV group) comprised the control groups. Eleven patients were treated with intravitreal TA (TA CNV group; n=6) or PDT and TA combined (PDT+TA CNV group; n=5) or PDT and TA monotherapy (PDT CNV group; n=5) or PDT and TA combined (PDT+TA CNV group; n=6) 3 to 9 days preoperatively. The CNV membranes were stained for cytokeratin 18, CD34, VEGF, intercellular adhesion molecule-1 (ICAM-1), E-selectin, CD68, CD45, Ki-67, and Thy-1.

Results: Treatment with TA and PDT+TA resulted in increased immunostaining of ICAM-1 in endothelial cells and the stroma and a higher percentage of Thy-1 expression than controls. The density of macrophages was significantly increased in PDT+TA CNV membranes. Leukocyte density and proliferative activity were lower in TA and PDT+TA CNV membranes. The total VEGF score was significantly increased in TA and PDT+TA CNV membranes compared with the control CNV membranes. Evidence of VEGF in the retinal pigment epithelium of PDT+TA CNV membranes was stronger than in control CNV membranes.

Conclusions: Triamcinolone acetonide has no inhibitory effect on macrophage infiltration or ICAM-1, Thy-1, or VEGF expression in CNV membranes in the early term. The clinical benefits of TA are probably not based on pure anti-inflammatory or VEGF-suppressing mechanisms.


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expression of Thy-1. Expression of CD45 was greater on blood-borne infiltrating leukocytes and monocytes rather than resident macrophages that express CD68. \(^{24,25}\) Thy-1 is a cell surface marker expressed on vascular EC that is upregulated by inflammatory cytokines interleukin (IL)–1 and tumor necrosis factor (TNF)–α, but remains unaffected by growth factors such as VEGF. \(^{26}\)

### METHODS

**SUBJECTS AND TREATMENTS**

We retrospectively reviewed 29 eyes of 29 consecutive patients with AMD who had been treated with full macular translocation surgery with CNV membrane removal at 10 distinct surgical sites between 1997 and 2005. Except for 14 patients without any preoperative therapy (control CNV group), surgery was performed 3 to 9 days after verteporfin PDT (PDT CNV group; \(n=4\)), TA monotherapy (TA CNV group; \(n=3\)), or PDT+TA combination therapy (PDT+TA CNV group; \(n=6\)). The clinical characteristics of patients treated with PDT, TA, or PDT+TA preoperatively are summarized in the **Table**.

Full macular translocation was offered when (1) visual acuity was worse than 20/200, being the minimum visual acuity to recommend the first PDT treatment, \(^2\) (2) visual deterioration progressed after initial PDT, (3) the patient refused (re)treatment with PDT, TA, or PDT+TA owing to continuous visual deterioration in the fellow eye in spite of therapy, and (4) (re)treatment with PDT was impossible owing to recurrent or massive submacular hemorrhage. Preoperative therapy with PDT, TA, and PDT+TA was intended to decrease intraoperative hemorrhage, postoperative CNV recurrence, and the proliferative vitreoretinopathy rate. \(^{5,17,21}\) Triamcinolone acetonide was prepared preservative-free through a sedimentation technique previously described. \(^{28,29}\) Each patient gave written informed consent after the experimental nature, risks, and benefits of all of the therapy options had been explained. The study followed the guidelines of the Declaration of Helsinki. The study and histological analysis of specimens were approved by the local institutional review board.

### IMMUNOHISTOLOGY

The CNV specimens were fixed in formalin and embedded in paraﬃn. Each specimen was sectioned into 5-µm sections and mounted on poly-L-lysine–coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining. After deparaffinization, antigen retrieval was performed through heat treatment in citrate buffer (0.01M; pH, 6.0) for 2 minutes for CD34, ICAM-1, E-selectin, CD45, CD68, and Ki-67. For cytokeratin 18 and Thy-1, antigen retrieval was accomplished by proteolytic digestion with 0.5% protease XXIV (Sigma, St Louis, Missouri) for 10 minutes, whereas pretreatment with proteinase K (Dako) for 10 minutes was used for VEGF. Owing to inadequate pretreatment stability of 2 sections, only 2 PDT CNV were stained for E-selectin, ICAM-1, CD68, and CD45.

Immunohistochemical staining with the primary mouse monoclonal antibodies specific for CD34 (Clone QBEnd-10; Immunotech, Hamburg, Germany), cytokeratin 18 (Clone:Ks 18.04; Progen, Heidelberg, Germany), ICAM-1 (Clone 23G12; Novocastra, Newcastle upon Tyne, England), E-selectin (Clone 16G4; Novocastra), CD45 (Clones 2B11 and PD7/26; Dako), and Ki-67 (Clone MIB-1; Dako) was performed using a horseradish peroxidase method previously described.\(^8\) For E-selectin, cytokeratin 18, and ICAM-1 staining, the brown chromogen 3,3’-diaminobenzidine was replaced with 3-amino-9-ethylcarbazole, a highly sensitive substrate chromogen (Cytochrome, Code K3461; Dako).

Immunohistochemical staining with the primary mouse antibodies specific for VEGF (sc-7269; Santa Cruz Biotechnology Inc, Santa Cruz, California), Thy-1 (clone SE10; BD Biosciences, Pharmingen, San Jose, California), and CD68 (Clone

### Table. Clinical Characteristics of Patients Treated With Intravitreal TA and/or Verteporfin PDT Before Surgical Removal of Subfoveal CNV Membrane

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Eye</th>
<th>CNV Type</th>
<th>CNV Size, mm²</th>
<th>Preoperative VA</th>
<th>TA Injection</th>
<th>Each PDT Treatment, d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/90</td>
<td>Right</td>
<td>Hemorrhagic occult</td>
<td>0.12</td>
<td>10/200</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2/F/78</td>
<td>Right</td>
<td>Occult</td>
<td>0.21</td>
<td>10/100</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3/F/84</td>
<td>Right</td>
<td>PED (RAP)</td>
<td>0.22</td>
<td>10/63</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4/F/70</td>
<td>Right</td>
<td>PED, occult</td>
<td>0.13</td>
<td>10/40</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5/F/80</td>
<td>Left</td>
<td>RAP</td>
<td>0.22</td>
<td>10/50</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>6/M/76</td>
<td>Left</td>
<td>Classic</td>
<td>0.27</td>
<td>10/400</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7/F/78</td>
<td>Right</td>
<td>Classic</td>
<td>0.10</td>
<td>10/500</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8/M/54</td>
<td>Left</td>
<td>Predominantly classic</td>
<td>0.04</td>
<td>10/160</td>
<td>113, 3</td>
<td></td>
</tr>
<tr>
<td>9/M/84</td>
<td>Left</td>
<td>Classic</td>
<td>0.46</td>
<td>10/400</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>10/M/83</td>
<td>Right</td>
<td>Classic</td>
<td>0.40</td>
<td>10/200</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>11/M/83</td>
<td>Left</td>
<td>Classic</td>
<td>0.39</td>
<td>10/400</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>12/F/82</td>
<td>Left</td>
<td>Occult</td>
<td>0.57</td>
<td>1/35 MV</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>13/F/85</td>
<td>Right</td>
<td>Occult</td>
<td>0.76</td>
<td>10/50</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>14/F/75</td>
<td>Right</td>
<td>Occult</td>
<td>0.16</td>
<td>10/50</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>15/M/74</td>
<td>Left</td>
<td>Occult</td>
<td>0.23</td>
<td>10/125</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CNV, choroidal neovascularization; MV, meter vision; PDT, photodynamic therapy; PED, pigment epithelium detachment; RAP, retinal angiomatous proliferation; TA, triamcinolone acetonide; VA, visual acuity.
PG-M1; Dako) was performed by an alkaline-phosphatase method previously described. Color was developed using chromogen red (ChemMate Detection Kit; Dako). Hematoxylin (ChemMate, Code S2020; Dako) was used for counterstaining. For negative controls, primary antibodies were substituted with either the appropriate or normal sera.

STATISTICAL ANALYSIS

Two serial sections were analyzed for each specimen independently by 2 masked observers (O.T. and S.G.) by light microscopy.

Immunoreactivity for VEGF, ICAM-1, and E-selectin was analyzed separately in the RPE, EC, and stroma by light microscopy. Numerals 3, 2, 1, and 0 were assigned to indicate intense (70%-100% positive cells), moderate (40%-69% positive cells), or weak labeling (1%-39% positive cells), or absence of any staining, respectively. The total score for VEGF, ICAM-1, and E-selectin (range, 0-9) was described for each CNV specimen by the sum of the staining scores in the RPE, EC, and stroma.

Immunoreactivity to ICAM-1 was intense in the RPE (70%-100% positive cells), moderate (40%-69% positive cells), or weak labeling (1%-39% positive cells), or absence of any staining, respectively. The total score for ICAM-1 expression between the TA and PDT + TA CNV membranes did not show significance (ANOVA P = .001, and P = .001 for all) as well as the total ICAM-1 score (ANOVA P = .001). No significant difference was found between any groups (ANOVA P = .35). The mean (SE) proliferative activity was 96.57 (37.48) cells/mm³ in the control and 22.18 (19.02) cells/mm³ in the PDT + TA CNV membranes (P = .0004, respectively) and PDT + TA CNV membranes (P = .0004, respectively). No significant change was detected in ICAM-1 expression between the TA and PDT + TA CNV membranes.

The control CNV membranes showed E-selectin immunoreactivity in the RPE of all, the EC of 8 (57.14%), and the stroma of 9 (64.29%) specimens (Figure, B). In the PDT CNV membranes, E-selectin was present in the RPE and EC of 1 CNV membrane (Figure, B). The TA CNV membranes showed E-selectin immunoreactivity in the RPE of 2 (40%) and the EC and stroma of 1 CNV membrane (20%) (Figure, B and eFigure 1E). Immunoreactivity to E-selectin was detected in the RPE of 4 (66.67%), the EC of 3 (50.00%), and the stroma of 1 PDT + TA CNV membrane (16.67%) (Figure, B and eFigure 1F). No significant difference in E-selectin expression was found between any of subgroups.

LEUKOCYTE AND MACROPHAGE DENSITY AND PROLIFERATIVE ACTIVITY IN CNV MEMBRANES

The mean (SE) density of macrophages in the control, PDT, and TA CNV membranes were 1028.33 (224.64), 235.95 (185.63), and 1433.26 (399.94) cells/mm³, respectively (Figure, C). In the TA CNV membranes, the mean (SE) density of leukocytes tended to decrease to 56.67 (17.69) cells/mm³ (Figure, C and eFigure 3E). Leukocytes were found in 5 of 6 PDT + TA CNV membranes, with a mean (SE) density of 131.13 (41.57) cells/mm³ (Figure, C and eFigure 3F). No significant difference was found between any groups (ANOVA P = .35).

The mean (SE) proliferative activity was 96.57 (37.48) cells/mm³ in the control and 22.18 (19.02) cells/mm³ in the PDT CNV membranes (Figure, C). The mean (SE) proliferative activity in the TA (38.91 [21.29] cells/mm³) (Figure, C and eFigure 3G) and PDT + TA CNV membranes (18.19 [4.96] cells/mm³) (Figure 1C and eFigure 3H) was smaller than in the control CNV membranes, but without significance (ANOVA P = .35).

ANGIOGRAPHIC FINDINGS, CD34, AND THY-1 IMMUNOREACTIVITY

Following PDT and PDT + TA therapy, a hypofluorescence suggesting nonperfusion of the irradiated area and CNV membrane was detected in fluorescein angiography on the day of surgery (eFigure 2A). In addition, CD34

RESULTS

Immunohistopathological findings are summarized in the Figure.

EXPRESSSION OF CELLULAR ADHESION MOLECULES ICAM-1 AND E-SELECTIN

Immunoreactivity to ICAM-1 was detected in the RPE, EC, and stroma of 92.86% (13 of 14), 35.72% (5 of 14), and 71.43% (10 of 14) of control CNV membranes, respectively (Figure, A and eFigure 1A; archophthalmol.com). Expression of ICAM-1 in the EC (ANOVA P = .001) and stroma (ANOVA P = .001) as well as the total ICAM-1 score (ANOVA P = .001) showed significant differences between subgroups.

Immunoreactivity to ICAM-1 was intense in the RPE of both PDT CNV membranes and was weak in the stroma of 1 PDT CNV membrane (Figure, A and eFigure 1B).

In all TA CNV membranes (n = 5), RPE, EC, and stromal cells displayed ICAM-1 intensely. It was also significantly stronger in the EC, stroma, and in the total score than in the control CNV membranes (P < .001 for all) and PDT CNV membranes (P = .004, P < .001, and P = .001, respectively) (Figure, A and eFigure 1C).

Intense ICAM-1 expression was detected in the RPE of all, the EC of 4 (66.67%), and the stroma of 5 (83.3%) PDT + TA CNV membranes (n = 6) (Figure, A and eFigure 1D). Also, ICAM-1 expression was significantly stronger in the EC, stroma, and total score than in both of the control CNV (P < .001, and P < .001, respectively) and PDT CNV membranes (P = .004, respectively). No significant change was detected in ICAM-1 expression between the TA and PDT + TA CNV membranes.
immunohistochemistry demonstrated mostly occluded vessels with damaged EC (eFigure 2B). In contrast, the control CNV membranes showed patent vessels lined with healthy EC.
All CNV membranes were vascularized. Thy-1 immunoreactivity was detected in all but 1 control CNV membrane, with a mean (SD) percentage of 73.93% (8.76%) of vessels (Figure D and eFigure 3C). In the PDT CNV membranes, 10% of vessels of only 1 CNV membrane displayed Thy-1 immunoreactivity, whereas other CNV membranes were immunonegative (Figure, D and eFigure 3D). All vessels in all TA CNV membranes were stained for Thy-1 (Figure, D and eFigure 3E). The mean percentage of Thy-1 expressing vessels was 98.33% (1.67%) in the PDT + TA CNV membranes (Figure 1D and eFigure 3F). The percentage of Thy-1–expressing vessels showed a significant difference between subgroups (ANNOVA P < .001), being significantly higher in the TA and PDT + TA CNV membranes than in both the control (P = .04 and P = .04, respectively) and PDT CNV membranes (P < .001 for both). The percentage of Thy-1–expressing vessels in the TA and PDT + TA CNV membranes was comparable with each other (P = .9).

**IMMUNOREACTIVITY OF VEGF IN CNV MEMBRANES**

Immunoreactivity to VEGF was detected in the RPE, EC, and stroma of 10 (71.43%), 8 (57.14%), and 13 (92.86%) control CNV membranes, respectively. Intense VEGF expression was found in the RPE of 4 (28.57%) CNV membranes (Figure, E and eFigure 4A). The PDT CNV group displayed VEGF in the RPE intensely in all CNV membranes, and either weakly or moderately in the EC of 3 and the stroma of all specimens (Figure, E and eFigure 5B). The RPE, EC, and stroma displayed VEGF in all TA CNV membranes and intensely in 3 (60%) (Figure, E and eFigure 4C). The PDT + TA CNV membranes displayed VEGF intensely in the RPE and stromal cells of 5 (83.33%) CNV membranes and in the EC of 3 (50%) (Figure, E and eFigure 4D). Expression of VEGF was significantly more intense in the RPE of the PDT + TA than the control CNV membranes (ANOVA P = .05, P = .02). The total VEGF scores in the TA (P = .05) and PDT + TA (P = .01) CNV were also higher than in the control CNV group (ANOVA P = .05). Expression of VEGF in the TA and PDT + TA CNV group did not show any significant changes with respect to each other or the PDT CNV specimens.

Knowledge about inflammation in the pathogenesis of neovascular AMD is increasing. Inflammatory constituents of drusen induce VEGF expression. In turn, VEGF stimulates expression of ICAM-1 and E-selectin on vascular EC and facilitates migration of inflammatory cells to neovascularization. There is a closed but amplifying circuit between VEGF and inflammatory cells because leukocytes produce VEGF and stimulate RPE and fibroblasts to produce VEGF. Enhanced VEGF expression in the RPE induces CNV. New neovascular AMD treatment strategies therefore target the complement system, ICAM-1, and macrophages. They inhibit leukocyte infiltration and VEGF expression and therefore CNV development. Triamcinolone ace-
VEGF expression in the RPE. Enhanced VEGF in ARPE-19 by cellular uptake of verteporfin was suppressed by TA in vitro. However, TA reduced VEGF expression in the RPE induced by either oxidative stress or IL-1, but did not affect hypoxia-stimulated VEGF expression. It is still unknown which is causative for increased VEGF expression after PDT. Furthermore, responses in vivo are different and influenced by cell types, vascularization, and perfusion. In addition, TA reduces VEGF in the ARPE-19 cell line, vascular smooth cells, EC, and Muller cells in vitro, but not in rat retina and hemangioma in vivo.

This study shows that TA and PDT+TA CNV membranes are inflammatory active, showing many macrophages. However, macrophages are not only proangiogenic. First, macrophages control vessel growth and are required for cell death and tissue remodeling in the eye. During vascular regression, macrophages regulate EC apoptosis. In the case of macrophage elimination, EC survive and capillaries persist. Similarly, in our specimens, macrophages might be recruited to remove the cellular debris early after the treatment. Second, macrophages inhibit angiogenesis through the release of proteolytic enzymes that activate endogenous angiogenesis inhibitors such as endostatin. Correspondingly, endostatin was enhanced in TA and PDT+TA CNV membranes. Third, macrophages are involved in CNV inhibition. Mice deficient in monocyte chemotactic protein–1 develop CNV. Inhibition of macrophage entry into the eye promotes CNV, whereas direct injection of macrophages inhibits CNV. Previously, macrophage try into the eye promotes CNV, whereas direct injection of macrophages inhibits CNV.63 Previously, macrophage infiltration is unknown. Proliferative activity and density of leukocytes tend to be lower in TA and PDT+TA CNV membranes. In contrast, CNV membranes treated with bevacizumab, a full-length recombinant humanized monoclonal antibody against VEGF, show higher proliferative activity and leukocyte density than control CNV membranes. Whether these are sufficient rationales for triple combination therapy including PDT, TA, and anti-VEGF agents, as recently introduced, needs to be further evaluated.

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17. Nauck M, Karakulaklis G, Perruchoud AP, Papatsonstantino E, Roth M. Corticoste-