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=5) or PDT and TA combined (PDT+TA CNV group; n=6) 3 to 9 days preoperatively. The CNV membranes were stained for cytokertatin 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 antiinflammatory 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. 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.

### 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.

<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</th>
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<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>
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<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>
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<td>Classic</td>
<td>0.27</td>
<td>10/400</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7/F/78</td>
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<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>
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<td>Classic</td>
<td>0.46</td>
<td>10/400</td>
<td>3</td>
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</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>
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</tr>
<tr>
<td>13/F/85</td>
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<td>Occult</td>
<td>0.76</td>
<td>10/50</td>
<td>5</td>
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<tr>
<td>14/F/75</td>
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<td>Occult</td>
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<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 angiomatosus proliferation; TA, triamcinolone acetonide; VA, visual acuity.

#### IMMUNOHISTOLOGY

The CNV specimens were fixed in formalin and embedded in paraffin. Each section was sectioned into 3-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. 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 (Cyto- mation, Code K3461; Dako).

Immunohistochemical staining with the primary mouse antibodies specific for VEGF (sc-7269; Santa Cruz Biotechnolog Inc, Santa Cruz, California), Thy-1 (clone 5E10; BD Biosciences, Pharmingen, San Jose, California), and CD68 (Clone 110030).
Immunohistopathological findings are summarized in the Figure.

EXPRESSION 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 = .004, P < .001, and P < .001, respectively) and PDT CNV membranes (P = .01, P = .004, and P = .002, 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). The mean (SE) macrophage density in the PDT + TA CNV membranes (2831.31 [481.29] cells/mm³) was significantly higher than in the control (P < .001), PDT (P = .002), and TA CNV membranes (P = .02 (ANOVA P = .002) (eFigure 2A-D). The higher macrophage density in the TA than in the control and PDT CNV membranes did not show significance (P = .41).

Leukocytes were present in all but 1 control CNV membrane, with a mean (SE) density of 180.92 (52.34) cells/mm³. The density of leukocytes in 2 PDT CNV membranes ranged from 0 to 3.73 cells/mm³ (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 2G) 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...
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 (ANOVA 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.

### COMMENT

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 acetonide inhibits experimental CNV and was suggested to alter inflammatory cell activity and/or numbers, reduce VEGF expression, or downregulate ICAM expression. It is efficacious in AMD treatment. Herein, we evaluated the early effects of TA on cellular adhesion molecules, inflammatory cell infiltration and activity, Thy-1, and proliferation in human CNV.

In our series, control CNV were mostly inflammatory active, with varying densities of leukocytes, macrophages, Thy-1, ICAM-1, and E-selectin expression. Leukocytes and macrophages were previously found to be present in CNV membranes. In concordance with Yeh et al., we found ICAM-1 expression mainly and intensely in the RPE. E-selectin was present in the EC, stroma, and RPE, as previously reported. Intense VEGF expression was detected in the RPE, EC, and stroma in less than 30% of the specimens. In TA or PDT+TA CNV membranes, the density of CD68 immunopositive peripheral leukocytes tended to decrease in comparison with control CNV membranes, similar to observations of Ciulla et al. Surprisingly, the density of CD68 immunoreactive resident macrophages was higher in both the TA or PDT+TA CNV membranes than in the controls, but significantly increased only in the PDT+TA CNV membranes. Macrophages synthesize IL-1 and TNF-α. Hence, increased macrophage density was associated with significantly higher Thy-1 expression, reflecting enhanced IL-1 and TNF-α activity. Both IL-1 and TNF-α further enhance ICAM-1 expression in EC and the RPE. Additionally, macrophages induce VEGF production in the RPE through IL-1 and TNF-α. The density of infiltrating inflammatory cells is correlated with VEGF levels in CNV. Increased density of macrophage infiltration and Thy-1 expression, therefore, is associated with an increased total VEGF expression score in TA and PDT+TA CNV. Enhanced VEGF expression might re-stimulate the cascade by enhancing ICAM-1 expression and macrophage infiltration.

The effect of TA on inflammation has been previously studied. Ishibashi et al. supposed that TA might inhibit experimental CNV through inhibiting infiltration of inflammatory cells, especially of macrophages. Penfold et al. suggested that TA diminished numbers of dendritiform microglia but not macrophage-like populations in neural retina overlying subretinal proliferation. Monotherapy with TA reduced permeability and expression of ICAM-1 in choroidal EC. However, E-selectin was unaffected. Macrophage infiltration was increased after subconjunctival injection of TA. Antoszky et al. suggested that the angiostatic, but not anti-inflammatory, effect of TA was significant in preventing neovascularization.

Age, maturity, pretreatment inflammatory activity, and VEGF expression in CNV cannot be predicted. In contrast, the time of PDT application acts as an artificial time zero. Photodynamic therapy induces a significant decrease in inflammatory cell infiltration and activity and a significant increase in VEGF expression by the RPE. Therefore, PDT CNV serves as an ideal control group for PDT+TA CNV. Their comparison also revealed increased macrophage infiltration, enhanced Thy-1 and ICAM-1 expression, and increased VEGF total score in the PDT+TA CNV group as well as persisting intense
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 ret 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.

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 depletion with liposomes was shown to inhibit CNV. However, reduction of neovascularization was recently suggested to be due to direct toxicity of liposomes on EC.

Proliferative activity is significantly higher in inflammatory active CNV. The TA and PDT+TA CNV membranes were highly infiltrated with macrophages; however, mean proliferative activity was lower than in the control CNV membranes, possibly owing to the antiproliferative effect of TA.

We are unaware of previous reports of clinicopathological evaluation of ICAM-1, inflammation, and proliferation in human CNV treated with TA or PDT+TA combination therapy. Proper interpretation of the study is limited by the small number of specimens and the possibility of selection bias. An absolute quantification of mRNA and/or protein expression by real time polymerase chain reaction and/or Western blot in further studies will surely supply additional valuable information. Nevertheless, TA and PDT+TA CNV membranes show infiltration with a significantly higher density of resident macrophages and intense VEGF expression early after therapy, although its duration 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 bevazumab, 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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REFERENCES
