Early Effects of Triamcinolone on Vascular Endothelial Growth Factor and Endostatin in Human Choroidal Neovascularization

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Objective: To evaluate the early effects of triamcinolone acetonide as monotherapy or as an adjuvant to ocular verteporfin photodynamic therapy (PDT) on angiogenesis in human choroidal neovascularization (CNV) secondary to age-related macular degeneration.

Methods: Retrospective review of an interventional series of 55 patients who underwent CNV extraction. Eleven patients were treated with intravitreal triamcinolone acetonide (4 mg) monotherapy (triamcinolone-treated CNV group [n=5]) or with PDT–triamcinolone combination therapy (PDT–triamcinolone-treated CNV group [n=6]) 3 to 9 days before surgery. Forty patients who underwent CNV extraction without previous therapy (control CNV group) and 4 patients who underwent CNV extraction 3 days after PDT (PDT CNV group) served as control subjects. The CNV samples were stained for CD34, endostatin, cytokeratin 18, and vascular endothelial growth factor (VEGF).

Results: Vascular endothelial growth factor expression was stronger in the PDT CNV samples (P < .001), triamcinolone CNV samples (P = .01), and PDT–triamcinolone CNV samples (P = .007) compared with the control CNV samples. There were no statistically significant differences in VEGF expression among the PDT CNV samples, triamcinolone CNV samples, and PDT–triamcinolone CNV samples. Endostatin expression was weaker in the PDT CNV samples than in the control CNV samples (P = .008). Endostatin expression was stronger in the triamcinolone CNV samples and the PDT–triamcinolone CNV samples compared with the control CNV samples (P = .001 and P < .001, respectively) and the PDT CNV samples (P < .001 for both).

Conclusion: To some extent, triamcinolone monotherapy seems to exert its angiogenesis inhibitory effects on CNV by enhancing endostatin expression rather than by suppressing VEGF expression.

Surgical intervention and removal of CNV were offered when indicated with 360° retinotomy, were discussed with each patient. Therapy options, including observation, PDT treatment or re-treatment, conventional thermal laser photocoagulation, intravitreal triamcinolone injection, and full macular translocation were discussed with each patient before CNV excision. In patients before CNV excision are summarized in the Table.

**Table. Clinical Characteristics of 15 Patients Treated With Triamcinolone, Verteporfin Photodynamic Therapy (PDT), or Both Before Surgical Removal of Choroidal Neovascularization (CNV) Membranes**

<table>
<thead>
<tr>
<th>Patient No./Sex/ Age, y</th>
<th>Eye</th>
<th>CNV Type</th>
<th>Time to Surgery From</th>
<th>Triamcinolone Injection, d</th>
<th>PDT, d</th>
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<tr>
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<td>Hemorrhagic plus occult</td>
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<tr>
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<td>OD</td>
<td>Occult</td>
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<td>3/F/84</td>
<td>OD</td>
<td>PED (RAP)</td>
<td>4</td>
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<td>PED, occult</td>
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<td>...</td>
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<tr>
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<td>OS</td>
<td>Occult</td>
<td>9</td>
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**Abbreviations:** OD, right; OS, left; PED, pigment epithelium detachment; RAP, retinal angiomatous proliferation; ellipsis, no therapy.

Effects of intravitreal triamcinolone acetonide (4 mg) monotherapy and of PDT–triamcinolone combination therapy on angiogenesis in human CNV membranes. An antibody specific for vascular endothelial growth factor A (VEGF-A) was used as a marker of angiogenic stimulation. Endostatin was evaluated as an endogenous angiogenesis inhibitor. CD34 and cytokeratin 18 were used to identify endothelial cells (ECs) and retinal pigment epithelium (RPE) cells, respectively. Immunohistologic findings in 11 patients treated with intravitreal triamcinolone acetonide (4 mg) monotherapy (triamcinolone-treated CNV group [n = 5]) or with PDT–triamcinolone combination therapy (PDT–triamcinolone-treated CNV group [n = 6]) 3 to 9 days before surgery were compared with the findings in 4 patients who underwent CNV extraction 3 days after PDT (PDT CNV group) and in 40 patients who underwent CNV extraction without previous therapy (control CNV group).

**METHODS**

We retrospectively reviewed 55 eyes of 55 consecutive patients with AMD in whom full macular translocation surgery with CNV extraction was performed at 10 surgical sites between January 15, 1997, and July 28, 2005. In 15 of these patients, surgery was performed after verteporfin PDT (n = 4), triamcinolone monotherapy (n = 5), or PDT–triamcinolone combination therapy (n = 6). Clinical characteristics of the patients before CNV excision are summarized in the Table. Therapy options, including observation, PDT treatment or re-treatment, conventional thermal laser photocoagulation, intravitreal triamcinolone injection, and full macular translocation with 360° retinotomy, were discussed with each patient. Surgical intervention and removal of CNV were offered when (1) visual acuity was less than 20/200 (the minimum visual acuity at which the first PDT is recommended according to the Treatment of Age-Related Macular Degeneration With Photodynamic Therapy Investigation); (2) visual deterioration progressed after the initial PDT; (3) the patient refused retreatment with PDT, triamcinolone, or PDT–triamcinolone because of continuous visual deterioration in the fellow eye despite therapy; and (4) re-treatment with PDT was impossible because of recurrent or massive submacular hemorrhage. In 4 patients, verteporfin PDT was performed 3 days before surgery to reduce bleeding from the lesion site at the time of surgical extraction. Preoperative therapy with triamcinolone and PDT–triamcinolone was administered to decrease intraoperative hemorrhage, postoperative CNV recurrence, and the proliferative vitreoretinopathy rate. After the experimental nature of the treatment procedures and the risks and benefits of all therapy options had been fully explained, each patient gave written informed consent. The study followed the guidelines of the Declaration of Helsinki as revised in Tokyo, Japan, and in Venice, Italy. The study and the histologic analysis of the specimens were approved by the local institutional review board.

**IMMUNOHISTOLOGIC EXAMINATION**

After serial paraffin sections were deparaffinized and rehydrated using a graded series of alcohol, various techniques for antigen retrieval were applied. For cytokeratin 18 and endostatin, antigen retrieval was performed by proteolytic digestion using 0.5% protease XXIV (Sigma-Aldrich Inc, St Louis, Missouri), whereas proteinase K (Dako) was used for VEGF. For CD34, the method of antigen retrieval was heat treatment in citrate buffer (0.01M [pH 6.0]) in a pressure cooker.

Immunohistochemical staining with the primary antibodies specific for CD34 (mouse monoclonal antibody; Immunotech, Hamburg, Germany) and cytokeratin 18 (mouse monoclonal antibody; Progen, Heidelberg, Germany) was performed using the horseradish peroxidase method as previously described. Hematoxylin (ChemMate, code S2020; Dako) was used for counterstaining. Immunohistochemical staining for VEGF and endostatin was performed using the alkaline phosphatase method according to the manufacturer’s instructions (ChemMate detection kit; alkaline phosphatase/red, rabbit/mouse, K5005; Dako) as previously described. An antihuman VEGF-A antibody (mouse monoclonal antibody, clone C-1; Santa Cruz Biotechnology, Santa Cruz, California) and an antihuman endostatin antibody (rabbit, polyclonal; Dianova GmbH, Hamburg, Germany) were used. For negative control samples, the primary antibodies were substituted by appropriate normal serum samples or were omitted.

**SPECIMEN ANALYSIS**

Serial sections from specimens were analyzed independently by 2 masked observers (O.T. and S.G.) using light microscopy. Immunoreactivity for VEGF and endostatin was analyzed separately in vessels, stroma, and RPE–Bruch membrane complex. A grading scheme indicating the degree of staining was used: grades of 3, 2, 1, and 0 were assigned to indicate intense labeling (70%–90%)...
100% positive cells), moderate labeling (40%-69% positive cells), weak labeling (1%-39% positive cells), and the absence of staining, respectively. The overall scores (range, 0-9) for VEGF and endostatin expression were obtained for each CNV sample by summing the staining scores in RPE, vessels, and stroma. The predominance scores of VEGF over endostatin were obtained for RPE cells, ECs, and stroma of each membrane separately by calculating the difference between the VEGF and endostatin staining scores in each component.

We comparatively analyzed the intensity of VEGF and endostatin immunostaining in RPE cells, ECs, and stroma and their predominance scores, as well as the overall VEGF and endostatin staining scores of the defined subgroups using analysis of variance (ANOVA) followed by Fisher protected least significant difference post hoc test. $P \leq .05$ was considered statistically significant.

The frequency of VEGF and endostatin immunoreactivity intensities, their mean immunoreactivity scores, mean predominance scores, and mean overall VEGF and endostatin staining scores were obtained for the subgroups of CNV samples. The results are summarized in the Figure.

**ANGIOGRAPHIC FINDINGS AND VASCULARIZATION**

Angiographic findings in 15 patients with CNV before treatment with PDT, triamcinolone, or PDT–triamcinolone are summarized in the Table. After PDT (data not shown) and PDT–triamcinolone combination therapy, hypofluorescence suggesting nonperfusion of the irradiated area and the CNV was detected by fluorescein angiography on the day of surgery (eFigure 1; available at http://www.archophthalmol.com). These angiographic findings were supported by CD34 immunostaining that demonstrated mostly occluded vessels with damaged ECs. In contrast, patients in the control CNV group had patent vessels lined with healthy ECs.
IMMUNOREACTIVITY OF VEGF IN CNV SAMPLES

Retinal pigment epithelium cells immunoreactive for cytokeratin 18 were found in all specimens. In control CNV samples, VEGF staining was detected in RPE cells in 48% (19 of 40) of the specimens (strongly in only 15% [6 of 40]). All specimens were vascularized. CD34-immunoreactive ECs demonstrated VEGF expression in 75% (30 of 40) of the samples. Cells within stroma revealed VEGF expression in 80% (32 of 40) of the membranes (intensely in 20% [8 of 40] of them) (Figure, A and eFigure 2A; available at http://www.archophthalmol.com).

Triamcinolone-treated CNV samples displayed VEGF immunoreactivity in stroma, ECs, and RPE cells in all specimens (intensely in 60% [3 of 5] of them) (Figure, A and eFigure 2B). Vascular endothelial growth factor demonstrated statistically significantly stronger immunostaining in RPE (P = .005, ANOVA P < .001), stroma (P = .03, ANOVA P = .003), and overall score (P = .01, ANOVA P = .005) in the triamcinolone-treated CNV samples compared with the control CNV samples (Figure, B).

The PDT CNV samples demonstrated statistically significantly enhanced VEGF expression in RPE cells (Figure, A and eFigure 2C) compared with the control CNV samples (P < .001, Figure, B). Vascular endothelial growth factor expression in the PDT CNV samples was comparable to that in the triamcinolone-treated CNV samples (P = .40, P = .1, P = .54, and P = .45 for RPE, vessels, stroma, and overall score, respectively).

All PDT–triamcinolone-treated CNV samples (n = 6) disclosed strong to moderate VEGF immunostaining in RPE and in stroma. Endothelial cells were present in all but 1 sample. Endothelial cells displayed strong VEGF immunoreactivity in 40% (2 of 5) of the samples (Figure, A and eFigure 2D). Vascular endothelial growth factor immunoreactivity was enhanced in RPE (P < .001) and in stroma (P < .001) compared with that in the control CNV samples. The overall VEGF staining score in the PDT–triamcinolone-treated CNV samples was also considerably higher than that in the control CNV samples (P = .007) (Figure, B). However, the VEGF staining intensities in RPE, vessels, and stroma, as well as the overall VEGF staining scores in these membranes, were comparable to those in the triamcinolone-treated CNV samples (P = .50, P = .45, P = .46, and P = .89, respectively) and in the PDT CNV samples (P = .80, P = .34, P = .19, and P = .38, respectively).

IMMUNOREACTIVITY OF ENDOSTATIN IN CNV SAMPLES

Endostatin immunoreactivity was detected in RPE–Bruch membrane complex, vessels, and stroma in 48% (19 of 40), 58% (23 of 40), and 88% (35 of 40) of the control CNV samples, respectively (Figure and eFigure 3; available at http://www.archophthalmol.com). Endostatin expression was found in vessels, and stroma in all specimens in the triamcinolone-treated CNV group. The endostatin staining intensity was intense to moderate in 80% (4 of 5) of the samples. Endostatin expression was stronger in RPE (P = .02, ANOVA P = .001) and in vessels (P = .01, ANOVA P < .001) in the triamcinolone-treated CNV samples and the PDT–triamcinolone-treated CNV samples compared with the control CNV samples (Figure, B). Although endostatin staining in stroma was comparable to that in the control CNV samples (P = .06, ANOVA P < .001), the overall endostatin staining score was statistically significantly higher in the triamcinolone-treated CNV samples (P = .001, ANOVA P < .001).

In the PDT CNV samples, endostatin expression was found in the RPE–Bruch membrane complex of only 2 specimens. None of the specimens demonstrated endostatin expression in vessels or in stroma (Figure and eFigure 3). Therefore, endostatin expression was statistically significantly weaker in vessels (P = .49), stroma (P < .001), and overall score (P = .008) in the PDT CNV samples than in the control CNV samples (Figure, B). Compared with the triamcinolone-treated CNV samples, endostatin expression in the PDT CNV samples was again weaker in RPE (P = .048), vessels (P = .002), stroma (P < .001), and overall score (P < .001).

In the PDT–triamcinolone-treated CNV samples, endostatin immunostaining was moderate to strong in RPE–Bruch membrane complex and in vessels. Except for 1 sample with mild expression (17%), all PDT–triamcinolone-treated CNV samples demonstrated strong (50% [3 of 6]) to moderate (33% [2 of 6]) endostatin expression in stroma (Figure, C and eFigure 3D). Compared with the control CNV samples, endostatin expression in the PDT–triamcinolone-treated CNV samples was considerably stronger in RPE (P < .001) and in vessels (P = .002), and the overall endostatin staining score was statistically significantly higher (P < .001) (Figure, B). Compared with the PDT CNV samples, endostatin expression in the PDT–triamcinolone-treated CNV samples was statistically significantly more intense in RPE (P = .007), vessels (P < .001), and stroma (P < .001), and correspondingly the overall endostatin staining score was higher (P < .001). However, endostatin expression in the PDT–triamcinolone-treated CNV samples was not statistically significantly increased compared with that in the triamcinolone-treated CNV samples (P = .47, P = .55, P = .89, and P = .53 in RPE, vessels, stroma, and overall score, respectively).

ANALYSIS OF VEGF PREDOMINANCE OVER ENDOSTATIN

In the PDT CNV samples, VEGF expression statistically significantly predominated over endostatin expression in RPE (P = .002, ANOVA P = .01), stroma (P = .002, ANOVA P = .01), and overall score (P = .003, ANOVA P = .02) compared with that in the control CNV samples (Figure, B). However, VEGF predominance over endostatin early after PDT was statistically significantly decreased in RPE (P = .02); in the overall predominance score (P = .006), PDT was combined with the triamcinolone-treated CNV. Furthermore, the predominance scores among the triamcinolone-treated CNV samples were statistically significantly lower in RPE (P = .03), stroma, (P = .03), and overall
Neovascularization such as that in CNV is a complex process controlled by the local balance between angiogenesis stimulators and inhibitors. A shift in the balance between stimulators and inhibitors may turn the ‘angiogenic switch’ on or off. Herein, we aimed to evaluate the effects of intravitreal triamcinolone administration on VEGF, endostatin, and the balance between them in human CNV.

Vascular endothelial growth factor is a major stimulator of CNV. Human CNV samples display VEGFexpression and anti-VEGF agents are beneficial in neovascular AMD treatment. To some extent, the angiogenesis inhibitory action of triamcinolone is believed to be due to decreased VEGF directly or indirectlythrough its anti-inflammatory effects. Therefore, we evaluated VEGF expression in human CNV samples excised following triamcinolone injection. In our series, VEGF expression was more intense in RPE and stroma in the triamcinolone-treated CNV samples compared with that in the control CNV samples. This seems to conflict with the findings of some previous in vitro studies showing that triamcinolone reduces VEGF in the cultured human retinal pigment epithelium (ARPE19) cell line, in isolated human vascular smooth cells, and in umbilical vein ECs. However, in another in vitro study, VEGF was not involved in triamcinolone-induced inhibition of capillary growth in cultures obtained from human hemangioma samples. Nevertheless, the same stimulus or inhibitor may act differently in vivo. In vivo studies revealed that triamcinolone does not alter basal VEGF expression in rat retina and does not suppress VEGF expression in human hemangioma. In our series, the age and maturity of the CNV and the intensity of VEGF expression before triamcinolone injection were unknown. Therefore, strong VEGF expression after triamcinolone injection may be related to preexisting high levels or (less likely) to induction by triamcinolone injection.

Vascular endothelial growth factor expression is enhanced in human CNV following verteporfin PDT. Vascular occlusion–related hypoxia and reactive oxygen intermediates induced by PDT enhance VEGF expression and lead to recurrences. In our PDT–triamcinolone-treated CNV samples, VEGF expression was stronger than that in the control CNV samples and was comparable to that in the PDT CNV samples. In our opinion, this suggests that increased VEGF levels induced by PDT remain unaffected by triamcinolone injection. In an in vitro study using the ARPE19 cell line, increased VEGF by the cellular uptake of verteporfin was suppressed by triamcinolone. Again, findings in the in vitro setting may not reflect the more complex mechanisms in vivo. In RPE cells, triamcinolone reduced VEGF expression induced by oxidative stress or by interleukin 1 but did not affect hypoxia-stimulated VEGF expression. Following PDT, it is unknown whether hypoxia stimulates VEGF expression more than oxidative stress, but this question is beyond the scope of our study. However, our results reveal that triamcinolone does not exert its antiangiogenic effect through decreased VEGF expression in human CNV. Whether triamcinolone modulates the VEGF downstream signaling or its receptors in CNV needs to be further investigated.

Endostatin inhibits experimental CNV and VEGF-induced neovascularization. Human CNV samples express endostatin. Folkman noted that endogenous expression of endostatin can be up-regulated by corticosteroids. In our triamcinolone-treated CNV samples, endostatin expression was stronger than that in our control CNV samples. Expression of endostatin was statistically significantly reduced in CNV membranes excised 3 days after PDT. In PDT CNV samples, VEGF predominates over endostatin and conceivably stimulates recurrences. This correlates with the fact that reduced endostatin production has a role in hypoxia-induced angiogenesis and in CNV formation. Our study revealed that triamcinolone treatment as an adjuvant to PDT enhanced endostatin expression in RPE and in stroma. As a consequence, VEGF predominance over endostatin was reduced. This explains the improved clinical outcome when PDT is combined with triamcinolone treatment. In addition, the antiangiogenic effects of endostatin were enhanced in vivo and in vitro when endostatin was used in combination with anti-VEGF therapy. Because triamcinolone seems to act through up-regulation of the angiogenesis inhibitor pathway, a synergistic effect might be expected when an existing angiogenesis promoter such as VEGF is simultaneously blocked by an aptamer or antibody. This rationale supports a recently proposed triple combination therapy of PDT, triamcinolone, and anti-VEGF agents that was shown to be clinically effective, but this modality requires further evaluation.

We are unaware of previous reports of clinicopathologic evaluation of VEGF and endostatin expression in human CNV membranes treated by triamcinolone monotherapy or by PDT–triamcinolone combination therapy. The interpretation of our study results is limited by the small number and heterogeneity of the examined specimens, which renders the immunohistochemical evaluation challenging. Although the histopathologic findings in patients with better therapeutic outcomes might differ from our case findings, it is conceivable that the antiangiogenic activity of triamcinolone is, at least in part, mediated by enhanced endostatin expression and by a shift in predominance between promoters and inhibitors of angiogenesis.


**eFigure 1.** Photomicrographs of 2 choroidal neovascularization (CNV) membranes extracted 4 days after intravitreal triamcinolone acetonide injection and 3 days after photodynamic therapy (PDT) (A and B from patient 10 in the Table and C from patient 11) and of a CNV membrane without prior therapy (D). A, Late phase of fluorescein angiography on the day of surgery displays nonperfusion of the CNV and laser spot area. B-D, Specimens were probed using antibody against CD34 and were stained using 3-diaminobenzidine, resulting in brown chromogen. The brown chromogen–labeling endothelial cells can be distinguished from the melanin granula (asterisk) in pigmented cells. Most of the vessels in CNV excised after PDT–triamcinolone acetonide combination therapy were occluded and were lined with damaged endothelial cells (B and C, arrows). Vessels in CNV without prior therapy are patent and are lined with healthy endothelial cells (D, arrows). Bar indicates 50 µm.
Figure 2. Photomicrographs of choroidal neovascularization (CNV) probed using antibody against vascular endothelial growth factor (VEGF) and stained with red chromogen. Hematoxylin was used as a counterstain. A, In control CNV without previous therapy (the same CNV as in eFigure 1D), VEGF staining was detected within some cells in stroma (arrow) but not in retinal pigment epithelium (RPE) (asterisk). B, In CNV excised 4 days after intravitreal triamcinolone acetonide monotherapy (from patient 3 in the Table), intense VEGF expression was found in RPE (asterisk) and in stromal cells (arrow). C, In CNV excised 3 days after photodynamic therapy (PDT) (from patient 6), intense VEGF expression is prominent in RPE cells. D, In another CNV membrane excised 3 days after PDT and 4 days after intravitreal triamcinolone acetonide injection (from patient 11), RPE (asterisks) and stromal cells (arrows) disclose VEGF intensely. Bar indicates 50 µm.
Figure 3. Photomicrographs of choroidal neovascularization (CNV) probed using endostatin stained with red chromogen. Hematoxylin-eosin was used as a counterstain. A, In control CNV without previous therapy (same CNV as in eFigure 1D and eFigure 2A), retinal pigment epithelium (RPE) (asterisk), vessels (black arrowhead), and stromal cells (arrow) display endostatin expression. B, Choroidal neovascularization excised 3 days after intravitreal triamcinolone acetonide monotherapy (from patient 1 in the Table) reveals strong endostatin expression in RPE (asterisk) and in stromal cells (arrow). C, Specimen treated with photodynamic therapy (PDT) 3 days before surgery (from patient 6) disclosed no endostatin expression (asterisk). D, A CNV membrane excised 3 days after PDT and 4 days after intravitreal triamcinolone acetonide injection (from patient 11) was strongly immunopositive for endostatin in RPE (asterisks), vessels (black arrowhead), and stroma (arrows). Bar indicates 50 µm.