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.

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NEOVASCULAR AGE-RELATED macular degeneration (AMD) is the leading cause of visual disability among older populations in Western countries.[1,2] The development of ocular verteporfin photodynamic therapy (PDT) to selectively destroy choroidal neovascularization (CNV)[3] has been an important milestone in the treatment of neovascular AMD.[4-6] However, its efficacy is limited by high recurrence rates following therapy.[4-6] Although submacular removal of CNV is not recommended alone,[7] CNV extraction with macular translocation seems to be a promising modality to improve near and distance visual acuity[8-10] and is effective in patients who do not sufficiently benefit from prior PDT.[11-13] Refinements in surgical techniques and instruments have improved the surgical outcomes. However, postoperative proliferative vitreoretinopathy and CNV recurrence threaten long-term favorable prognoses. Recently, angiogenesis-modulating drugs have been used as monotherapy or as adjuvant therapy to overcome these problems.

The corticosteroid triamcinolone acetonide is an angiostatic drug that inhibits laser-induced CNV.[14-16] Consequently, triamcinolone is a promising treatment option for CNV as monotherapy[17-19] or as an adjuvant to PDT.[20-24] Intravitreal injection of triamcinolone improved visual acuity, decreased the re-treatment rate of PDT, and inhibited CNV growth in pilot clinical studies.[17-24]

Triamcinolone mediates antiangiogenic, anti-inflammatory, and antipermeability effects.[25-31] The anti-inflammatory effects of triamcinolone on neovascularization have been studied previously.[31-33] However, its effectiveness against angiogenesis in human CNV has not been clarified, to our knowledge.

This immunohistopathologic study was undertaken to evaluate the short-term ef-

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

Patient No./Sex/ Age, y	Eye	CNV Type	Time to Surgery From Triamcinolone Injection, d	Time to Surgery From PDT, d
1/M/90	OD	Hemorrhagic plus occult	3	...
2/F/78	OD	Occult	3	...
3/F/84	OD	PED (RAP)	4	...
4/F/70	OD	PED, occult	7	...
5/F/80	OS	RAP	8	...
6/M/76	OS	Classic	...	3
7/F/78	OD	Classic	...	3
8/M/54	OS	Predominantly classic	...	113 and 3
9/M/84	OS	Classic	...	3
10/M/83	OD	Classic	4	5
11/M/83	OS	Classic	4	5
12/F/82	OS	Occult	5	5
13/F/85	OD	Occult	5	5
14/F/75	OD	Occult	6	7
15/M/74	OS	Occult	9	3

Abbreviations: OD, right; OS, left; PED, pigment epithelium detachment; RAP, retinal angiomatous proliferation; ellipsis, no therapy.

fects 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.[34] Endostatin was evaluated as an endogenous angiogenesis inhibitor.[35] CD34 and cytokeratin 18 were used to identify endothelial cells (ECs)[36] and retinal pigment epithelium (RPE) cells,[37] 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

SUBJECTS AND TREATMENTS

We retrospectively reviewed 33 eyes of 33 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 retreatment, 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[2,3]), (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.[25,27,38] 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.[39] The study and the histologic analysis of the specimens were approved by the local institutional review board.

TISSUE PREPARATION

Within minutes after surgery, excised CNV samples were fixed in 3.7% formalin and subsequently embedded in paraffin. Each specimen was serially mounted on poly-L-lysine–coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining.

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.[40] 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.[40] 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%-

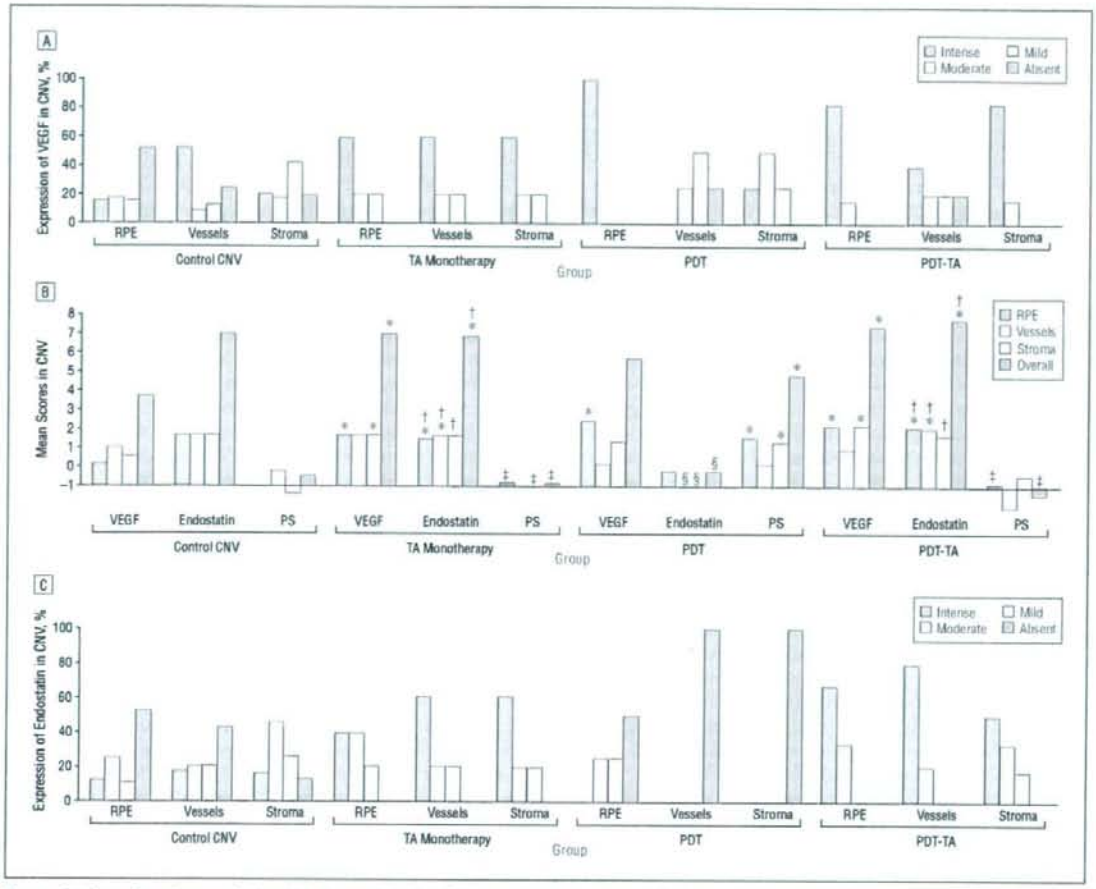

Figure. Data from the subgroups of choroidal neovascularization (CNV) samples. A, Vascular endothelial growth factor (VEGF) immunoreactivity. B, Mean VEGF and endostatin staining scores and predominance scores (PS). *Statistical significance higher than that in control CNV samples; †, higher than that in the photodynamic therapy (PDT) CNV samples; ‡, lower than that in the PDT CNV samples; and §, lower than that in control CNV samples. C, Endostatin immunostaining intensity in CNV without previous therapy (control CNV), extracted after verteporfin PDT, triamcinolone acetonide (TA) monotherapy, and PDT–triamcinolone combination therapy. RPE indicates retinal pigment epithelium.

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.

RESULTS

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 RPE, 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 ($P=.049$), 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

score ($P = .01$) compared with those among the PDT CNV samples. The predominance scores among the PDT–triamcinolone-treated CNV samples were not statistically significant different in RPE, vessels, and stroma compared with those among the triamcinolone-treated CNV samples ($P = .97$, $P = .31$, and $P = .53$, respectively) or the control CNV samples ($P = .75$, $P = .09$, and $P = .20$, respectively).

COMMENT

Neovascularization such as that in CNV is a complex process controlled by the local balance between angiogenesis stimulators and inhibitors.[41] 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.[34,42,43] Human CNV samples display VEGF[40,44,46] and anti-VEGF agents are beneficial in neovascular AMD treatment.[50,51] To some extent, the angiogenesis inhibitory action of triamcinolone is believed to be due to decreased VEGF directly[54,58] or indirectly[32,33,59,60] through 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[55-57] 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,[61] 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[62] and does not suppress VEGF expression in human hemangioma.[63] 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.[40,44,49] Vascular occlusion–related hypoxia[64-66] and increased reactive oxygen intermediates[67] induced by PDT enhance VEGF expression and lead to recurrences.[68] 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[38] 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.[38] 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[69] and VEGF-induced neovascularization.[70] Human CNV samples express endostatin.[44,48] Folkman[71] 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.[48] 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[72,73] and in CNV formation.[74] 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.[20-24] In addition, the antiangiogenic effects of endostatin were enhanced in vivo and in vitro when endostatin was used in combination with anti-VEGF therapy.[75,76] 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,[77] 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.[57,72]

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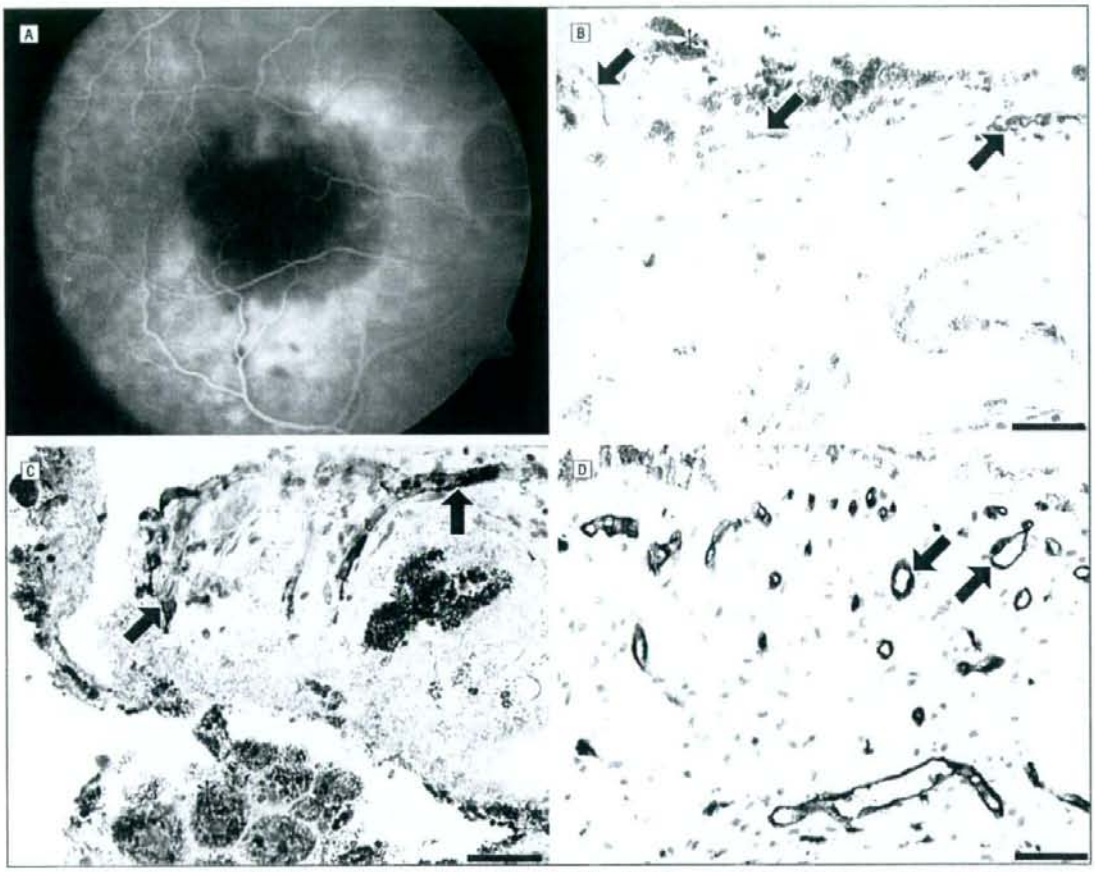
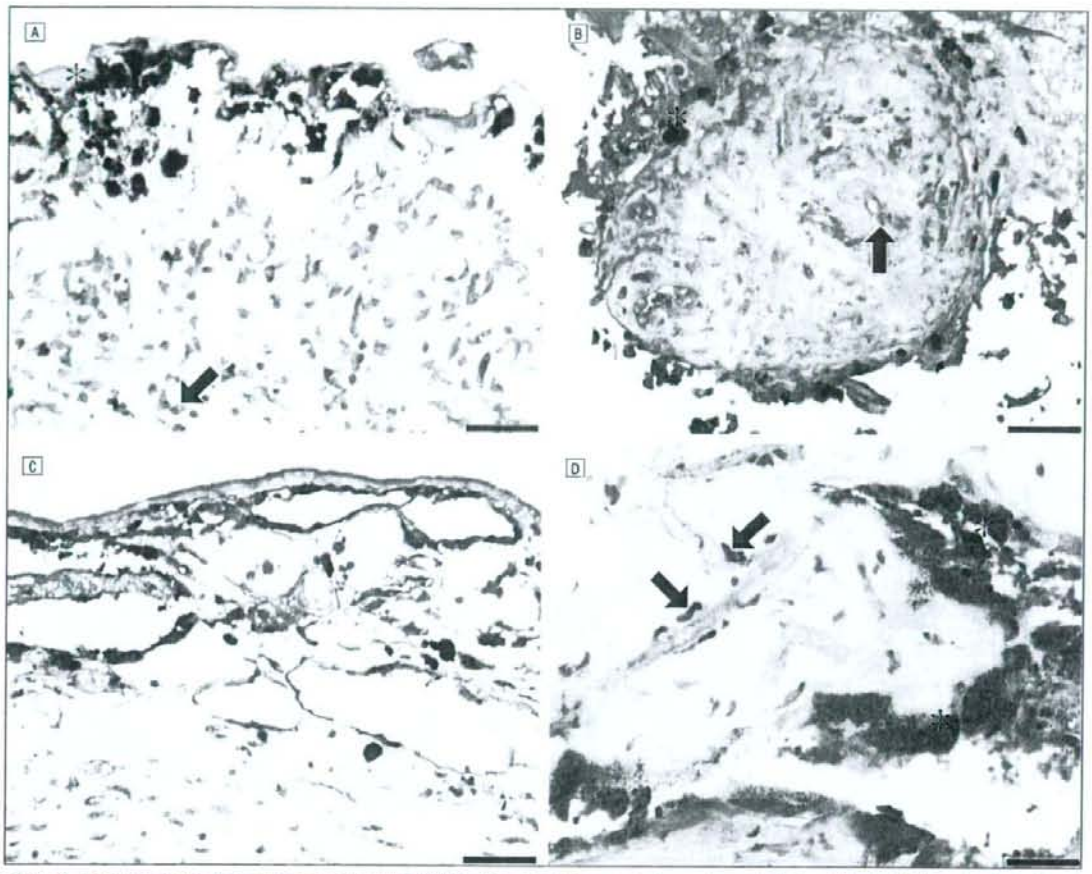
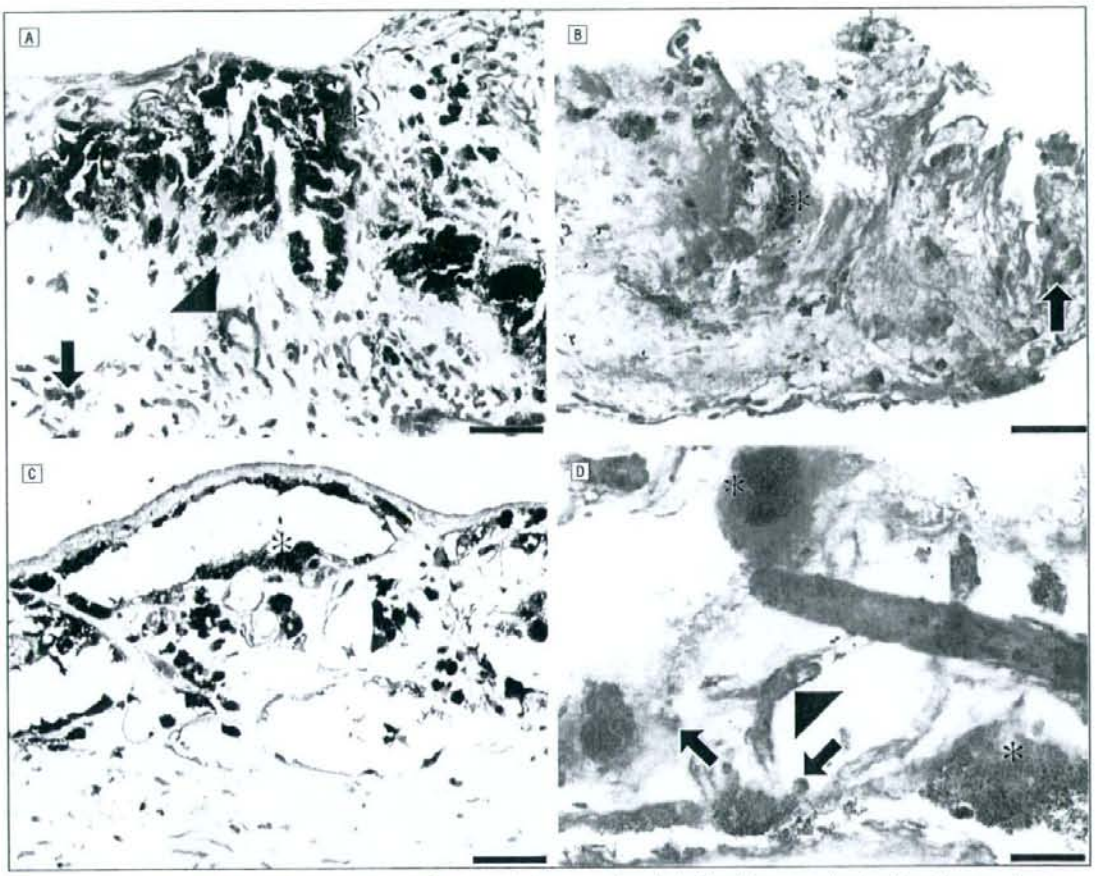


eFigure 1. Photomicrographs of 2 choroidal neovascularization (CNV) membranes extracted 4 days after intravitreal triamcinolone acetonide injection and 3 days after photodynamic therapy (PDT) (A and B from patient 10 in the Table and C from patient 11) and of a CNV membrane without prior therapy (D). 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.



eFigure 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.



eFigure 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.

Letters to the Editor

Ptosis after Sub–Tenon's Capsule Triamcinolone

Dear Editor:

An intravitreal or sub–Tenon's capsule injection of triamcinolone acetonide (TA) has been shown to be efficacious for major retinal vascular conditions. However, secondary ptosis after sub–Tenon's capsule TA injection has recently been described.[1–4] We reviewed the incidence of ptosis in patients after treatment for retinal vascular diseases, and we discuss the possible pathogenesis of the secondary ptosis based on our assessment of the intraoperative findings.

The medical records of 148 eyes of 136 patients that had a total of 260 sub–Tenon's capsule TA injections between 2004 and 2006 for retinal vascular occlusion, age-related macular degeneration (AMD), or diabetic macular edema were reviewed. Written informed consent was obtained from all patients after a full explanation of the purpose and possible complications of the sub–Tenon's capsule TA injection. After application of topical 4% lidocaine anesthetic drops, the patient was asked to look inferonasally. A 26-gauge needle was inserted superotemporally into the sub–Tenon's capsule space at the equator by gently moving the tip of the needle to avoid ocular penetration. Then, 10 mg or 20 mg of TA (40 mg/ml; Kenacort-A, Bristol Myers KK, Tokyo, Japan) was injected.

None of 24 eyes developed symptomatic ptosis after bilateral sub–Tenon's capsule TA injection. Among 124 patients with unilateral sub–Tenon's capsule TA injection, 6 eyelids (4.1% [6/148 eyes]) developed symptomatic ptosis after the sub–Tenon's capsule injection of TA for AMD in 3 eyes, central retinal vein occlusion in 2 eyes, and branch retinal vein occlusion in 1 eye, and none recovered in the following 6 months. Ptosis was considered to be present when the palpebral fissure was >2 mm narrower than that of the fellow eye at the primary eye position. Patients with myopathic, congenital, or senile ptosis before the TA injection were not involved. The period from the initial sub–Tenon's capsule TA injection to the symptomatic ptosis varied from 6 to 24 months (mean ± standard deviation, 15.0±6.7). Total volumes of injected TA before ptosis were 40 mg in 2 eyes, 30 mg in 2 eyes, 20 mg in 1 eye, and 10 mg in 1 eye. Five of 6 patients underwent eyelid plastic surgery, whereas the other patient declined surgery. Levator functions in the affected eye were 11.8±1.5 mm preoperatively and 12.3±1.3 mm postoperatively (n = 5), whereas those in the fellow eye were 12.4±1.5 mm preoperatively and 12.5±1.7 mm postoperatively. Margin reflex distances in the affected eye were −1.3±1.5 mm preoperatively and 2.2±0.4 mm postoperatively ($P = 0.043$, Wilcoxon rank test; n = 5), whereas those in the fellow eye were 2.2±0.4 mm preoperatively and 2.2±0.4 mm postoperatively ($P>0.999$).

Intraoperatively, the preaponeurotic fat and soft connective tissue appeared normal without anatomical defects in the levator aponeurosis and Müller's muscle in all eyes. However, a dehiscence of the levator aponeurosis from the tarsal plate was found in all eyes (Fig 1 [available at http://aaojournal.org]). The detached levator aponeurosis was sutured onto the tarsal plate without advancing Müller's muscle or tucking the levator muscle.

The incidence of blepharoptosis after posterior sub–Tenon's capsule TA injection has been reported to be from 4% to 15%.[1,2] Dal Canto et al[3] described a recruitment of inflammatory histocytes with phagocytosed materials in the pathological sections obtained from the biopsy of the herniated orbital fat in patients with orbital fat prolaspe and ptosis 2 weeks after last sub–Tenon's capsule TA injection. They concluded that paradoxically low-grade inflammation developed after local TA injection, and this was the cause of the eyelid abnormalities such as rarefaction of the orbital septum, frail deconstruction of the levator muscle, and dehiscence of levator aponeurosis.[3] In our cases, levator function was well preserved and the intraoperative findings were consistent with aponeurotic ptosis but not with the idea of low-grade paradoxical inflammation. Local injections of TA into rabbit Achilles tendons has been described to weaken tendons, which decreased failure stress both within the tendon and the space between the heel bones.[5]

These results indicate that dehiscence of levator aponeurosis was most likely to cause the ptosis after TA injection—probably not related to the injection trauma but, rather, a pharmacological effect of the drug. A pathological evaluation of the levator aponeurosis beneath the conjunctival fornix will be necessary to prove this hypothesis. However, the ptosis after sub–Tenon's capsule TA injections can be successfully treated by a conventional method as used for aponeurotic ptosis.

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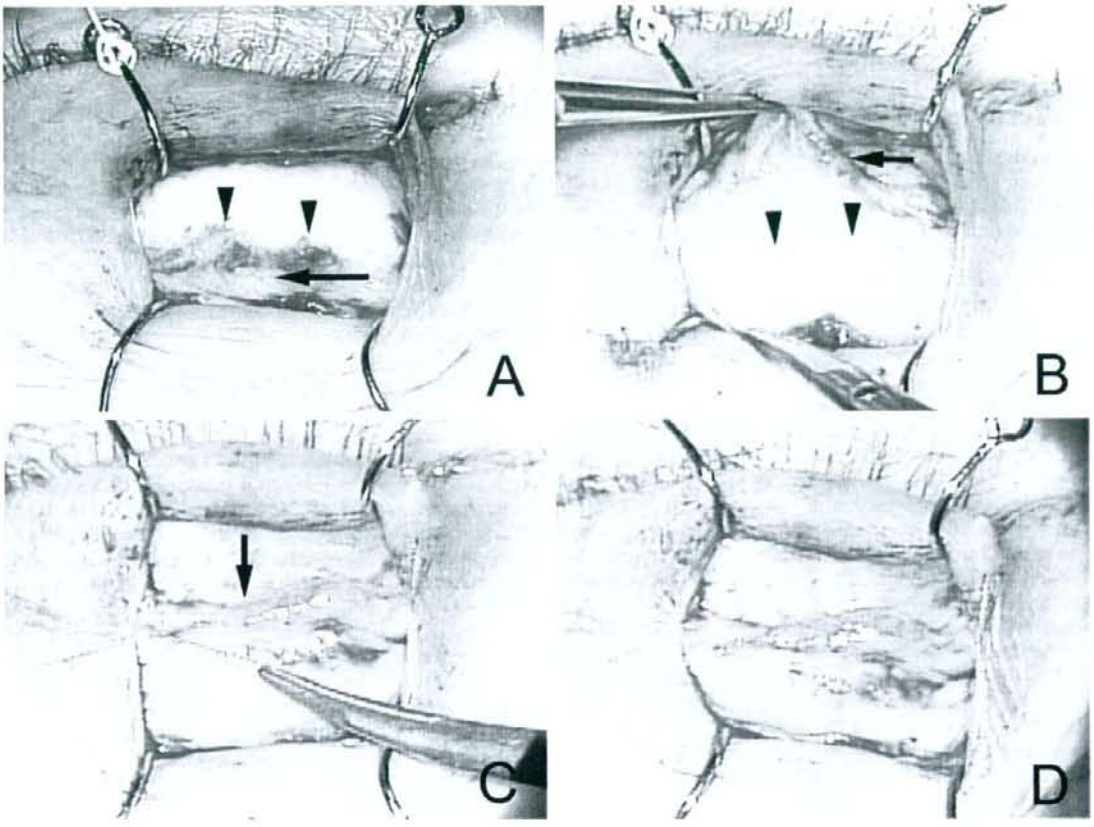

Figure 1. Intraoperative photograph through the operating microscope. **A**, Müller's muscle (arrowheads) is seen at the upper rim of the tarsus. The adjunction of aponeurosis (arrow) is deviated superiorly to the attached location of Müller's muscle. **B**, The levator aponeurosis (arrow) was identified and pulled down by forceps, then the orbital fat (arrowheads) above the levator muscle was exposed. **C, D**, The detached levator aponeurosis was sutured onto the tarsal plate (arrow) without advancing Müller's muscle or tucking the levator muscle.

REDUCED CHOROIDAL BLOOD FLOW CAN INDUCE VISUAL FIELD DEFECT IN OPEN ANGLE GLAUCOMA PATIENTS WITHOUT INTRAOCULAR PRESSURE ELEVATION FOLLOWING ENCIRCLING SCLERAL BUCKLING

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Purpose: To determine the cause of the progressive glaucomatous visual field defects in three patients after an encircling scleral buckle for a rhegmatogenous retinal detachment (RRD).

Methods: Scanning laser Doppler flowmetry and visual field tests were performed on three cases with unilateral progressive visual field defect after an encircling scleral buckling for a RRD. Similar measurements were made after the buckle was removed.

Results: After implanting the scleral buckle, the intraocular pressure was normal and chamber angle was open. The blood flow in the neuroretinal rim of the optic disk was lower than that in the healthy fellow eye. After removing the buckle, the blood flow improved to normal levels and a further worsening of the visual field was not detected.

Conclusions: These results suggest that an encircling scleral buckle may impair choroidal circulation and lead to visual field defects similar to eyes with normal tension glaucoma.

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The development of glaucoma is well-known to be a complication of scleral buckling.[1] In general, angle-closure glaucoma results from the anterior displacement of the lens-iris diaphragm by expansile gases[2,3] or by a movement of the ciliary body following the scleral buckling.[4,5] An impairment of blood flow by the buckle has also been suggested to have a role for unknown visual field defect.[6]

We have followed three cases who developed the characteristics of open angle glaucoma following an encircling scleral buckle. In these three cases, the intraocular pressure (IOP) and chamber angle were normal, but the glaucomatous visual field defects and disk cupping worsened. We hypothesized that the mechanism causing the glaucomatous changes was a reduction of tissue blood flow in the optic nerve head similar to that in patients with normal tension glaucoma (NTG), especially the type with significantly low intraocular pressure.

To test this hypothesis, the ocular blood flow at the neuroretinal rim of the optic disk was measured by scanning laser Doppler flowmetry in eyes with an encircling scleral buckle. The blood flow was significantly lower than in the same region of the unaffected fellow eye. After removal of the buckle, the blood

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flow improved and no further progression of the visual field defect was observed.

Case Reports

Case 1

In May 1993, a 61-year-old man underwent scleral buckling surgery for a superior peripheral rhegmatogenous retinal detachment (RRD) in his left eye (Figure 1A) at the Keio University Hospital. Preoperatively, his left optic disk appeared glaucomatous, and the cup to disk ratio (C/D ratio) was 0.6. Following sclerotomy for subretinal fluid drainage, an encircling buckle with a silicone band (#240, MIRA) combined with a silicone tire (#279, MIRA) was placed on the eye. The tire extended for approximately 150°, and a silicone sleeve (#270, MIRA) was placed as an exoplant. Then, 0.5 cc of pure sulfur hexafluoride gas was injected intravitreally as a tamponade. No intraoperative complications were encountered.

The retina was successfully reattached but 6 months later, Goldmann perimetry revealed a temporal-superior visual field defect in the left eye and normal visual field in the right eye. The C/D ratio was 0.5 in the right eye and 0.8 in the left eye. The best-corrected visual acuities were 20/20 in the right eye and 20/40 in the left, and the IOP ranged between 10 and 16 mm Hg bilaterally throughout the pre- and postoperative period. Gonioscopy revealed neither a narrowed angle nor peripheral anterior synechia in both eyes.

A topical beta blocking agent (carteorol) was prescribed as in eyes with NTG, but the glaucomatous visual field defect progressed during the following 6 years (Figure 1B). The IOP remained between 10 and 16 mm Hg throughout the follow-up period. Ophthalmoscopy showed no recurrence of the RRD, but the glaucomatous cupping worsened. No obstruction of the branch retinal vessels was detected in the area corresponding to the visual field defect.

Tissue blood flow was measured at the superior and inferior disk rim areas and superior and inferior macular areas in both eyes with

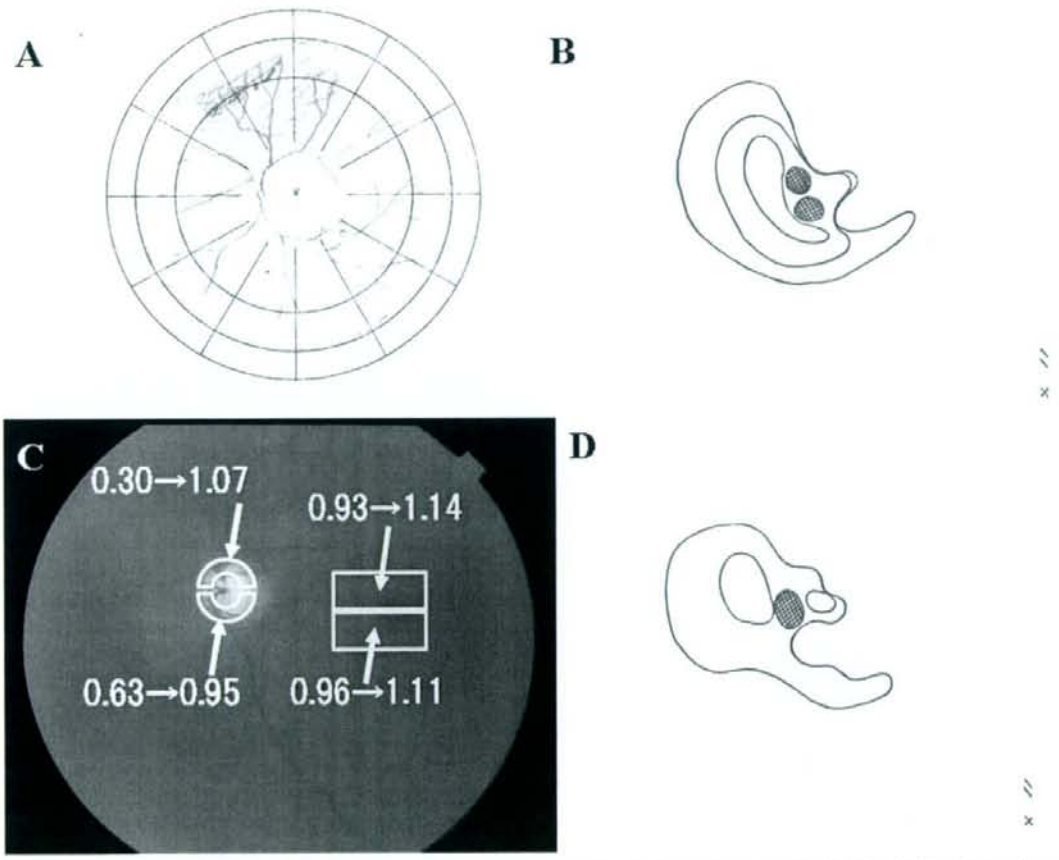

Fig. 1. Clinical findings in Case 1. **A,** Fundus schema of the left eye. A superior retinal detachment associated with retinal breaks can be seen at the edge of the lattice retinal degeneration. **B,** Goldmann visual field 6 years after the encircling buckle showing developed visual field defect which cannot be explained by previously detached retina. **C,** Blood flow (affected eye/fellow eye: a/f) ratios before buckle removal and 4 months after. Measurements before buckle removal show a reduction of blood flow in the disk rim area. The mean ratio of each area shows an improvement of blood flow after buckle removal. **D,** Goldmann visual field 4 years after removal of the encircling buckle. The visual field defect has progressed only slightly.

Table 1. Mean Blood Flow (MBF) Values at Each Measurement Area

	Neuroretinal Rim Area						Macular Area					
	Superior			Inferior			Superior			Inferior		
Case	a	f	a/f	a	f	a/f	a	f	a/f	a	f	a/f
1												
Preop	63.5	210.9	0.30	124.5	197.0	0.63	191.6	205.4	0.93	184.0	192.4	0.96
Postop	331.2	308.2	1.07	270.1	284.8	0.95	276.3	243.2	1 14	300.3	269.7	1 11
2												
Preop	268.0	386.5	0.69	244.7	364.9	0.67	183.8	249.2	0.74	204.6	257 1	0.80
Postop	396.4	385.1	1.03	259.8	290.4	0.89	243.9	269.9	0.90	233.3	255.9	0.91
3												
Preop	123.9	213.4	0.58	195.6	290.1	0.67	206.6	225.6	0.92	218.2	177.4	1.23
Postop	160.9	196.5	0.82	249.6	300.7	0.83	215.7	227.7	0.95	242.8	245.3	0.99

The values were calculated as an average of three measurements of MBF and are expressed in arbitrary units.
a = affected eye; f = fellow eye; a/f = a ratio of MBF in affected eye to that of fellow eye; op = operation of removing encircling buckle.

the Heidelberg Retina Flowmeter[7] (HRF, Heidelberg Engineering GmbH, Heidelberg, Germany). The mean blood flow (MBF) in each area was obtained using the automatic full-field perfusion image analyzer[8] (version 3.3, Heidelberg Engineering GmbH), which enables the manual control for the width of the analyzing window. And for the measurement of the MBF in the optic disk rim, the width was properly fitted to the width of optic disk rim in each measurement.

In each subject, at least three good quality images, suitable for evaluation of blood flow, were selected and analyzed. Coefficients of variation (CV = SD/mean × 100%) were calculated to evaluate the reproducibility of the MBF measurements.

The ratio of the MBF of the affected eye to the fellow eye (a/f ratio) was calculated for each area to minimize interexamination variations (Table 1).[9] The a/f ratios at superior and inferior disk rim areas were 0.30 and 0.63, respectively, and the ratios at the superior and inferior macular areas were 0.93 and 0.96, respectively. These findings indicated a decrease of blood flow at the disk rim especially in the superior area (Figure 1C). These findings indicated a decrease of blood flow especially at the disk rim, suggesting that defects may have resulted from the compromised choroidal circulation.

In August 2000, the scleral buckle was removed with the intention of improving ocular circulation. Four months later, the a/f ratios were 1.07 and 0.95 at superior and inferior disk rim areas, and 1 14 and 1 11 at superior and inferior macular areas, respectively. These findings indicated an improvement of blood flow (Figure 1C), and a worsening of the visual field defect was not observed even at the last visit 5 years after the removal of the buckle and without medication (Figure 1D).

Case 2

In October 1998, a 47-year-old man was diagnosed with a superior peripheral RRD in the left eye (Figure 2A). An encircling scleral buckle with a silicone tire (#240, MIRA) and a silicone tire (#277, MIRA) was inserted. The band extended for 120°, and a silicone sleeve (#270, MIRA) was placed as an exoplant. No intravitreal gas injection was used. No signs of angle closure, IOP elevation, or

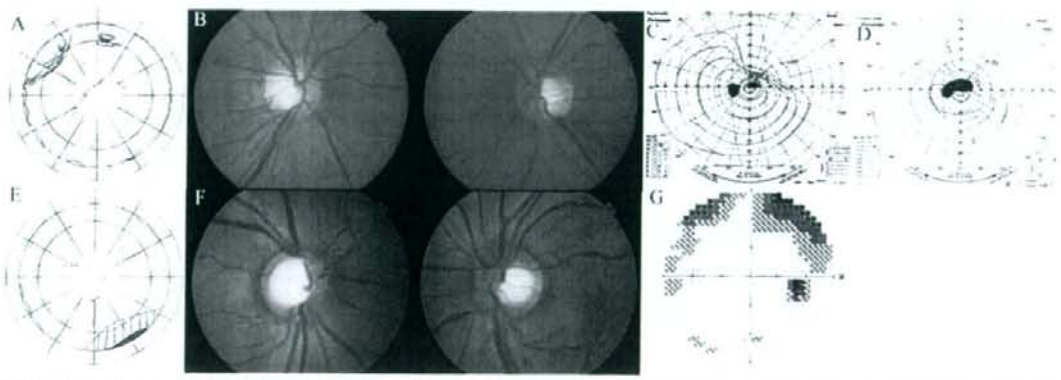

Fig. 2. Clinical findings in Cases 2 (upper) and Case 3 (lower). **A,** Fundus schema of the left eye in Case 2. **B,** Optic disk of both eyes (left side: right eye, right side: left eye). **C,** Goldmann visual field 6 months after encircling buckling showing superior visual field defect which cannot be explained by previously detached area. **D,** After removal of the encircling buckle, visual field defect has progressed only slightly during the 2-year period. **E,** Fundus schema of the right eye in Case 3. **F,** Optic disk of both eyes (left side: right eye, right side: left eye). **G,** Humphrey visual field 32 months after encircling buckle showing newly developed superior visual field defect that is not associated with previously detached area.

Table 2. The a/f Ratios in Each Case

	Disc Rim		Macular Area	
	Before*	After*	Before	After
Case 1				
Superior area	0.3	1.07	0.93	1.14
Inferior area	0.63	0.95	0.96	1.11
Case 2				
Superior area	0.69	1.03	0.74	0.9
Inferior area	0.67	0.89	0.8	0.91
Case 3				
Superior area	0.58	0.9	0.92	1.03
Inferior area	0.67	0.91	1.23	0.89

*P = 0.0003, Bonferroni procedure.
Before = before removal of buckle; after = after removal of buckle.

vascular obstruction was observed, and the C/D ratio was 0.6 (Figure 2B). The C/D ratio is larger in the left eye than the right eye. However, 6 months later, Goldmann perimetry revealed a superior visual field defect in the left eye. His visual acuity was 20/20 bilaterally.

After 3 years, a relative paracentral scotoma developed (Figure 2C), and the a/f ratios at the superior and inferior disk rim areas were 0.69 and 0.67, respectively, and the ratios at superior and inferior macular areas were 0.74 and 0.80, respectively. In October 2002, the encircling buckle was removed, and 6 months later, the a/f ratios were 1.03 and 0.89 at superior and inferior disk rim areas, and 0.90 and 0.91 at superior and inferior macular areas, respectively. The visual field defect had not changed at the last visit in January 2005(Figure 2D). The IOP in the left eye did not change after the buckle was removed and ranged between 10 and 14 mm Hg throughout the follow-up period. The a/f ratios of flow values at superior and inferior optic disk rim were 1.03 and 1.03 in the second measurement, and 1.06 and 1.21 in the third measurement, respectively. We found that the blood flow change persisted after buckle removal.

Case 3

In November 1999, a 13-year-old boy underwent a successful encircling scleral buckle procedure with a silicone band (#240, MIRA) in combination with a silicone tire (#220, MIRA). The tire extended for 150°, and a silicone sleeve (#270, MIRA) was placed for a peripheral nasal-inferior RRD in the right eye (Figure 2E). No intravitreal gas was given. Despite the normal anterior chamber angle and normal IOP (11 to 17 mm Hg), a unilateral visual field defect developed on superior Bjerrum area together with glaucomatous cupping with a C/D ratio of 0.8 in the right eye (Figure 2F). Visual acuity was 25/20 bilaterally. The a/f ratios at superior and inferior disk rim areas were 0.58 and 0.67, respectively, while the ratios at superior and inferior macular areas were 0.92 and 1.23, respectively.

In December 2002, the visual acuity decreased to 20/40 in the right eye and the encircling buckle was removed. Eight months later, the a/f ratios were 1.03 and 0.89 at superior and inferior disk rim areas, and 0.90 and 0.91 at superior and inferior macular areas, respectively. The visual field in the right eye remained unchanged until the last visit in December 2004 (Figure 2G).

Summary of the Three Cases

In these three cases, the anterior chamber angle remained opened and the IOP was not elevated, and yet, the a/f ratios were reduced in each case after the buckle was implanted. The CV of the MBF in the paramacular area ranged between 0.8% and 14.9% in the three patients. The CV of the MBF in the optic disk rim area ranged from 2.7% to 18.8%.

The a/f ratios recovered after the removal of the encircling buckle (Table 2). In each case, the MBF tended to be more reduced at the disk rim compared to that at macular area. The mean a/f ratio at optic disk rim in three cases after buckle removal was 0.932 ± 0.104 and significantly higher (P = 0.0003, Bonferroni procedure) than that before buckle removal (0.590 ± 0.147). The pre- and postoperative mean a/f ratio at the paramacular area was 0.930 ± 0.170 and 1.000 ± 0.170, respectively. It was not significantly increased after surgery (P = 0.376, Bonferroni procedure).

Discussion

Our three cases developed the characteristics of normal-tension glaucoma following the buckling procedure. The IOPs were not elevated after the buckling, and we suggest that the mechanism for the glaucomatous changes did not depend on the IOP but on the altered blood flow. It has been reported that choroidal and retinal blood flow are significantly reduced in rabbits following encircling buckling.[10] Additional clinical evidence has shown the adverse effects of an encircling buckle on the circulation of retina,[11–13] choroid,[14,15] and optic disk.[6] Thus, a compressive mechanism was considered to be the causative factor for the reduced choroidal blood flow following scleral buckling.

In good accordance with those reports, our cases demonstrated reduced tissue blood flow at disk rim area that improved after removal of the buckle while the tissue blood flow at macular area was normal.

Eshita et al investigated tissue blood flow in the paramacular region before and after buckling surgery including 17 local buckling and 11 encircling for RRD in 28 patients. They found that the blood flow was significantly reduced compared with healthy fellow eye but increased to the normal level 1 month after buckling surgery irrespective of the extent of silicone buckle sutured.[13] Therefore there is subclinically transient disturbance in the microcirculation in the eye with RRD, which normally subsides 1 month after surgery. But in the current cases the microcirculation impairment lasted longer and plausibly induced glaucomatous visual field change. The blood flow to the inner retinal layers and nerve fibers of the optic disk is supplied by the central retinal artery and its branches, while the blood flow to the prelaminar region adjacent to nerve fiber layer is supplied by branches of the juxtapapillary choroidal vessels.[16] The blood flow values in the disk rim area obtained by HRF are considered to be a mixture of retinal and choroidal blood flow because the penetration of the laser beam is 400 μm,[17] and the depth of the HRF scan is to the level of the nerve fiber layer and prelaminar region. Thus, the

findings in our cases indicate that an encircling buckle disturbs the choroidal circulation predominantly while leaving retinal circulation relatively intact.

The question then arises on the effect of a segmental buckle. It can be expected that the adverse effect on the ocular circulation, especially on the choroidal circulation, is less with segmental buckle than an encircling buckle. This is supported by the report of Nagahara et al, who reported that segmental scleral buckling with encircling elements decreased the tissue blood flow velocity in the choroid and retina on the buckled side and caused no significant change on tissue circulation in other areas of the fundus or optic nerve head.[14] Investigations of ocular circulatory changes in eyes with segmental buckle in comparison to encircling buckles must be performed.

Because only three cases were studied, our findings should be interpreted carefully and cannot be used to justify treating patients by either encircling buckling or vitrectomy. However, the information obtained from this study is useful in that it brings to light the possible existence of secondary normal tension glaucoma after scleral buckling surgery due to choroidal circulation disturbance. Clinicians should be aware that in cases with unknown visual field deficit after an encircling buckle, an impairment of the choroidal circulation might be responsible even if the IOP is normal, though these data we showed are preliminary, and further studies are needed.

Key words: choroidal blood flow, encircling scleral buckling, glaucoma, neuroretinal rim, rhegmatogenous retinal detachment.

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EARLY EFFECTS OF INTRAVITREAL TRIAMCINOLONE ACETONIDE ON INFLAMMATION AND PROLIFERATION IN HUMAN CHOROIDAL NEOVASCULARIZATION

Running Title: Effects of Triamcinolone on Inflammation and Proliferation in CNV

Key words: Triamcinolone Acetonide, Choroidal Neovascularization, Verteporfin Photodynamic Therapy, Inflammation, Proliferation

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ABSTRACT

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

Methods: Retrospective review of interventional case series of 29 patients who underwent macular translocation. Fourteen CNV without previous therapy (control CNV) and 4 CNV excised 3 days after photodynamic therapy (PDT CNV) composed control groups. Eleven patients were treated with intravitreal TA (TA CNV, n=5) or PDT+TA therapy (PDT+TA CNV, n=6) 3 to 9 days preoperatively. CNV were stained for cytokeratin18, CD34, VEGF, intercellular adhesion molecule-1 (ICAM-1), E-selectin, **CD68, CD45**, Ki-67 and thy-1.

Results: TA and PDT+TA CNV disclosed **increased immunostaining** of ICAM-1 in endothelial cells and stroma and higher percentage of thy-1 expression **than in controls**. Density of macrophages was significantly increased in PDT+TA CNV. Leukocyte density and proliferative activity were lower in TA and PDT+TA CNV. Total VEGF score was significantly increased in TA and PDT+TA CNV than control CNV. VEGF in RPE of PDT+TA CNV was stronger than control CNV.

Conclusions: TA has no inhibitory effect on macrophage infiltration, ICAM-1, thy-1 and VEGF expression in CNV in early term. Clinical benefits of TA are probably not based on pure anti-inflammatory or VEGF suppressing mechanisms.

INTRODUCTION

Neovascular age-related macular degeneration (AMD) is the leading cause of visual disability in elderly population in industrialized countries.[1] Ocular verteporfin photodynamic therapy (PDT) is an established treatment modality.[2] However, high recurrence rate compromises its success.[2] Pilot studies, therefore, combine PDT with adjuvants to overcome these limitations. Intravitreal triamcinolone acetonide (TA) either alone or in combination with PDT appears to be beneficial in neovascular AMD treatment, especially in reducing PDT re-treatment rate.[3,4]

TA mediates anti-angiogenic, anti-inflammatory and anti-permeability effects.[5-21] Vascular endothelial growth factor (VEGF) is a major stimulator of choroidal neovascularization (CNV).[22] Anti-angiogenic action of TA was supposed to be due to decreased VEGF expression either directly[16-21] or indirectly through its anti-inflammatory effects.[8,10,13-15]

However, VEGF expression was not decreased in human CNV excised after TA monotherapy or PDT+TA combination therapy.[23] Since VEGF expression in CNV was found to be correlated with inflammatory cell infiltration, this study was undertaken to evaluate early effects of intravitreal TA either as monotherapy or as adjuvant to PDT on inflammation and proliferation in human CNV membranes. CD34, cytokeratin 18 and Ki-67 were used to identify endothelial cells (EC), retina pigment epithelium (RPE) and proliferating cells, respectively. Inflammation was evaluated by expression of cellular adhesion molecules, namely intercellular adhesion molecule 1 (ICAM-1) and E-selectin, density of CD45 and CD68 immunoreactive inflammatory cells, and expression of thy-1. **CD45 was shown to be expressed more on blood born infiltrating leukocytes and monocytes rather**