Consequences of Verteporfin Photodynamic Therapy on Choroidal Neovascular Membranes

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Objective: To examine the impact of photodynamic therapy (PDT) on angiogenesis in human choroidal neovascular membranes with respect to vascular endothelial growth factor (VEGF) expression, proliferation, and vascularization.

Methods: Retrospective review of an interventional case series of 50 patients (50 eyes) who underwent removal of choroidal neovascular membranes. Choroidal neovascularization was secondary to age-related macular degeneration. Twenty patients were treated with PDT 3 to 655 days before surgery. Choroidal neovascular membranes were stained for CD34, CD105, Ki-67, cytokeratin 18, and VEGF. Thirty choroidal neovascular membranes secondary to age-related macular degeneration without previous treatment were used as controls.

Results: Specimens without pretreatment disclosed varying degrees of vascularization, proliferative activity, and VEGF expression by different cells. Specimens treated with PDT 3 days earlier showed mostly occluded vessels, damaged endothelial cells, and low proliferative activity. In contrast, specimens excised at later time points after PDT were highly vascularized and proliferating. This chronology was associated with an impressive VEGF immunoreactivity unique to retinal pigment epithelial cells shortly after PDT that also shifted to other cells at later time points.

Conclusions: Photodynamic therapy induces selective vascular damage in choroidal neovascular membranes. The effectiveness and selectivity of this treatment, however, seem to be jeopardized by a rebound effect initiated by enhanced VEGF expression in retinal pigment epithelial cells.

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Age-related macular degeneration (AMD) is the leading cause of legal blindness in patients older than 60 years in the Western world. The exudative form of the disease is characterized by the development of choroidal neovascularization (CNV) in the macular area that leads to irreversible damage to the neurosensory retina and severe loss of visual acuity. Numerous treatment modes have been attempted to destroy the pathological blood vessels using thermal photocoagulation, ionizing radiation, or photosensitizing dyes or to surgically remove the neovascular tissue with or without replacement of the damaged retinal pigment epithelium (RPE) or translocation of the fovea. Although some modalities are still experimental, large randomized clinical trials have shown the value of laser photocoagulation and photodynamic therapy (PDT). The major handicap of laser photocoagulation, however, is the inevitable damage to the neurosensory retina that is associated with a sudden decrease in visual acuity. This problem was overcome by the introduction of PDT.

Photodynamic therapy is a nonthermal process based on the targeted photoactivation of an intravenously administered photosensitive drug. The activated dye results in the creation of oxygen intermediates and free radicals affecting the exposed endothelial cells. Photodynamic therapy seems to be an ideal treatment approach for CNV, allowing selective photothermolysis of CNV without damage to the overlying neurosensory retina.

After randomized clinical trials demonstrated that PDT with verteporfin (Visudyne; Novartis AG, Buelach, Switzerland) is an effective treatment for subfoveal CNV secondary to AMD, it was accepted as a routine procedure under certain circumstances. The potential and success of verteporfin PDT, however, are considerably compromised by a recurrence rate of about 90% within 3 months and a mean visual acuity loss of 2 Early Treatment Diabetic Retinopathy Study lines within 6 months.
Although many preclinical studies have demonstrated that PDT with verteporfin induces vascular occlusion both angiographically and histologically, information about the effect of this treatment on pathological tissues is quite poor and the understanding of the clinical results and the high rate of recurrences is still limited.

We describe our results of a clinical and immunohistological study of surgically extracted CNV membranes due to AMD following verteporfin PDT. This analysis focuses on the angiogenesis, vascularization, and proliferative activity within the specimens extracted after different intervals and numbers of PDT treatments. The chronology and quality of the observed changes suggest the need for a critical reassessment of the actual treatment modalities and the implementation of adjuvant therapies.

METHODS

SUBJECTS AND TREATMENTS

We retrospectively reviewed 50 eyes of 50 consecutive patients with AMD for whom surgery for CNV was performed. In 20 of these patients, surgery was performed after verteporfin PDT (Table). In addition to the complete ophthalmological examination, in patients receiving verteporfin PDT, stereoscopic fluorescein angiography was performed before the treatment and thereafter on the day of surgery. Choroidal neovascular membranes were classified according to the guidelines of the Treatment of Age-Related Macular Degeneration With Photodynamic Therapy and Verteporfin in Photodynamic Therapy studies. Subjects and treatments including observation, conventional thermal laser photocoagulation, PDT retreatment, macular translocation with 360° retinotomy, and CNV membrane extraction were discussed with the patients. Surgical intervention was offered when visual acuity was below 20/200 (as that was the minimum visual acuity to recommend the first PDT session according to the Treatment of Age-Related Macular Degeneration With Photodynamic Therapy study and when visual deterioration progressed after initial PDT. Clinical characteristics of the patients treated with verteporfin PDT are summarized in the Table.

Four eyes underwent CNV extraction 3 days after PDT. Three of these 4 eyes had subfoveal classic CNV. The visual acuity of these 3 eyes was between 4/200 and 5/200, less than the 20/200 that was the lowest permissible visual acuity for PDT in the Treatment of Age-Related Macular Degeneration With Photodynamic Therapy study. The fourth patient with predominantly classic CNV experienced a decrease in visual acuity from 60/200 to 10/160 accompanied by a leakage in fundus fluorescein angiography 3 months after the first PDT session. He opted to proceed with macular surgery rather than PDT retreatment. Photodynamic therapy 3 days prior to surgery was intended to reduce the risk of bleeding at the time of surgical extraction. Each patient gave written informed consent after the experimental nature of the treatment procedure and the risks and benefits of all of the therapeutic options were discussed in detail. The study followed the guidelines of the Declaration of Helsinki as revised in Tokyo, Japan, and Venice, Italy, and adhered to requirements of the local institutional review board. The histological analysis of the specimens was approved by the institutional ethics committee.

TISSUE PREPARATION AND IMMUNOHISTOLOGICAL ANALYSIS

Within minutes after surgery, excised CNV membranes were fixed in 3.7% formalin and embedded in paraffin. After serial sections were deparaffinized and rehydrated, antigen retrieval was accomplished by proteolytic digestion with 0.5% pronase (Sigma-Aldrich, St Louis, Mo) for cytoketatin 18 and with proteinase K (Dako, Glostrup, Denmark) for vascular endothelial...
growth factor (VEGF) and by heat treatment in citrate buffer in a pressure cooker for Ki-67, CD34, and CD105.

Immunohistochemical staining was performed using the horseradish peroxidase method for all of the antigens excluding VEGF according to the manufacturer’s protocol ( Vectastain Universal Elite ABC PK-6200 kit; Vector Laboratories, Burlingame, Calif). To block endogenous peroxidase activity, 3% hydrogen peroxide and 0.1% sodium azide were applied. After preincubation with horse serum, sections were probed with the primary antibodies specific for human CD105 (mouse monoclonal antibody, clone SN6h; Dako), CD34 (mouse monoclonal antibody; Immunotech, Hamburg, Germany), Ki-67 (mouse monoclonal antibody, clone Ki-55; Dako), and cytokeratin 18 (mouse monoclonal antibody; Progen, Heidelberg, Germany). Incubation with the biotinylated horse-antimouse secondary antibody and the ABC complex (Vectastain Universal Elite ABC PK-6200 kit) was followed by development with a 3-diaminobenzidine (Fluka, Buchs, Germany) solution combined with hydrogen peroxide. For cytokeratin 18 staining, the chromogen was replaced with a 3-amino-9-ethylcarbazole highly sensitive substrate chromogen (Dako). Immunohistochemical staining for VEGF was performed with the alkaline phosphatase method according to the manufacturer’s instructions (Alkaline Phosphatase/RED Rabbit/Mouse ChemMate Detection Kit, K5005; Dako). A monoclonal mouse-antihuman VEGF antibody (clone C-1; Santa Cruz Biotechnology, Santa Cruz, Calif), non–cross-reactive with VEGF-C, VEGF-D, or placental growth factor but specific for VEGF-A, was used. The biotinylated goat-antimouse secondary antibody was followed by streptavidin conjugated to alkaline phosphatase and chromogen red. Levamisole was applied to inhibit endogenous alkaline phosphatase activity. Hematoxylin III according to Gill (Merck, Darmstadt, Germany) was used as a counterstain. For negative controls, the primary antibodies were substituted with appropriate normal sera or were omitted.

ANALYSIS

Two serial sections from each specimen were analyzed by light microscopy 3 times independently by 2 masked observers (O.T. and S.G.).

Vascularization was calculated by counting the number of CD34- and CD105-positive vascular-like patterns in the most vascularized area under 200× magnification.

All of the Ki-67–positive nuclei in RPE, endothelial, and stromal cells were counted separately in each specimen. The percentages of Ki-67–expressing RPE, endothelial, and stromal cells with regard to the total number of proliferating cells in the treated and nontreated subgroups of membranes were determined. Proliferative activity (nuclei per square millimeter) of a membrane was defined as the number of Ki-67–expressing nuclei in a 1-mm² area of a specimen. Proliferative activity in each specimen was determined quantitatively by calculating the ratio of the total number of Ki-67–positive nuclei in CNV to the area of the membrane (in square millimeters).

Immunoreactivity for VEGF was analyzed separately in RPE, endothelial, and stromal cells. A grading scheme indicating the degree was used as follows: grades of 3, 2, 1, and 0 were assigned to indicate intense VEGF labeling (70%-100% positive cells), moderate VEGF labeling (40%-69% positive cells), weak VEGF labeling (1%-39% positive cells), and absence of any staining, respectively.

Based on previous studies, 9,10 the CNV membranes were classified semiquantitatively as inflammatory active when inflammatory cells were more dominant than fibrosis or as inflammatory inactive when fibrosis was dominant with minor or absent inflammatory response.

The proliferative activity and intensity of VEGF immunostaining between groups were analyzed with a Mann-Whitney U test. P≤.05 was considered statistically significant.

ANGIOGRAPHIC CLASSIFICATION AND CHARACTERIZATION

The angiographic features classified according to the Treatment of Age-Related Macular Degeneration With Photodynamic Therapy and Verteporfin in Photodynamic Therapy study reports differed depending on the post-PDT interval (Table). In all of the 4 membranes extracted 3 days after PDT, a hypofluorescence suggesting nonperfusion of the irradiated area and the CNV was seen in early phases of angiography on the day of surgery (Figure 1A). Late phases of fluorescein angiography revealed hyperfluorescence and leakage at the fovea consistent with choroidal ischemia (Figure 1B). In CNV membranes extracted at longer post-PDT intervals, fluorescein angiography on the day of surgery disclosed a membrane (Figure 1C) with leakage in late phases (Figure 1D).

HISTOLOGICAL CHARACTERIZATION WITH CD34, CD105, AND KI-67 LABELING

All but 1 membrane in each group of treated and untreated cases were vascularized as evidenced by CD34-positive vessels (Figure 2A). In CNV membranes devoid of PDT, all of the vessels stained positively for CD34 (Figure 3A) but stained only partially for CD105 (Figure 3A and B). In membranes extracted 3 days after PDT, immunohistological analysis with CD34 and CD105 showed not only many collapsed vessels (mean percentage of occluded vessels, 81.1%) but also patent ones (Figure 3D). The endothelial cells lining the patent vessels appeared damaged. In contrast, vessels in CNV membranes extracted at longer post-PDT intervals were all patent with endothelial cells displaying prominent nuclei. The specimens were hypercellular and highly vascularized. The vessels strongly expressed CD105 (Figure 3E), reflecting very vital and active endothelial cells.

In nontreated CNV membranes, a differing number of Ki-67–positive proliferating cells could be detected (Figure 2A). The Ki-67–positive cells (n=544) were rarely endothelial cells (n=31 [5.7%]) or RPE cells (n=15 [2.8%]) but most often appeared to be stromal cells (n=498 [91.5%]) (Figure 3C), especially in the inflammatory infiltration. In fact, specimens classified as inflammatory active (n=20) had significantly higher proliferative activity (median proliferative activity, 59.85 nuclei/mm²; range, 0-113.69 nuclei/mm²) than inflammatory-inactive specimens (n=10; median proliferative activity, 6.81 nuclei/mm²; range, 0-514.08 nuclei/mm²) (P= .001). In specimens treated with PDT, Ki-67–positive nuclei (n=1222) were rarely detected in RPE cells (n=74 [6.1%]) or endothelial cells (n=51 [4.2%]) but were mostly detected in stromal cells (n=1097 [89.8%]). However, in membranes extracted 3 days after PDT, Ki-67–expressing cells (n=37) were completely absent.
in 2 cases and relatively abundant (n=36) only in 1 specimen with the lowest percentage of occluded vessels (Figure 2A). The proliferative activity in specimens extracted 3 days after PDT (median proliferative activity, 4.85 nuclei/mm²; range, 0-9.71 nuclei/mm²) was smaller than that in the CNV membranes without prior PDT (median proliferative activity, 53.20 nuclei/mm²; range, 0-514.08 nuclei/mm²), but this difference did not reach statistical significance (P=.13). However, at longer intervals following PDT, proliferative activity increased significantly (median proliferative activity, 78.28 nuclei/mm²; range, 0-829.29 nuclei/mm²) (P=.04) (Figure 2A and Figure 3F).

**EXPRESSION OF VEGF**

In CNV membranes not treated with PDT, VEGF staining was absent in the RPE cells of 18 (60.0%) of 30 specimens (Figure 2B). In the remaining 12 (40.0%) of 30 specimens, VEGF staining was mostly weak to moderate (Figure 4A and B). A moderate amount of VEGF expression was found in 16 (53.3%) of 30 vascular endothelial cells. Only 4 (13.3%) of 30 specimens, however, displayed intense staining (Figure 2B). The VEGF staining within the stroma appeared in both fibroblast-like and inflammatory cells (Figure 2B and Figure 4A and B).

In all of the membranes extracted 3 days after PDT (n=4), cytokeratin 18–positive RPE cells (Figure 4C) showed an intense staining for VEGF (Figure 2B and Figure 4C and D). At longer posttreatment intervals, the VEGF staining in RPE cells persisted to different degrees (Figure 2B and Figure 4F), being absent in only 2 cases. Three days after PDT, only 1 membrane had endothelial cells with a moderate expression of VEGF. The other 3 specimens were either negative for staining (n=1) or were just weakly stained (n=2). At greater intervals (n=16), the VEGF staining in endothelial cells appeared to increase, with only 1 case of endothelial cells without VEGF staining, and 10 (60.7%) of 15 specimens had moderate to intense staining at the vessels (Figure 2B and Figure 4E and F). The VEGF staining was significantly increased in RPE cells (P<.001), endothelial cells (P=.008), and stromal cells (P=.02) after PDT (Figure 2B and Figure 4E and F). The VEGF staining in-
tensity showed no predilection according to the localization in the CNV.

Lately, PDT has gained an important role in the treatment of neovascular AMD. The potential and benefit of this therapy, however, are compromised by high recurrence rates and a reduced functional prognosis. To promote this treatment concept and to reduce its limitations, knowledge of the biological effects of PDT in CNV is crucial.

Choroidal neovascularization is most likely the result of neoangiogenesis, and VEGF is thought to play a pivotal role within this process.\(^9\)\(^6\)\(^7\) Vascular endothelial growth factor has been shown to be required for normal vascular development,\(^16\) survival and morphology of choriocapillaris,\(^17\) and retinal neovascularization\(^18\) in experimental animal models. It plays a key role in the pathogenesis of ischemia-associated retinal neovascularization\(^19\) and is a major stimulator of CNV development and growth.\(^14\) Vascular endothelial growth factor is present both in surgically excised CNV membranes from human eyes with AMD\(^9\)\(^12\) and in experimental CNV membranes.\(^13\) It can induce CNV

Figure 2. The distribution of endothelial cells (EC) (with vascularization calculated by counting the number of CD34- and CD105-positive vascular-like patterns in the most vascularized area under 200× magnification) and proliferation marker (with proliferative activity evaluated by counting the absolute number of Ki-67–positive nuclei within the specimen) (A) and of vascular endothelial growth factor (with vascular endothelial growth factor immunostaining in retinal pigment epithelial [RPE] cells, EC, and stromal cells evaluated separately and semiquantitatively) (B) in choroidal neovascularization without pretreatment, 3 days after photodynamic therapy (PDT), and 1 or more months after PDT.
in animal models, and CNV has been suppressed by anti-VEGF therapy in primates.

To understand the changes that might be related to PDT, we first examined CNV membranes that did not receive PDT before surgery. In our study, VEGF expression by RPE cells could be detected in fewer than 50% of the cases, with intense expression in only 13% of the membranes. In contrast, expression by stromal cells was

Figure 3. Photomicrographs of a surgically excised choroidal neovascular membrane. The specimen of a case that did not receive photodynamic therapy was probed with antibodies against CD34 (A) and CD105 (B), stained with 3-diaminobenzidine (resulting in a brown chromogen), and counterstained with hematoxylin. The endothelial cell markers CD34 and CD105 are selectively expressed in vascular structures (arrows). Some endothelial cells do not stain for CD105 (arrowhead). The brown chromogen can be distinguished from the melanin granula (asterisk) contained in pigmented cells. C, In the same case that did not receive photodynamic therapy, several cell nuclei express the proliferation marker Ki-67 (arrows). The brown chromogen can be distinguished from the melanin granula (asterisk) contained in pigmented cells. D, The choroidal neovascular membrane from case 2 extracted 3 days after photodynamic therapy was probed with CD34. Some of the vessels shown by the brown chromogen are still patent (arrow) whereas others appear collapsed (arrowhead). The brown chromogen can be distinguished from the melanin granula (asterisk) contained in pigmented cells. A choroidal neovascular membrane (case 7) extracted 40 days after photodynamic therapy shows the brown chromogen from the stain by CD105 with patent and vital looking vessels (E) and by Ki-67 with several proliferating cells (brown nuclei) (F).
found in 90% of the cases. These results confirm the findings by Kvanta et al.\textsuperscript{10} who detected VEGF staining and VEGF messenger RNA expression particularly in fibroblast-like cells but only occasionally in RPE cells of human CNV membranes. Lopez et al.\textsuperscript{12} described VEGF production by RPE cells, endothelial cells, fibroblasts, macrophages, and monocytes in CNV membranes. In our membranes, VEGF staining in endothelial, RPE, and stromal cells can be detected within inflammatory and stromal cells whereas RPE cells are negative for staining. A choroidal neovascular membrane from case 2 was extracted 3 days after photodynamic therapy and stained (red chromogen) for cytokeratin 18 (C) and VEGF (where the RPE cells [arrows] were strongly positive for VEGF as compared with the choroidal neovascular membranes that were not treated with photodynamic therapy) (D). Specimens from cases 7 (E) and 11 (F) were stained for VEGF (red chromogen), and VEGF was expressed by endothelial cells (arrows), stromal cells (arrowheads), and RPE cells (asterisk).

Figure 4. Photomicrographs of surgically excised choroidal neovascular membranes. A, A specimen that did not receive photodynamic therapy was stained for vascular endothelial growth factor (VEGF) (red chromogen) and showed a retinal pigment epithelial (RPE) cell layer (asterisk), vascularization (arrow), fibroblastic stromal cells, and different degrees of an inflammatory infiltration (arrowhead). The VEGF staining can be detected within inflammatory, endothelial, and stromal cells. B, Another specimen that did not receive photodynamic therapy was stained for VEGF (red chromogen) and showed an RPE cell layer (asterisk), vascularization (arrow), fibroblastic stromal cells, and different degrees of an inflammatory infiltration (arrowhead). The VEGF staining can be detected within endothelial and stromal cells whereas RPE cells are negative for staining. A choroidal neovascular membrane from case 2 was extracted 3 days after photodynamic therapy and stained (red chromogen) for cytokeratin 18 (C) and VEGF (where the RPE cells [arrows] were strongly positive for VEGF as compared with the choroidal neovascular membranes that were not treated with photodynamic therapy) (D). Specimens from cases 7 (E) and 11 (F) were stained for VEGF (red chromogen), and VEGF was expressed by endothelial cells (arrows), stromal cells (arrowheads), and RPE cells (asterisk).
mal cells at different intensities was in concordance with their findings.

In our series of 20 patients receiving presurgical PDT, 4 membranes were extracted 3 days after PDT. Fluorescein angiography on the day of surgery revealed nonperfusion of the CNV within the area of the laser treatment as demonstrated by the occlusion of most of the vessels both within the CNV and in the surrounding normal choroid. Histological analysis of PDT-treated human eyes supported these findings by identifying an occluded choriocapillaris layer within the spot produced by the laser. We detected as an early change many collapsed vessels as well as several patent vessels with presumably damaged endothelial cells. The proliferative activity within these specimens was low. Interestingly, in all of these membranes, intense VEGF staining was extremely prominent in RPE cells whereas it varied in endothelial and stromal cells. Vascular endothelial growth factor is known to be strongly induced by hypoxia in RPE cells as well as by reactive oxygen intermediates. Either aggravated hypoperfusion of the choroid and/or reactive oxygen intermediates released by the photochemical effect of PDT might be the angiogenic stimuli inducing VEGF secretion by RPE cells after PDT.

Whatever reason might be responsible, enhanced VEGF expression by RPE cells, even when temporary, is an important angiogenic stimulus that leads to increased vascular leakage and development of CNV. In fact, CNV membranes extracted at longer intervals after PDT showed patent vessels lined by healthy endothelial cells that were highly positive for CD34 and CD105. None of these 16 specimens had occluded or collapsed vessels. The proliferative activity was significantly increased and associated with a cell-rich fibrocellular stroma and an inflammatory response. The VEGF expression by RPE cells persisted in most of these cases. In contrast, the VEGF expression in endothelial and stromal cells appeared to be enhanced compared with the early post-PDT cases. The high inflammatory activity appearing in the longer post-PDT intervals may also be a factor in sustaining increased VEGF expression.

Bula et al examined VEGF expression in 6 CNV membranes extracted 3 months after PDT. Four CNV membranes without prior PDT composed the control group. Contrary to our results, no significant difference in VEGF expression was detected between the treated and untreated CNV membranes. The small number of specimens as well as the long post-PDT interval in their work might be responsible for their results.

The number of retreatments did not seem to affect the chronology of the process that is induced by the last treatment. Although it is a mild and selective modality, PDT is still a traumatizing event inducing a wound-healing cascade with the initial characteristics of angiogenesis within an inflammatory setting. In fact, although all of the membranes appear to be inflammatory inactive and nonproliferative 3 days after PDT, there seems to be a rebound activity thereafter.

When applied to normal chorioretinal structures, PDT increased VEGF expression in endothelial cells of the choriocapillaris, but not at the level of that in the RPE cells. However, in our study, PDT applied to CNV induced an early VEGF response by RPE cells.

Our results may explain the clinical observation of a decreased retreatment rate and an increased visual improvement when PDT is combined with anti-VEGF treatment. Intravitreal injections of anti-VEGF molecules should interrupt the vicious cycle induced by PDT. In a phase II study, anti-VEGF aptamer was injected intravitreally 5 to 10 days after PDT. Prominent VEGF expression in RPE cells already 3 days after PDT in our samples, however, suggests an earlier anti-VEGF intervention.

To our knowledge, this is the first clinicopathological correlation of changes regarding VEGF expression, proliferative activity, and vascularization in CNV membranes treated with PDT. The proper interpretation of this study, however, is limited by the fact that our cases may represent a negative selection. Although the histopathological findings in patients who benefit from verteporfin PDT might differ, it is conceivable that PDT causes trauma followed by enhanced VEGF expression and angiogenesis associated with an inflammatory wound-healing process. With regard to the reintiation of the angiogenic cascade, the need for a counteracting adjutant therapy started at the proper time becomes more and more obvious.


