cularization, as in wet AMD, is suggested to occur because AQ: 18 of decreased production of inhibitors and/or increased production of angiogenic stimulators.[20,21] VEGF is a major stimulator of CNV.[22] VEGF is expressed in surgically excised CNV membranes from human AMD eyes.[23-26] Immunoreactivity of VEGF is enhanced in human CNV membranes following PDT,[26] however, the impact of PDT on the expression of counteracting angiogenesis inhibitory factors in CNV is still uncovered.

AQ: 19 PEDF is one of the most potent endogenous angiogenesis inhibitory factors expressed in adult human retina and choroid.[27] However, senescent cells do not express PEDF in vitro[28]; consequently, in AMD, PEDF expression is significantly reduced in retinal pigment epithelium cells, AQ: 20 Bruch's membrane, choriocapillaris,[27] and vitreous[29] in comparison to the age-matched controls. These findings reveal the existence of a critical balance between VEGF and PEDF in physiologic conditions. Therefore, a shift in the equilibrium towards angiogenesis by the decreased PEDF expression in retinal pigment epithelium cells and laser injury sites has been suspected to be the CNV promoting factor in AMD in histopathologic[27] and experimental studies.[28,30,31] Furthermore, experimentally induced CNV membranes can be inhibited either by intravitreal injection of the PEDF vectors[31,32] or periocular gene transfer of PEDF.[33]

In our work, to understand the changes that might be related to PDT, we first examined CNV membranes that did not receive PDT before surgery. We have detected VEGF production by retinal pigment epithelium cells, EC, and stromal cells in concordance with the findings of Lopez and associates.[25] In our series, VEGF expression by retinal pigment epithelium cells was detected in nearly 35% of the cases but with an intense expression in only 15% of the membranes. In contrast, expression by stromal cells was encountered in 93% of the cases. These results confirm with the findings of Kvanta and associates[23] who also detected VEGF staining and VEGF mRNA expression par- AQ: 21 ticularly in fibroblast-like cells but only occasionally in retinal pigment epithelium of the human CNV membranes.

In our series of untreated CNV membranes (n = 27), PEDF, expressed in 92.5% of the specimens, was detected in retinal pigment epithelium cells, EC and cells within stroma in 70%, 63%, and 78% of the specimens, respectively. PEDF mRNA and PEDF expression has been detected in retinal pigment epithelium cells, fibroblasts, and macrophages in mouse CNV induced by photocoagulation.[30,34] To the best of our knowledge, PEDF was found to be expressed in retinal pigment epithelium cells, stromal cells and EC in CNV membranes secondary to AMD in a few studies[35,36] (Bula DV, ARVO Meeting, 2004, Abstract), but these series consisted of a limited number of membranes. Therefore, our series of untreated CNV secondary to AMD remains, so far, the only study evaluating PEDF expression in a relative big number of specimens so that incidence of PEDF expression separately in retinal pigment epithelium cells, EC, and stroma can be evaluated.

In our series of 15 patients receiving presurgical PDT, four membranes were extracted 3 days after PDT. FA on the day of surgery revealed nonperfusion of the CNV within the area of the laser treatment. Histology of PDT treated human eyes supported these findings by identifying an occluded choriocapillary layer within the spot produced by the laser.[37-39] Confocal ICG angiographic studies revealed that verteporfin-PDT induced an intense, early onset but long-lasting and irreversible choroidal nonperfusion in the treated area.[40,41] In CNV, we detected as an early change many collapsed vessels but only a few patent vessels, however, still with presumably damaged EC.[18] VEGF is known to be strongly induced by hypoxia in retinal pigment epithelium cells.[42,43] Therefore, Schlingemann,[44] Michels,[41] and Schmidt-Erfurth and associates[37,40] suggested that aggravated hypoperfusion of the choroid and the CNV following PDT might be the angiogenic stimulus which induces enhanced VEGF secretion by retinal pigment epithelium. In accordance with their suggestion, in all of these membranes, intense VEGF staining was extremely prominent in retinal pigment epithelium cells. Contrary to VEGF, PEDF is suppressed by hypoxia in retinal pigment epithelium cells.[45,46] Additionally, reactive oxygen intermediates (ROI) released by the photochemical effect of PDT may also induce VEGF expression.[47] Once again, contrary to VEGF, PEDF mRNA, and protein levels are markedly decreased in differentiated retinal pigment epithelium cells during oxidative stress induced by H_2O_2 treatment.[48] Therefore, either possible hypoxia and/or released reactive oxygen intermediates might be the cause underlying increased VEGF predominance score in retinal pigment epithelium 3 days after PDT.

Our series of CNV membranes extracted at longer time intervals after PDT (n = 11) disclosed patent vessels lined by healthy EC that were highly positive for CD 105 which is a marker for activated EC. VEGF is shown to upregulate PEDF expression in human retinal pigment epithelium cells in vitro.[49] However, in our series, although VEGF expression by retinal pigment epithelium cells persisted to be significantly higher than the untreated CNV group, PEDF expression remained mostly weak or negative. In EC and stromal cells, PEDF expression was significantly reduced.

Consequently, predominance score of VEGF over PEDF was significantly higher in retinal pigment epithelium and cells within stroma than in the control group. Bula and associates examined VEGF and PEDF expression in six CNV extracted 3 months after PDT (Bula DV, ARVO Meeting, 2004, Abstract). Four CNV without prior PDT composed their control group. Although they have marked a slight decrease in PEDF expression, this difference did not reach a statistical significance. They reported also no significant difference in VEGF expression between the treated and untreated CNV. The small number of speci-

mens in their work might be responsible for their controversial results.

PDT, when applied to normal chorioretinal structures, enhanced VEGF and PEDF expression in EC of the choriocapillaris, but not at the level of retinal pigment epithelium.[50] However, in our study, PDT applied to CNV induced an early VEGF response by retinal pigment epithelium and decreased PEDF response in EC and stroma.

Our data show that in CNV membranes following PDT, whatever reason might be responsible (either hypoxia or AQ: 22 oxidative stress attributable to reactive oxygen species), the balance between VEGF and PEDF is disturbed more than that in untreated CNV in favor of significant VEGF predominance to PEDF in retinal pigment epithelium and stroma. Enhanced VEGF expression by retinal pigment epithelium cells, even when temporary, is an important angiogenic stimulus which leads to increased vascular leakage and development of CNV.[51] 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.[16,17] Concerning PEDF, phase I clinical trials of intravitreal injection of adenoviral vectors containing PEDF cDNA are on the way.[52] PEDF seems to be ideal as antiangiogenic therapy agent as[1] it is a natural angiogenesis inhibitor that[2] targets only new vessel growth,[3] can be administered either as a soluble protein or by viral-mediated gene transfer,[4] is stable and nontoxic when injected, and[5] is more potent than other well-characterized angiogenesis inhibitors.[53,54] However, PEDF delivered by subcutaneous miniosmotic pumps was shown to inhibit the experimental CNV only in low doses but stimulate in high doses.[55] Therefore, further experimental and/or clinical studies are needed to determine AQ: 23 whether upregulation or replacement of PEDF improves the efficiency of PDT and the optimal dosage to be used.

The authors are unaware of previous reports of clinicopathological correlation demonstrating increased VEGF predominance over PEDF in CNV membranes treated by PDT and could find no reference to it in a computerized search utilizing Pubmed. The proper interpretation of this study, however, is limited by the fact that our cases may represent a negative selection. Though histopathologic findings in patients who profit from verteporfin-PDT might differ, it is conceivable that PDT causes a trauma followed by enhanced VEGF and decreased PEDF expression leading to angiogenesis. With regard to the disturbance of balance in growth factors in favor of angiogenesis stimulation, the need for a counteracting adjunctive therapy becomes more and more obvious.

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AQ: 24 All the authors contributed to conception, critical revision and final approval of the article. Dr Tatar, Dr Stalmans, Dr Eckardt, Dr Lüke, Dr Bartz-Schmidt, and Dr Grisanti are also the treating physicians. Analysis and interpretation of data, literature search, administrative, and technical work has been done by Tatar, Adam, Shinoda, and Grisanti. Data are statistically analyzed by Tatar and Shinoda. Article was written by Tatar and Grisanti. Tatar, Grisanti and Bartz-Schmidt have worked in obtaining funding for personnel and material costs. AQ: 25

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Expression of endostatin in human choroidal neovascular membranes secondary to age-related macular degeneration

Olcay Tatar [a], Kei Shinoda [b], Annemarie Adam [c], Jens Martin Rohrbach [a], Klaus Lucke [d], Sigrid Henke-Fahle [a], Karl Ulrich Bartz-Schmidt [a], Salvatore Grisanti [a,*]

[a] *Department of Ophthalmology I, Division of Vitreoretinal Surgery, Eberhard-Karls University Tuebingen, Schleichstrasse 12-15, 72076 Tuebingen, Germany*
[b] *Laboratory of Visual Physiology, National Institute of Sensory Organs, National Hospital Organization, Tokyo Medical Center, Japan*
[c] *Department of Pathology, Eberhard-Karls University, Tuebingen, Germany*
[d] *Eye Clinic Universitätsallee, Bremen, Germany*

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Abstract

Endostatin is an endogeneous angiogenesis inhibitor which requires E-selectin for its antiangiogenic activity. The aim of this study was to investigate the expression of endostatin in human choroidal neovascular membranes (CNV) secondary to age-related macular degeneration (AMD) with regard to vascularization and proliferative activity. An interventional case series of 36 patients who underwent removal of CNV were retrospectively investigated. Thirty-six CNV were analyzed by light microscopic immunohistochemistry for the expression of CD34 (endothelial cells, EC), CD105 (activated EC), Ki-67 (cell proliferation), Cytokeratin 18 (epithelial cells), VEGF (vascular endothelial growth factor), E-selectin and endostatin. Donor eyes ($n = 7$) including one with AMD were used as controls. Endostatin immunoreactivity was present in choroidal vessels of five as well as in the retinal pigment epithelium (RPE)-Bruch's membrane complex of two donor eyes without AMD. In one eye with AMD, endostatin was detected in RPE, Bruch's membrane and choroidal vessels. Ninety-two percent (33/36) of CNV disclosed endostatin staining. RPE-Bruch's membrane complex, choroidal vessels and stroma were positive in 50% (16/36), 72% (26/36), and 78% (28/36) of the membranes, respectively. Both control eyes and CNV expressed all the investigated markers except E-selectin being positive only in membranes. Endostatin, an endogenous angiogenesis inhibitor, is expressed in CNV and its therapeutic up-regulation may be a new strategy in the treatment of neovascular AMD.
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Keywords: Age-related macular degeneration; choroidal neovascularization; angiogenesis; endostatin; E-selectin

1. Introduction

Choroidal neovascularization (CNV) is responsible for the majority of severe visual loss due to age-related macular degeneration (AMD), the most common cause of visual morbidity in elderly (The Macular Photocoagulation Study Group, 1991). Recent studies have clarified the importance of a balance between local inhibitory and stimulatory factors in the pathogenesis of angiogenesis (Pepper, 2001), but some of these factors still remain to be defined.

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, has been identified as an endogenous angiogenesis inhibitor (O'Reilly et al., 1997). Collagen XVIII is the core protein of a heparan sulfate proteoglycan in vascular and epithelial basement membranes (Zatterstrom et al., 2000). Endostatin is bound to collagen XVIII by a protease-sensitive hinge. Some proteases, such as matrix metalloproteases (MMPs), can cleave the hinge so that endostatin can be released and becomes available (Sasaki et al., 1998; Ferreras et al., 2000).

* Corresponding author. Tel.: +49 7071 2984004; fax: +49 7071 295215.
E-mail address: salvatore.grisanti@med.uni-tuebingen.de (S. Grisanti).

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The antiangiogenic effect of endostatin is both at the transcriptional and cellular level. At the transcriptional level, endostatin down-regulates proangiogenic genes related to vascular endothelial growth factor (VEGF) signalling (e.g. thrombin receptors, Stats, and HIF-1), but up-regulates antiangiogenic genes (such as Vasostatin, Kininogen) in human microvascular endothelial cells (EC) (Abdollahi et al., 2004). On the cellular level, endostatin was shown to inhibit EC proliferation and migration stimulated by FGF-2 (Eriksson et al., 2003), EC chemotactic migration towards VEGF (Eriksson et al., 2003; Yamaguchi et al., 1999) and EC invasion by blocking the activation and catalytic activity of MMP-2 (Lee et al., 2002; Kim et al., 2000). Additionally, endostatin induces EC apoptosis (Dhanabal et al., 1999; Dixelius et al., 2000). Lately, E-selectin, a cellular adhesion molecule, was proven to be required for the antiangiogenic activity of endostatin (Yu et al., 2004).

EC in different parts of the body have different characteristics (Lanzer and Raff, 1987). Variations in surrounding cells and extracellular matrix may also contribute to the tissue-specific aspects of neovascularization (Mori et al., 2001). The altered local balance between stimulators and inhibitors is accepted to be the cause of neovascularization (Pepper, 2001; Mori et al., 2001); however, the involved players may vary depending on the setting.

All the aforementioned angiogenic factors (FGF, VEGF, MMP, HIF-1) seem to have well-defined roles in the pathogenesis and development of CNV (Amin et al., 1994; Kvanta et al., 1996; Martin et al., 2004; Lambert et al., 2003). Endostatin, shown to inhibit their activities and known to up-regulate antiangiogenesis genes, therefore, is likely to be an endogenous inhibitor of angiogenesis also in CNV. Intravitreal or intravenous delivery of endostatin by viral vectors was shown to inhibit diabetic retinopathy and choroidal neovascularization in experimental studies. (Mori et al., 2001; Auricchio et al., 2002). Bhutto et al. evaluated endostatin expression in five donor eyes with advanced AMD—four eyes with disciform scar with small CNV and one eye with geographic atrophy with sub-RPE neovascularization—and reported nearly absent endostatin expression. However, to the best of our knowledge, expression of this endogenous angiogenesis inhibitor in surgically excised, active CNV membranes extracted surgically before they reach the disciform scar stage has not been defined experimentally or clinically yet.

The aim of this study was to analyze the endostatin expression in surgically extracted active human CNV with regard to vascularization and proliferative activity. The level of vascularization was determined by the expression of CD 34, a pan-endothelial cell marker, and CD105 (Endoglin), a marker for activated endothelial cells (Grisanti et al., 2004).

Ki-67, Cytokeratin18 (CK18) and an antibody specific for VEGF-A were used to detect the proliferative activity (Grisanti et al., 2004), to identify RPE cells (Martin et al., 2004) and to evaluate the angiogenic stimulation, respectively. Expression of E-selectin which is required for the antiangiogenic activity of endostatin (Yu et al., 2004) was analyzed both in control eyes and CNV.

2. Materials and methods

2.1. Subjects and treatment

We retrospectively reviewed 36 CNV, secondary to neovascular AMD, from 36 eyes of 36 consecutive patients, in which three-port vitrectomy and extraction of CNV was performed. None of the patients had disciform scar but CNV with submacular leakage or hemorrhage leading to progressive visual deterioration. CNV membranes previously treated with photodynamic therapy, laser coagulation or any kind of anti-angiogenesis therapy including intravitreal triamcinolone injection were not included in our series. Each patient gave written informed consent after the nature of the procedure and alternatives had been fully explained. The study followed the guidelines of the declaration of Helsinki as revised in Tokyo and Venice and adhered to the requirements of the local Institutional Review Board. The histological analysis of the specimens was approved by the Institutional Ethic Committee. Normal donor eyes ($n = 6$) and a donor eye with drusen but without neovascularization ($n = 1$) received for keratoplasty were used as controls.

2.2. Tissue preparation

Within minutes after surgery, excised CNV and donor eyes were fixed in 3.7% formalin and subsequently embedded in paraffin. Each specimen was serially sectioned into 5-μm sections and mounted on poly-L-lysine coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining. Hematoxylin–eosin and PAS staining were performed to determine the histologic orientation. PAS staining was primarily used to confirm the location of the diffuse drusen and to help the overall orientation of the specimens.

2.3. Immunohistology

After serial paraffin sections were de-paraffinized and rehydrated with a graded series of alcohol, different techniques for antigen retrieval were applied. For CK18 and endostatin, antigen retrieval was accomplished by proteolytic digestion with 0.5% protease type XXIV (Bacterial, Sigma, St. Louis, MO) whereas proteinase K (Dako) was used for VEGF. For Ki-67, CD 34, CD 105 and E-selectin staining, antigen retrieval was accomplished with heat treatment in citrate buffer in a pressure cooker for 2 min.

Immunohistochemical staining for CD 105, CD 34, Ki-67, CK 18 and E-selectin was performed using the horseradish peroxidase method according to the manufacturer's protocol (Vectastain Universal Elite ABC PK-6200 kit, Vector Laboratories, Burlinghame, CA). To block endogenous peroxidase activities, 3% hydrogen peroxide and 0.1% sodium acid were applied to each section. Subsequently, the sections were incubated with horse serum (30 min). Thereafter, specimens were incubated with the primary antibodies specific for human CD 105 (mouse, Mab, Clone SN6h, Dako), CD 34 (mouse, Mab, Immunotech, Hamburg, Germany), Ki-67

(mouse, Mab, Clone Ki-S5, Dako), and CK 18 (mouse, Mab, Progen, Heidelberg, Germany) and E-selectin (mouse, Mab, Novocastra, UK) for 1 h at room temperature. After incubating with the biotinylated horse anti-mouse anti-rabbit secondary antibody and the ABC Complex (Vectastain Universal Elite ABC PK-6200 kit, Vector Laboratories, Burlinghame, CA) for 15 min, followed by washing in-between and afterwards, the antibody-treated sections were developed with a 3-diaminobenzidine (Fluka, Buchs, Germany) solution combined with H_2O_2 for CD 34, CD 105 and Ki-67 staining. For CK 18 and E-selectin staining, the chromogen was replaced with AEC high sensitive substrate chromogen (Cytomation, Code K3461, Dako).

Immunohistochemical stainings for VEGF and endostatin were performed by the alkaline-phosphatase method according to the manufacturer's instructions (ChemMate Detection Kit, Alkaline Phosphatase/RED, Rabbit/Mouse, K5005; Dako). Briefly, after proteolytic digestion with proteinase K (Dako) for 10 min, the sections were incubated with the anti-VEGF primary antibody (mouse, Mab, clone C-1; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 37 °C. For endostatin immunostaining, following proteolytic digestion with protease (Protease XXIV, Sigma) for 10 min, the sections were incubated with the primary endostatin antibody (rabbit, polyclonal, Dianova GmbH, Hamburg, Germany) for 1 h at 37 °C. They were then subsequently incubated with the linking biotinylated goat anti-mouse anti-rabbit secondary antibody (ChemMate Link, Biotinylated Secondary Antibodies, AB2, Dako), and with the streptavidin conjugated to alkaline phosphatase for 30 min each. Chromogen Red (ChemMate Detection Kit, Dako) was used as a chromogenic substrate. 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 either by appropriate normal sera or omitted.

For better visualization of positive areas, donor eyes and CNV were examined after melanin bleaching. Melanin bleaching was performed according to the protocol established by (Kivela (1995) and Makitie et al. (1998).

2.4. Analysis

Slides were analyzed by light microscopy. Intensity and presence of staining were determined three times independently by two masked observers (OT, SG) and the median score for each specimen was obtained. Inter- and intraobserver agreement was found in about 98% of the cases.

Based on the previous studies (Kvanta et al., 1996; Grossniklaus et al., 2002), the CNV membranes were classified as "inflammatory active (IA)" when inflammatory cells were more dominant than fibrosis (i.e. >50% inflammatory cells) or "inflammatory inactive (II)" when fibrosis was more dominant (<50% inflammatory cells) with minor or absent inflammatory response in CNV membranes.

Vascularization was evaluated by analyzing the specimen stained for CD34 and CD105 and counting the numbers of stained vessels in the most vascularized area under ×200 magnification. Every positive-staining endothelial cell or cell cluster that was separate from other vessels was counted irrespective of the staining intensity or the presence of a vascular lumen.

All Ki-67 positive nuclei in RPE, EC and stromal cells were counted separately in each specimen. The percentages of the Ki-67 expressing RPE, EC and stromal cells with regard to the total number of proliferating cells were determined. Proliferative activity (nuclei/mm^2) of a membrane was defined as the number of Ki-67 expressing nuclei in 1 mm^2 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 (mm^2).

Immunoreactivity for VEGF, endostatin and E-selectin were analyzed separately in RPE-Bruch's membrane complex, vessels and stroma. A grading scheme indicating the degree of staining was used. The values 3, 2, 1, 0 were assigned to indicate intense (70–100% positive cells), moderate (40–69% positive cells), weak VEGF labelling (1– 39% positive cells) and absence (–) of any staining, respectively. An "endostatin overall staining score", "E-selectin overall staining score" and "VEGF overall staining score" (range 0–9) were assigned to each membrane by summing up the staining scores in the three structures (RPE, stroma, EC) evaluated separately.

Statistics for the evaluation of proliferative activity and staining scores in the defined subgroups was performed with Mann–Whitney U test due to high variability of specimens. For correlation analysis, Spearman's correlation test was used; $p \leq 0.05$ was considered significant.

3. Results

3.1. Histologic characterization of control eyes

Six of the donor eyes ($n = 7$) were normal eyes without any ocular pathology, especially those associated with angiogenesis. One of the seven control eyes, however, disclosed drusen and sub-RPE deposits but no neovascularization. Demographic features and the immunohistologic findings of the control eyes are shown in Table 1 and Fig. 1, respectively.

For a better visualization and evaluation of the immunoreactivity within the choroid-Bruch's membrane-RPE complex, the melanin pigment was bleached. The staining pattern did not change after bleaching. Choroidal vessels displayed endostatin in 5 of 6 normal control eyes (Fig. 1A). Most of the choroidal vessels were endostatin positive in four of these

Table 1
Demographic features of the control eyes

Control eye	Age, sex	Eye	AMD
1	39, f	Right	–
2	62, m	Right	–
3	43, m	Right	–
4	52, f	Left	–
5	39, f	Left	–
6	49, f	Left	–
7	59, f	Right	+

AMD, age-related macular degeneration; f, female; m, male.

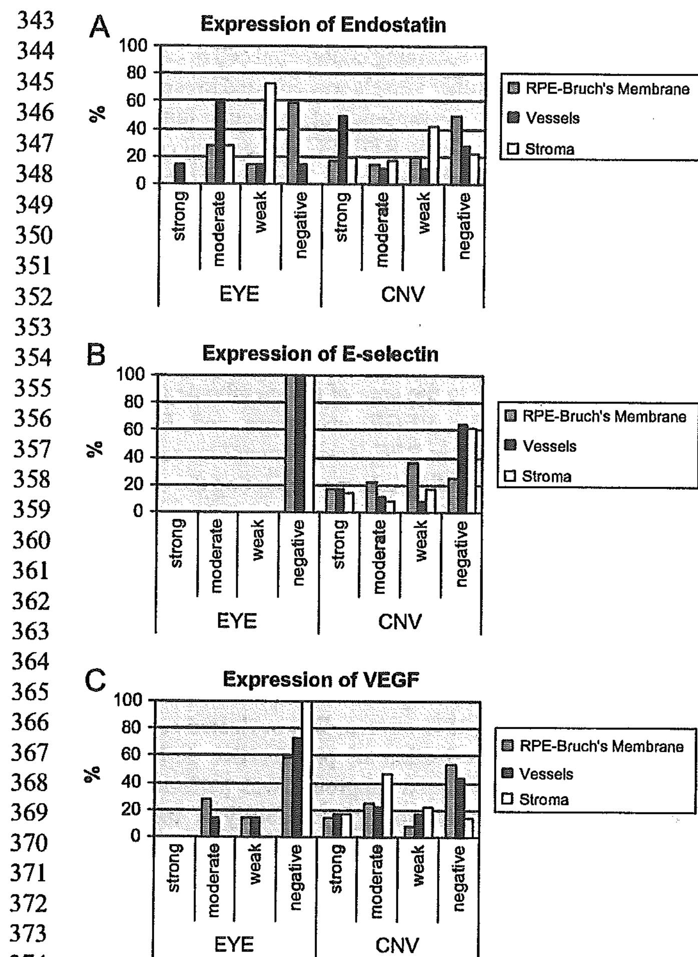

Fig. 1. Immunohistopathologic characteristics of the control eyes and CNV by analysis of the specimens for endostatin (A), E-selectin (B), and VEGF (C) separately in RPE-Bruch's membrane complex, vessels and stroma. For semi-quantitative evaluation, a grading scheme was used indicating the degree of staining: "strong" (70–100% positive cells); "moderate" (40–69% positive cells); "weak" (1–39% positive cells), "negative" (no positive cells).

eyes whereas in one eye only a few of the vessels expressed endostatin (Fig. 2A). The negative control of the same specimen by omitting the primary endostatin antibody displayed no staining in any of the structures (Fig. 2B). Endostatin staining was not specific to vessels. In 2 of 6 control eyes, RPE cells and Bruch's membrane were also stained for endostatin (Figs. 1A and 2C). Endostatin was not diffusely stained throughout a specimen as some endostatin expressing vessels (Fig. 2A) and RPE-Bruch's membrane complex (Fig. 2C) are detected beside the endostatin negative ones in the same specimen (Fig. 2A,D). Endostatin expression and variability in its pattern may be due to the local activity of the proteases in the setting which cleave only some of the endostatin from collagen XVIII. The absence of endostatin expression in RPE-Bruch's membrane complex in four of the control donor eyes (age range 39–62) leads us to assume that this commercial endostatin antibody detects only the free (cleaved) but not the bound form of endostatin. Staining for endostatin in varying intensities was seen in the choroidal stroma of all eyes. In the eye with drusen, endostatin expression was found in RPE-Bruch's membrane complex, stroma and choroidal vessels.

We next sought to investigate the expression of E-selectin and VEGF in the choroid. We found that none of the control eyes displayed E-selectin at all (Figs. 1B and 2E). In contrast, VEGF expression was present but still weak. RPE cells expressed VEGF moderately in two of the eyes. Weak to moderate expression of VEGF in choroidal vessels was also detected only in two eyes (Figs. 1C and 2F).

3.2. Histologic characterization of the CNV

All but one CNV were vascularized as evidenced by CD34 positive vessels. CD105 staining was specific to EC (Fig. 3A). CD105 was expressed in all the vascularized specimens ($n = 35$) to some extent (Fig. 3A). Within 13 of 35 vascularized CNV membranes (34%), some vascular ECs positive for CD34 did not express CD105. Contrary to control eyes, E-selectin was detected in RPE-Bruch's membrane, EC and some stromal cells in 75%, 36%, and 39% of CNV, respectively (Figs. 1B and 3B–D). Some CD34 positive EC as well as some RPE cells did not express E-selectin (Fig. 3B–D). CD 105 expressing EC were not always E-selectin positive (Fig. 3A,B).

Hematoxylin–eosin and PAS staining (Fig. 4A) were performed to determine the histologic orientation and to confirm the location of the diffuse drusen

CK18 expressing RPE cells were found to be present in all of the CNV samples (Fig. 4B).

Positive staining for endostatin in RPE-Bruch's membrane was found in 50% of the membranes at varying intensities (Figs. 1A and 4C). In some CNV, endostatin expression was not evenly distributed in the RPE cell layer but was only partially expressed. Positive immunostaining in vessels was observed in 72% of the specimens (Figs. 1A and 4C–F). Endostatin staining in CNV specimens also persisted after bleaching of the specimen for melanin (Fig. 4D) Vessels in a CNV were only partially stained for endostatin (Fig. 4E). A few vessels expressing CD105 but no endostatin were detected. Endostatin expressing vessels were not always E-selectin positive (Figs. 3B and 4E). Staining intensity between vessels in the same CNV also varied. Endostatin staining was also observed in stromal cells and in fibrous stroma itself (Fig. 4F).

Endostatin and E-selectin were co-expressed in many CNV specimens (Figs. 3C,D and 4C,D). However, expression of endostatin in RPE-Bruch's membrane, vessels and stroma evaluated separately or as "the overall expression score" were not correlated with the corresponding E-selectin expression in CNV membranes ($p = 0.3482$, $\rho = 0.3130$; $p = 0.6294$, $\rho = -0.1610$; $p = 0.5658$, $\rho = -0.1820$; $p = 0.3978$, $\rho = -0.2670$, respectively)

In CNV, VEGF staining was absent in RPE cells of 53% of the specimens. When expressed, VEGF staining intensity in RPE varied from weak to strong. Similarly, VEGF expression in EC was seen in 55% of the CNV. Weak to moderate expression was found in 17% and 28% of the examined membranes, respectively. In 86% of the cases, VEGF expression could be

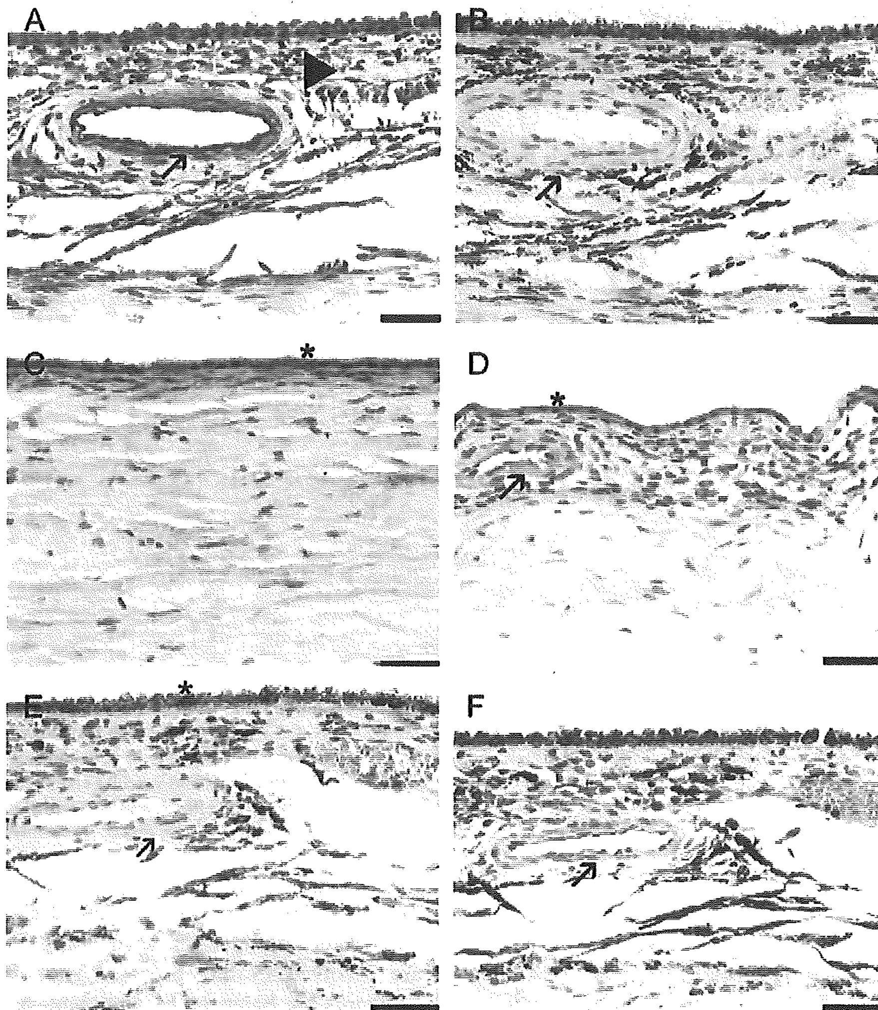

Fig. 2. Immunohistochemistry of a control eye (Case 4 in Table 1) probed with an antibody against endostatin (A, C, D), E-selectin (E) and VEGF (F). Sections were stained with either red alkaline phosphatase substrate (A–D, F) or AEC chromogen (E) and counterstained with hematoxylin. (A) Some vessels within the choroid disclose a prominent staining (arrow) whereas others are completely negative (arrowhead). (B) A serial section from the same specimen is used as a negative control by omitting the primary antibody "endostatin". None of the choroidal structures display false positive staining. (C) Endostatin labelling is present in part of the RPE-Bruch's membrane complex (*) whereas endostatin negative RPE-Bruch's membrane complex is also seen in the same eye (D, *). (D) A weakly stained choroidal vessel is apparent in the same section (arrow). (E) E-selectin is not expressed in any of the structures of the same eye whereas VEGF is expressed weakly in some of the choroidal vessels (F, arrow). Scale bars: 50 μm.

encountered within the stroma of the membranes, especially in fibroblast-like and inflammatory cells (Figs. 1C and 5A). CNV membranes and vessels strongly stained both for VEGF and endostatin were also present (Figs. 4F and 5A).

3.3. Ki-67 labelling, proliferative and inflammatory activity in CNV

In CNV, a differing number of Ki-67 positive proliferating cells were detected. Ki-67 positive cells ($n = 971$), were rarely EC ($n = 51$, 5.3%) or RPE cells ($n = 36$, 3.7%) but mostly appeared to belong to stromal cells ($n = 874$, 91%), especially inflammatory infiltrate (Fig. 5B).

Proliferative activity in CNV membranes varied strongly from 0 to 514.01 nuclei/mm^2 (median 55.13, mean 105.72, SE 24.71). When "overall endostatin staining score" was concerned, there was no statistically significant difference in the median proliferative activity between the group with the high endostatin staining score (median 59.7, SE 31.67 overall, endostatin score 5–9, $n = 18$) and with the lower one (median 43.3, SE 38.88, overall endostatin staining score <5, $n = 18$) ($p = 0.4961$).High proliferative activity was observed in some CNV with strong endostatin expression (Figs. 4F and 5B). No correlation was found between the proliferative activity and the overall endostatin staining by Spearman's coefficient correlation test ($p = 0.321$, $\rho = 0.159$). Proliferative activity was also not correlated with either "E-selectin overall staining score" ($p = 0.622$, $\rho = 0.092$) or "VEGF overall staining score" ($p = 0.659$, $\rho = -0.075$).

Of the 36 CNV, 64% (23/36) was classified as inflammatory active (IA) whereas 36% (13/36) of the specimens were inflammatory inactive (II). There was no statistically significant

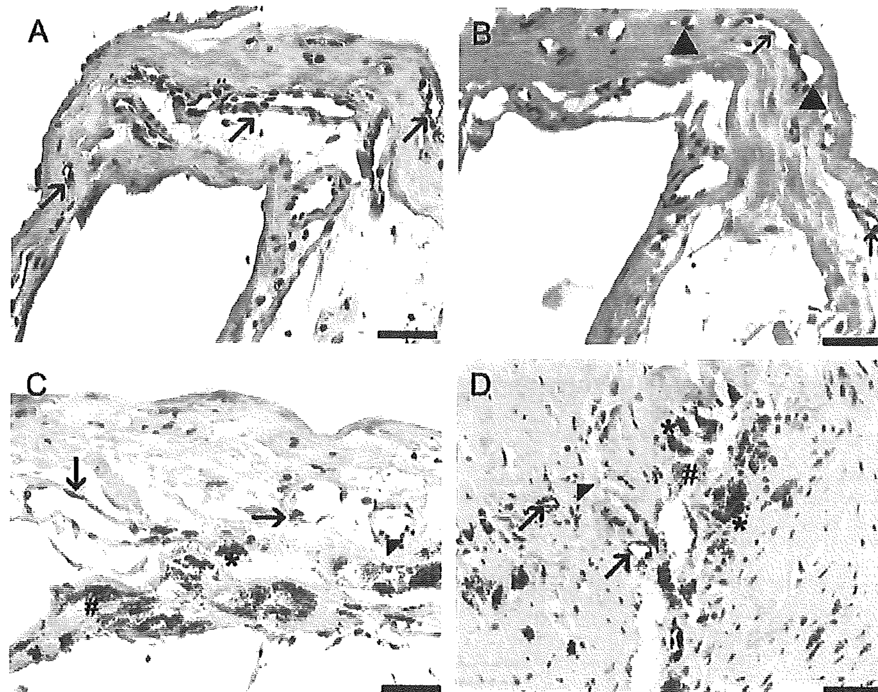

Fig. 3. Immunohistochemistry of the three different CNV membranes probed with antibodies against CD105 (A) and E-selectin (B–D). Sections were stained with 3-diaminobenzidine (A) and AEC (B–D) resulting in a brown and red chromogen, respectively and counterstained with hematoxylin. (A) CD105 is selectively expressed in endothelial cells (arrows). (B) CD105 expressing vessels do not always express E-selectin. Arrowheads point to some of the E-selectin negative vessels expressing CD105 in the parallel section (A). (B–D) E-selectin is positive in some but not all RPE cells and vascular endothelial cells. Arrows and asterisk indicate E-selectin expressing EC and RPE cells, respectively. However, in the same specimens, some RPE cells (#) and EC (arrowheads) are E-selectin negative. Scale bar: 50 μm.

difference either in endostatin staining intensity of RPE-Bruch's membrane (median 1, $p = 0.0789$), vessels (median 3, $p = 0.9715$), stroma (median 1, $p = 0.0719$) when evaluated separately (range 0–3) or in the "overall endostatin staining score" (median 5, range 0–9, $p = 0.1727$) between IA and II CNV. Similarly, E-selectin staining intensity in RPE-Bruch's membrane complex, vessels, and stroma evaluated separately (range 0–3) as well as the "E-selectin overall staining score"(range 0–9) did not vary significantly between IA (median 1, 0, 0, 3, respectively) and II membranes (median 1, 0, 0, 2; $p = 0.922$, $p = 0.8946$, $p = 0.9171$, $p = 0.7928$, respectively). Furthermore, in II CNV, VEGF expression either in RPE cells, vessels and stroma evaluated separately (median 0, 1, 2, respectively, range 0–3 for all) or in terms of "overall VEGF staining score" (median 4, range 0–9) were also not significantly different from those in IA CNV (median 0, 1, 2, 3; $p = 0.8428$, $p = 0.7274$, $p = 0.7119$, $p = 0.6657$, respectively).

However, the proliferative activity in IA CNV (median 72.8912, range 0–514.081, mean 149.125, SE 35.4) was significantly higher than in II CNV (median 12.8517 nuclei/mm^2, range 0–113.693, mean 28.931, SE 12.8517) ($p = 0.0059$).

4. Discussion

Endothelial cell activation, proliferation, tube formation and development of the basement membrane are among the successive steps during neovascularization (Pepper, 2001). Numerous angiogenesis stimulating factors such as VEGF are involved in this process. The effects of angiogenesis activators are balanced by angiogenesis inhibitors such as endostatin. Herein, we aimed to evaluate the presence and co-expression of endostatin with other markers representing different aspects of the process in order to understand the role of this endogenous inhibitor in the pathogenesis of CNV.

Collagen XVIII is a component of Bruch's membrane as well as the basement membrane of both active and quiescent vessels. In contrast, endostatin was shown to be selectively expressed in newly formed vessels in pathological angiogenesis but not in the quiescent vessels of some non-angiogenic and/or non-pathologic control organs other than eye (Ergun et al., 2001). However, in our series, similar to the findings of Bhutto et al. (2004), endostatin was expressed in the RPE-Bruch's membrane complex and choriocapillaris of some apparently normal control eyes, even in those from young donors. In order to evaluate further if endostatin was positive only in vessels lined by activated EC in CNV, we examined the expression of CD105, a marker for activated/proliferating endothelial cells (Grisanti et al., 2004). Although vessels were mostly expressing both of them, it was still possible to detect a few vessels stained for only one of the markers. Additionally, some endostatin expressing vessels were not positively stained for E-selectin which is expressed in proliferating EC under non-inflammatory conditions (Luo et al., 1999). Moreover,

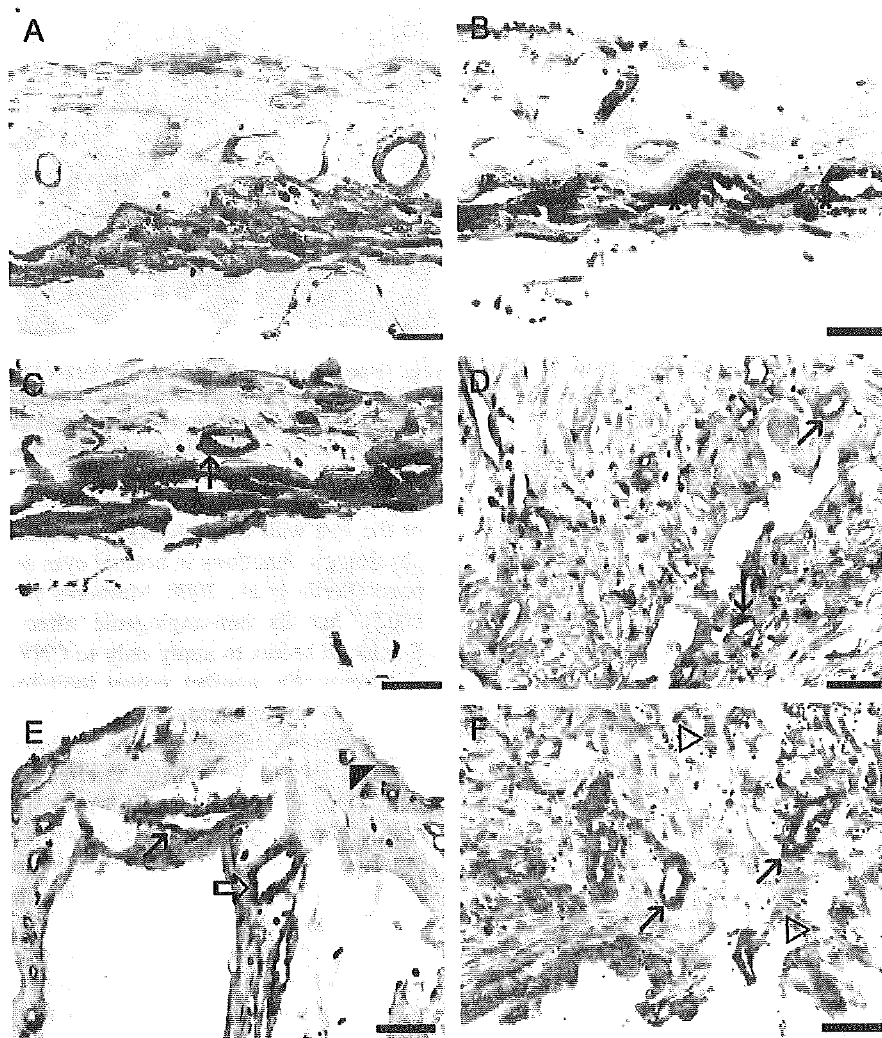

Fig. 4. Serial sections from those specimens in Fig. 3 (A-E) and another CNV (F). Sections are stained with PAS (A) or probed with antibodies against cytokeratin18 (B), endostatin (C–F). (A) PAS staining helps the overall orientation of the specimen. (B) RPE cells express Cytokeratin18 (*) in the same membrane. (C–F) RPE-Bruch's membrane complex (*) as well as vessels (arrows) are strong endostatin positive. (C, D) The specimens stained for E-selectin in Fig. 3C and D are also strong positive for endostatin in the serial sections. (D) Endostatin immunoreactivity is still detected after bleaching of the specimen for melanin. (E) Endostatin positive vessels (arrows) as well as endostatin negative vessels (arrowhead) are seen in the same specimen. Some endostatin positive vessels (white arrow) are not E-selectin positive as shown in the parallel section in Fig. 3B. (F) Stromal cells also display endostatin (white arrowhead). Sections were stained with the immunoperoxidase (B) or alkaline phosphatase (C–F) techniques with AEC (B) and red alkaline phosphatase substrate chromogen (C–F) and counterstained with hematoxylin. Scale bar: 50 μm.

the EC in endostatin expressing vessels were not always Ki-67 positive. Therefore, endostatin expression seems not to be limited to the newly formed vessels in the neovascularized tissue when normal choroid and CNV membranes are concerned.

During angiogenesis, activated endothelial cells release proteolytic enzymes like MMPs that break up the peptide bonds within the protease sensitive region of collagen XVIII (Lee et al., 2002; Kim et al., 2000). Consequently, local inhibitors of angiogenesis like endostatin fragments are released. Variations in intensity and pattern of endostatin expression in CNV as well as negative or limited staining in some control eyes may be, therefore, due to microenvironmental variations in the level of proteolytic enzymes.

The effect of endostatin on the proliferative activity of EC is still controversial. Contrary to the studies showing its inhibitory effect (Wang et al., 2004; Abdollahi et al., 2003), Skovseth et al. (2005) and Wang et al. (2005) reported that endostatin has no effect on the proliferative activity of the EC although it dramatically inhibits EC migration and perivascular cell recruitment. In our series, in some specimens, proliferative activity was remarkably high in spite of strong endostatin expression. When the CNV membranes were classified according to the "endostatin overall staining score", the difference in proliferative activity was not significant between the group with high (5–9) and low score (<5). In the specimens investigated, the proliferative activity did not seem to be

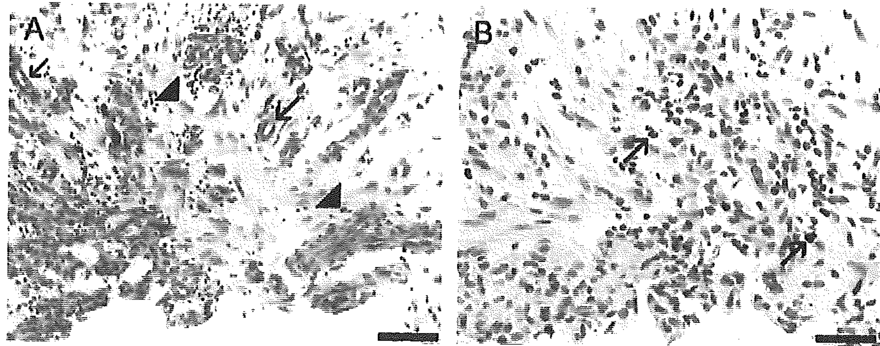

Fig. 5. Immunohistochemistry of serial sections from the CNV specimen shown in Fig. 4F probed with antibodies against VEGF (A) and Ki-67 (B). Sections were stained with red alkaline phosphatase substrate (A) and 3-diaminobenzidine (B) resulting in a red and brown chromogen, respectively, and counterstained with hematoxylin. (A) This specimen displays strong VEGF expression in EC (arrow) and stromal cells (arrowheads) such as fibroblasts and inflammatory cells despite strong endostatin expression shown in the serial section in Fig. 4F. (B) Several Ki-67 positive nuclei are detected (arrows) in this specimen. None of these nuclei appear to be associated with endothelial cells. Scale bar: 50 μm.

correlated with endostatin staining intensity but with inflammation; the Ki-67 positive cells were mostly stromal and inflammatory cells. We detected significantly higher proliferative activity in IA CNV than II CNV; however, there was no significant difference in the endostatin overall staining score between IA and II CNV. Proliferative activity did not seem to correlate with either E-selectin or VEGF expression scores in the CNV.

Collagen XVIII/endostatin was suggested to be critical for normal ocular blood vessel formation, vision and RPE function. Lack of collagen XVIII/endostatin resulted in ocular as well as functional RPE abnormalities with the formation of excess basal laminar-like deposits similar to drusen (Sertie et al., 2000; Marneros et al., 2004; Fukai et al., 2002). Still, it was not clear if collagen XVIII or its endostatin part was functionally important in AMD pathogenesis. Recent studies revealed that the level of endostatin was reduced in Bruch's membrane, RPE basal lamina and choriocapillaris in eyes with AMD whereas collagen XVIII immunostaining was similar in pattern and intensity in comparison to the normal donor eyes. It was concluded that reduced endostatin expression in choroid might be a factor contributing to AMD and perhaps choroidal neovascularization pathogenesis (Bhutto et al., 2004).

In our CNV specimens, E-selectin was detected in RPE and stromal cells as well as EC similar to the observations of others (Yeh et al., 2004; Shen et al., 1998). Interestingly, E-selectin was expressed only in CNV membranes but not in any of the control eyes. This finding is also concordant with the findings from Yeh et al. (2004). Taken together, these observations might point out to a possible role of endostatin in CNV pathogenesis as E-selectin was recently suspected to be required for the antiangiogenic activity of endostatin (Yu et al., 2004). Yu et al. showed that in E-selectin −/− mice, FGF-induced corneal angiogenesis and VEGF-stimulated endothelial sprout formation was not inhibited by endostatin and cells were endostatin-resistant in contrast to the normal controls. Our immunohistopathologic evaluation revealed that endostatin is co-expressed with E-selectin in RPE-Bruch's membrane complex, vessels and stroma in CNV membranes where pathological angiogenesis is active but not in either normal control eyes or the eye with early AMD. Endostatin seems to have other physiologic functions in normal eyes as stated by previous reports (Sertie et al., 2000; Marneros et al., 2004; Fukai et al., 2002), but its anti-angiogenic effect in conjunction with E-selectin seems to apply only to CNV membranes.

Angiostatin, another potent inhibitor of angiogenesis was shown to up-regulate E-selectin in proliferating EC, and was, therefore, suggested to be inhibiting selectively the pathological angiogenesis without effects on quiescent endothelium (Luo et al., 1998). However, information concerning such a correlation between endostatin and E-selectin is lacking. In our series, no correlation was found between endostatin and E-selectin expression, but, it should be sought by further experimental studies.

In our specimens, expression of E-selectin, an endothelial specific adhesion molecule especially for leukocytes, did not vary significantly between IA and II CNV membranes. E-selectin, however, is known to be induced not only by inflammatory stimuli but also by the proliferative state of the endothelium in non-inflammatory pathway (Luo et al., 1999). Although no correlation was found between proliferative activity of the membranes and E-selectin expression in our specimens, it must be considered that Ki-67 expressing proliferating cells in CNV were mostly stromal cells but not EC.

Grossniklaus et al. (2002) proposed that the development of CNV membrane is based on a dynamic process with sequential initiation, maintenance and involution stages. There is clinical (Green, 1996) and experimental (Wada et al., 1999) evidence for spontaneous involution of CNV. Grossniklaus et al. suggested that a decreased production of cytokines, especially VEGF, and an increased fibrosis may be responsible for the involution as CNV matures. Besides its inhibitory effect on EC stimulated by VEGF and FGF-2, endostatin was shown to stabilize the newly formed endothelial tubes in the early phase by constructing inter-endothelial junctions and attaching EC to the basement membrane (Ergun et al., 2001; Yu et al., 2004; Dixelius et al., 2002). Endostatin is also known to reduce VEGF induced retinal vascular permeability and neovascularization (Takahashi et al., 2003). Therefore, we suppose that endogenous endostatin expression may participate in

the spontaneous involution of CNV. RPE cells have a dual and regulatory role in CNV pathogenesis. RPE cells may promote progression of CNV in early stages; however, in the late stages, they have been suggested to promote regression (Lutty et al., 1999). The presence of endostatin and E-selectin within the RPE-Bruch's membrane complex in CNV may have a role in the involution process.

Vitreous levels of VEGF and endostatin were shown to be correlated with the activity of angiogenesis in diabetic retinopathy (Noma et al., 2002). In CNV, this may also be the fact but distinct stages of the angiogenesis process within this pathological tissue may act rather concomitantly than subsequently. The collagen XVIII/endostatin system limits its self activation by blocking the activation and the catalytic activity of MMP-2 (Ferreras et al., 2000; Lee et al., 2000). This suggests that angiogenic and antiangiogenic factors are active concomitantly and dependent on each other in CNV until they come into a balance and stabilize. This may explain the concomitant expression of intense VEGF as well as intense endostatin staining within some angiogenic specimens in our series and almost negative expression of endostatin in disciform scars examined by Bhutto et al.

Experimental trials of exogenous endostatin treatment for CNV have been already conducted and results seem to be promising (Mori et al., 2001). To the best of our knowledge, our results are the first to describe endostatin and E-selectin expression in a relatively high number of CNV membranes. Physiological levels of endostatin expression, although strong, may be insufficient to inhibit the existing CNV totally as is the case in tumors (Fukai et al., 2002). However, endogeneous endostatin levels are proven to be increased by orally administered drugs (Folkman, 2004). Therefore, the knowledge gained from our work may underline a new perspective for the future trials in CNV treatment.

The proper interpretation of this study is limited by the fact that age and 'maturity' of the surgically excised CNV specimens cannot be determined accurately. It is, therefore, not possible to determine the onset of endostatin expression according to time and phase of angiogenesis. Nevertheless, it is conceivable that endostatin is among the endogenous antiangiogenic factors expressed in human CNV. Its co-expression with E-selectin in CNV suggests that endostatin may act as an anti-angiogenesis factor in modulating neovascularization in CNV. Therapeutic up-regulation of endostatin as an alternative to its exogenous delivery, may be an important strategy in the future treatment of neovascular AMD to stabilize the course of neovascularization.

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CONSEQUENCES OF VERTEPORFIN PHOTODYNAMIC THERAPY ON CHOROIDAL NEOVASCULAR MEMBRANES

Key words: Age Related Macular Degeneration, Choroidal Neovascular Membranes, Verteporfin Photodynamic Therapy, Vascular Endothelial Growth Factor

Authors: [1]Olcay Tatar, MD, [2]Edwin Kaiserling, MD, [2]Annemarie Adam, [1]Faik Gelisken, MD, [3]Kei Shinoda, MD, PhD, [1]Michael Völker, MD, [4]Bart A. Lafaut, MD, [1]Karl Ulrich Bartz-Schmidt, MD, [1]Salvatore Grisanti, MD.

[1]Department of Ophthalmology, Eberhard-Karls University Tuebingen, Germany,

[2]Department of Pathology, University of Tuebingen, Germany,

[3]Department of Ophthalmology, Keio University, Tokyo, Japan

[4]Department of Ophthalmology, AZ StJan, Bruges, Belgium

Corresponding author: Ass. Prof. Dr. Salvatore Grisanti, Department of Ophthalmology, Division of Vitreoretinal Surgery, Eberhard-Karls University Tuebingen

Schleichstrasse 12-15, 72076 Tuebingen, Germany

Fax: 011/49/7071/295215, E-Mail: Salvatore.Grisanti@med.uni-tuebingen.de

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ABSTRACT

Objective: To examine the impact of photodynamic therapy (PDT) on angiogenesis in human choroidal neovascularization (CNV) membranes with respect to Vascular Endothelial Growth Factor (VEGF) expression, proliferation and vascularization.

Methods: Retrospective review of interventional case series of fifty patients (fifty eyes) who underwent removal of CNV. CNV was secondary to age-related macular degeneration (AMD). Twenty were treated with PDT 3 to 655 days before surgery. CNV were stained for CD34, CD105, Ki-67, cytokeratin18 and VEGF. Thirty CNV secondary to AMD without previous treatment were used as control.

Results: Specimens without pre-treatment disclose varying degrees of vascularization, proliferative activity and VEGF expression by different cells. Specimens treated by PDT three days previously show 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 is associated with an impressive VEGF immunoreactivity unique to retinal pigment epithelial cells (RPE) shortly after PDT that shifts also to other cells at later time points.

Conclusions: PDT induces a selective vascular damage in CNV. The effectiveness and selectivity of this treatment, however, seem to be jeopardized by a rebound effect initiated by an enhanced VEGF expression in RPE cells.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of legal blindness in patients older than 60 years of age **in the western world**.[1,2] The "exudative" form of the disease is characterized by the development of a 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. Though some modalities are still experimental, large randomized clinical trials have shown the value of laser photocoagulation and photodynamic therapy (PDT). The major handicap of the 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 the PDT.

PDT is a nonthermal process based on the targeted photoactivation of the intravenously given photosensitive drug. The activated dye results in the creation of oxygen intermediates and free radicals affecting the exposed endothelial cells.[3] PDT seems to be an ideal treatment approach for CNV allowing selective photothrombosis of the CNV without damage to overlying neurosensory retina.

After randomized clinical trials have demonstrated that PDT with Verteporfin (Visudyne, Novartis AG, Buelach, Switzerland) is an effective treatment for subfoveal choroidal neovascularization secondary to AMD, it is accepted as a routine procedure under certain circumstances.[4-8] 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 loss of two ETDRS lines within 6 months.[3,4]

Though a large scale of preclinical studies 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.

Herein, we present 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 time 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 AMD patients, in which surgery for CNV was performed. In 20 of these patients, surgery was performed after Verteporfin PDT (Table 1). In addition to the complete ophthalmological examination, in patients receiving Verteporfin PDT, stereoscopic fluorescein angiography (FA) was performed before the treatment and thereafter on the day of surgery. CNV were classified according to the guidelines of the TAP and VIP studies.[4-8] Therapy options, 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 a) visual acuity was below 20/200 being the minimum visual acuity to recommend the first PDT according to the TAP-Investigation [4,5] and b) visual deterioration progressed after initial PDT. Clinical characteristics of the patients treated with Verteporfin-PDT are summarized in Table 1.

Four eyes underwent CNV extraction three days after PDT. Three of these for eyes patients had subfoveal classic CNV. The visual acuity of these three eyes was between 4/200 and 10/200, less than the 20/200 that was the lowest permissible visual acuity for PDT in the TAP-Investigation.[4,5] The fourth patient with predominantly classic CNV has experienced decrease in visual acuity from 60/200 to 10/160 accompanied by leakage in FA 3 months after the first PDT. He opted to proceed with macular surgery rather than PDT retreatment. PDT 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 therapeutical options were discussed in details. The study followed the guidelines of the declaration of Helsinki as revised in Tokyo and Venice 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 Immunohistology

Within minutes after surgery, excised CNV membranes were fixed in 3.7% formalin and embedded in paraffin. After serial sections were de-paraffinized and re-hydrated, antigen retrieval was accomplished by proteolytic digestion with 0.5% pronase (Sigma, St. Louis, MO) for CK18 and with Proteinase K (Dako) for 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 the antigens excluding VEGF according to the manufacturer's protocol (Vectastain Universal Elite ABC PK-6200 kit, Vector Laboratories, Burlinghame, CA). To block endogenous peroxidase activities, 3% hydrogen peroxide and 0.1% sodium acid were applied. After preincubation with horse serum, sections were probed with the primary antibodies specific for human CD105 (Mouse, Mab, Clone SN6h, Dako), CD34 (Mouse, Mab, Immunotech, Hamburg, FRG), Ki-67 (Mouse, Mab, Clone Ki-S5, Dako), and CK 18 (Mouse, monoclonal, Progen, Heidelberg, Germany). Incubation with the biotinylated horse anti-mouse secondary antibody and the ABC Complex (Vectastain Universal Elite ABC PK-6200 kit, Vector Laboratories, Burlinghame, CA) was followed by the development with a 3-Diaminobenzidine (Fluka, Buchs, FRG)