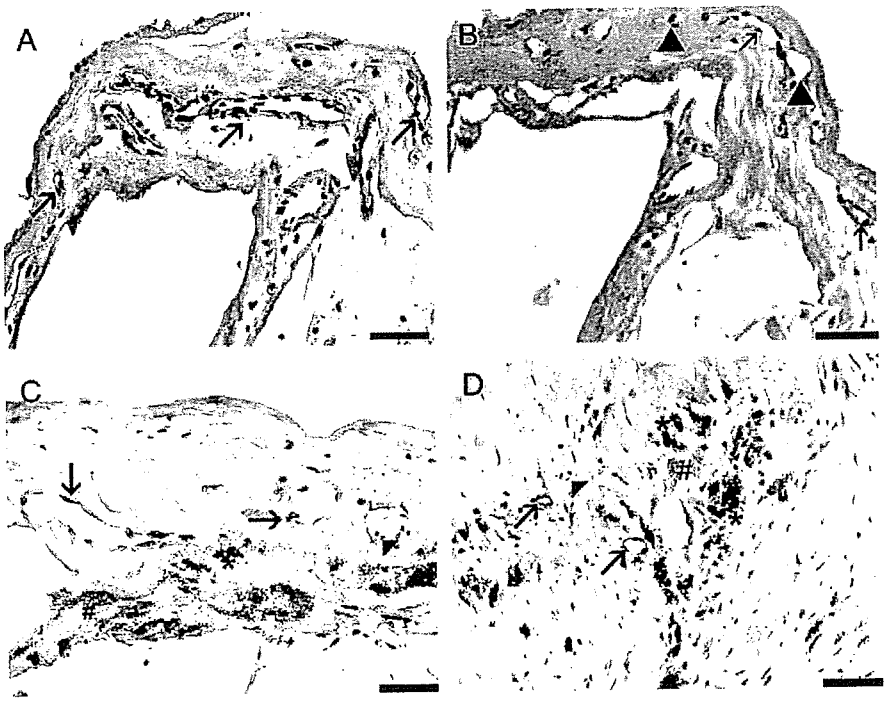

Fig. 3. Immunohistochemistry of the three different CNV membranes probed with antibodies against CD105 (A) and E-selectin (B–D). Sections were stained with 3-diaminobenzidine (A) and AEC (B–D) resulting in a brown and red chromogen, respectively and counterstained with hematoxylin. (A) CD105 is selectively 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 asterisks 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 72.89, range 0–514.081, mean 149.125, SE 35.4) was significantly higher than in II CNV (median 12.85 nuclei/mm^2, range 0–113.693, mean 28.931, SE 12.85) ($p = 0.0059$).

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 EC (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,

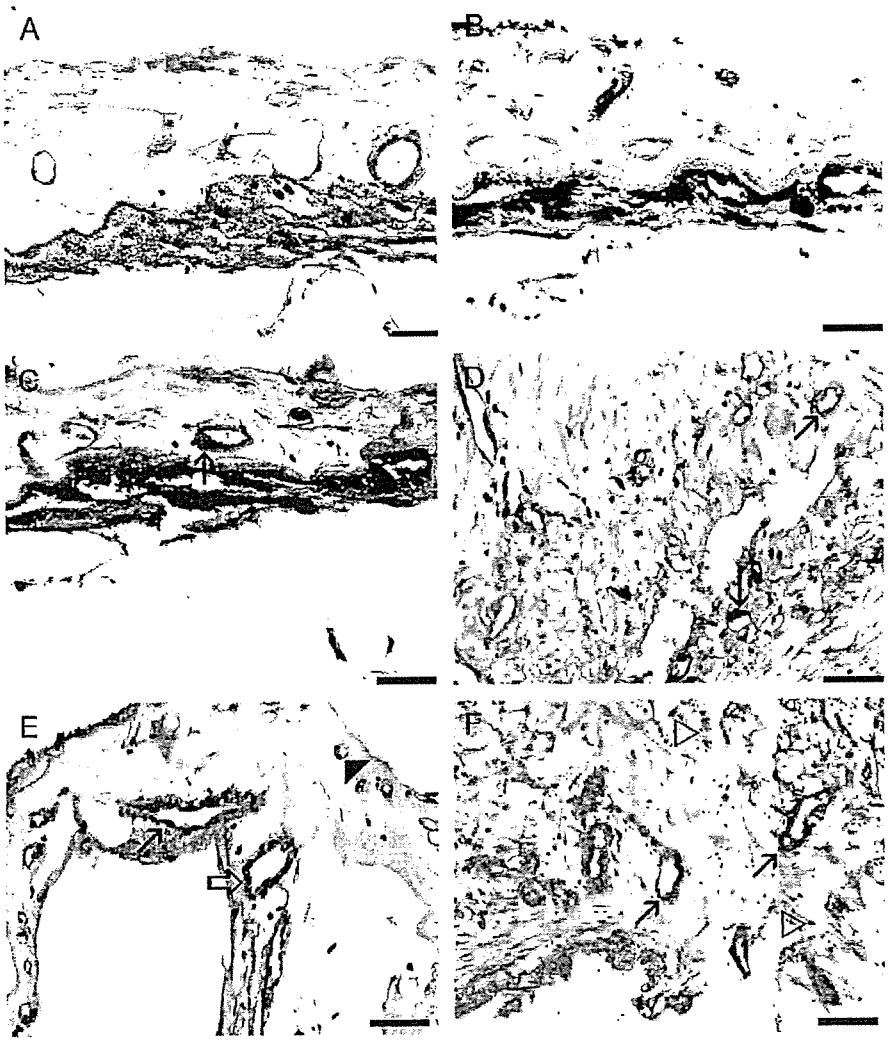

Fig. 4. Serial sections from those specimens in Fig. 3 (A-E) and another CNV (F). Sections are stained with PAS (A) or probed with antibodies against cytokeratin18 (B), endostatin (C–F). (A) PAS staining helps the overall orientation of the specimen. (B) RPE cells express Cytokeratin18 (*) in the same 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 EC 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

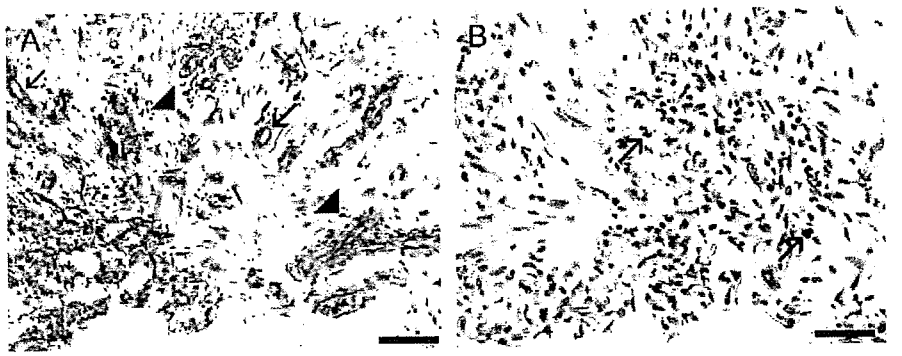

Fig. 5. Immunohistochemistry of serial sections from the CNV specimen shown in Fig. 4F probed with antibodies against VEGF (A) and Ki-67 (B). Sections were stained with red alkaline phosphatase substrate (A) and 3-diaminobenzidine (B) resulting in a red and brown chromogen, respectively, and counterstained 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 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., 2002). 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. (2004).

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, endogenous 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 antiangiogenesis 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.

Acknowledgements

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References

Abdollahi, A., Lipson, K.E., Sckell, A., Zieher, H., Klenke, F., Poerschke, D., Roth, A., Han, X., Krix, M., Bischof, M., Hahnfeldt, P., Grone, H.J., Debus, J., Hlatky, L., Huber, P.E., 2003. Combined therapy with direct and indirect angiogenesis inhibition results in enhanced antiangiogenic and antitumor effects. Cancer Res. 63, 8890–8898.

Abdollahi, A., Hahnfeldt, P., Maercker, C., Grone, H.J., Debus, J., Ansorge, W., Folkman, J., Hlatky, L., Huber, P.E., 2004. Endostatin's antiangiogenic signaling network. Mol. Cells 13, 649–663.

Amin, R., Pulkin, J.E., Frank, R.N., 1994. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 35, 3178–3188.

Auricchio, A., Behling, K.C., Maguire, A.M., O'Connor, E.M., Bennett, J., Wilson, J.M., Tolentino, M.J., 2002. Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. Mol. Ther 6, 490–494.

Bhutto, I.A., Kim, S.Y., McLeod, D.S., Merges, C., Fukai, N., Olsen, B.R., Lutty, A., 2004. Localization of collagen XVIII and the endostatin portion of collagen XVIII in aged human control eyes and eyes with age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 45, 1544–1552.

Dhanabal, M., Ramchandran, R., Waterman, M.J., Lu, H., Knebelmann, B., Segal, M., Sukhatme, V.P., 1999. Endostatin induces endothelial cell apoptosis. J. Biol. Chem. 274, 11721–11726.

Dixelius, J., Larsson, H., Sasaki, T., Holmqvist, K., Lu, L., Engstrom, A., Timpl, R., Welsh, M., Claesson-Welsh, L., 2000. Endostatin-induced tyrosine kinase signaling through the Shb adaptor protein regulates endothelial cell apoptosis. Blood 95, 3403–3411.

Dixelius, J., Cross, M., Matsumoto, T., Sasaki, T., Timpl, R., Claesson-Welsh, L., 2002. Endostatin regulates endothelial cell adhesion and cytoskeletal organization. Cancer Res. 62, 1944–1947.

Ergun, S., Kilic, N., Wurmbach, J.H., Ebrahimnejad, A., Fernando, M., Sevinc, S., Kilic, E., Chalajour, F., Fiedler, W., Lauke, H., Lamszus, K., Hammerer, P., Weil, J., Herbst, H., Folkman, J., 2001. Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. Angiogenesis 4, 193–206.

Eriksson, K., Magnusson, P., Dixelius, J., Claesson-Welsh, L., Cross, M.J., 2003. Angiostatin and endostatin inhibit endothelial cell migration in response to FGF and VEGF without interfering with specific intracellular signal transduction pathways. FEBS Lett. 536, 19–24.

Folkman, J., 2004. Endogenous angiogenesis inhibitors, 2004. APMIS 112 (7–8), 496–507.

Ferreras, M., Felbor, U., Lenhard, T., Olsen, B.R., Delaisse, J., 2000. Generation and degradation of human endostatin proteins by various proteinases. FEBS Lett. 486, 247–251.

Fukai, N., Eklund, L., Marneros, A.G., Oh, S.P., Keene, D.R., Tamarkin, L., Niemela, M., Ilves, M., Li, E., Pihlajaniemi, T., Olsen, B.R., 2002. Lack of collagen XVIII/endostatin results in eye abnormalities. EMBO J 21, 1535–1544.

Green, W.R., 1996. The retina. In: Spencer, W.H. (Ed.), Ophthalmic Pathology: An Atlas and Textbook. WB Saunders, Philadelphia, pp. 982–1051.

Grisanti, S., Canbek, S., Kaiserling, E., Adam, A., Lafaut, B., Gelisken, F., Szurman, P., Henke-Fahle, S., Oficjalska-Mlynczak, J., Bartz-Schmidt, K.U., 2004. Expression of endoglin in choroidal neovascularization. Exp. Eye. Res. 78, 207–213.

Grossniklaus, H.E., Ling, J.X., Wallace, T.M., Dithmar, S., Lawson, D.H., Cohen, C., Elner, V.M., Elner, S.G., Sternberg Jr., P., 2002. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. Mol. Vis 21, 119–126.

Kim, Y.M., Jang, J.W., Lee, O.H., Yeon, J., Choi, E.Y., Kim, K.W., Lee, S.T., Kwon, Y.G., 2000. Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase 2. Cancer Res. 60, 5410–5413.

Kivela, T., 1995. Immunohistochemical staining followed by bleaching of melanin: a practical method for ophthalmic pathology. Br. J. Biomed. Sci. 52, 325–326.

Kvanta, A., Algvere, P.V., Berglin, L., Seregard, S., 1996. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest. Ophthalmol. Vis. Sci. 37, 1929–1934.

Lambert, V., Wielockx, B., Munaut, C., Galopin, C., Jost, M., Itoh, T., Werb, Z., Baker, A., Libert, C., Krell, H.W., Foidart, J.M., Noel, A.,

Rakic, J.M., 2003. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. FASEB J. 17, 2290–2292.

Lanzer, R.C., Raff, M.C., 1987. Astrocytes induce blood-brain barrier properties in endothelial cells. Nature 325, 253–257.

Lee, S.J., Jang, J.W., Kim, Y.M., Lee, H.I., Jeon, J.Y., Kwon, Y.G., Lee, S.T., 2002. Endostatin binds to the catalytic domain of matrix metalloproteinase-2. FEBS Lett. 519, 147–152.

Luo, J., Lin, J., Paranya, G., Bischoff, J., 1998. Angiostatin upregulates E-selectin in proliferating endothelial cells. Biochem. Biophys. Res. Commun 245 (3), 906–911.

Luo, J., Paranya, G., Bischoff, J., 1999. Noninflammatory expression of E-selectin is regulated by cell growth. Blood 93, 3785–3791.

Lutty, G., Grunwald, J., Majji, A.B., Uyama, M., Yoneya, S., 1999. Changes in choriocapillaris and retinal pigment epithelium in age-related macular degeneration. Mol. Vis. 5, 35.

Makitie, T., Tarkkanen, A., Kivela, T., 1998. Comparative immunohistochemical oestrogen receptor analysis in primary and metastatic uveal melanoma. Graefes Arch. Clin. Exp. Ophthalmol. 236, 415–419.

Marneros, A.G., Keene, D.R., Hansen, U., Fukai, N., Moulton, K., Goletz, P.L., Moiseyev, G., Pawlyk, B.S., Halfter, W., Dong, S., Shibata, M., Li, T., Crouch, R.K., Bruckner, P., Olsen, B.R., 2004. Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. EMBO J. 14, 89–99.

Martin, G., Schlunck, G., Hansen, L.L., Agostini, H.T., 2004. Differential expression of angioregulatory factors in normal and CNV-derived human retinal pigment epithelium. Graefes Arch. Clin. Exp. Ophthalmol. 242, 321–326.

Mori, K., Ando, A., Gehlbach, P., Nesbitt, D., Takahashi, K., Goldsteen, D., Penn, M., Chen, C.T., Mori, K., Melia, M., Phipps, S., Moffat, D., Brazzell, K., Liau, G., Dixon, K.H., Campochiaro, P.A., 2001. Inhibition of choroidal neovascularization by intravenous injection of adenoviral vectors expressing secretable endostatin. Am. J. Pathol. 159, 313–320.

Noma, H., Funatsu, H., Yamashita, H., Kitano, S., Mishima, H.K., Hori, S., 2002. Regulation of angiogenesis in diabetic retinopathy: possible balance between vascular endothelial growth factor and endostatin. Arch. Ophthalmol. 120, 1075–1080.

O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R., Folkman, J., 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88, 277–285.

Pepper, M.S., 2001. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler. Thromb. Vasc. Biol. 21. 1104–1017.

Sasaki, T., Fukai, N., Mann, K., Gohring, W., Olsen, B.R., Timpl, R., 1998. Structure, function and tissue forms of the C-terminal globular domain of collagen XVIII containing the angiogenesis inhibitor endostatin. EMBO J. 17, 4249–4256.

Sertie, A.L., Sossi, V., Camargo, A.A., Zatz, M., Brahe, C., Passos-Bueno, M.R., 2000. Collagen XVIII, containing an endogenous inhibitor of angiogenesis and tumor growth, plays a critical role in the maintenance of retinal structure and in neural tube closure (Knobloch syndrome). Hum. Mol. Genet. 9, 2051–2058.

Shen, W.Y., Yu, M.J., Barry, C.J., Constable, I.J., Rakoczy, P.E., 1998. Expression of cell adhesion molecules and vascular endothelial growth factor in experimental choroidal neovascularisation in the rat. Br. J. Ophthalmol. 82, 1063–1071.

Skovseth, D.K., Veuger, M.J., Sorensen, D.R., De Angelis, P.M., Haraldsen, G., 2005. Endostatin dramatically inhibits endothelial cell migration, vascular morphogenesis and perivascular cell recruitment in vivo. Blood 105, 1044–1051.

Takahashi, K., Saishin, Y., Saishin, Y., Silva, R.L., Oshima, Y., Oshima, S., Melia, M., Paszkiet, B., Zerby, D., Kadan, M.J., Liau, G., Kaleko, M., Connelly, S., Luo, T., Campochiaro, P.A., 2003. Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment. FASEB J. 17, 896–898.

The Macular Photocoagulation Study Group, 1991. Argon laser photocoagulation for neovascular maculopathy: five year results from randomized clinical trials. Arch. Ophthalmol. 109, 1109–1114.

Wada, M., Ogata, N., Otsuji, T., Uyama, M., 1999. Expression of vascular endothelial growth factor and its receptor (KDR/flk-1) mRNA in experimental choroidal neovascularization. Curr. Eye Res. 18, 203–213.

Wang, W., Niu, X.G., Xie, L.X., Dong, X.G., 2004. Liposomes mediated plasmids encoding human endostatin inhibits endothelial cell proliferation. Zhonghua Yan Ke. Za. Zhi. 40, 321–325.

Wang, Y.S., Eichler, W., Friedrichs, U., Yafai, Y., Hoffmann, S., Yasukawa, T., Hui, Y.N., Wiedemann, P., 2005. Impact of endostatin on bFGF-induced proliferation, migration, and matrix metalloproteinase-2 expression/secretion of bovine choroidal endothelial cells. Curr. Eye Res. 30, 479–489.

Yamaguchi, N., Anand-Apte, B., Lee, M., Sasaki, T., Fukai, N., Shapiro, R., Que, I., Lowik, C., Timpl, R., Olsen, B.R., 1999. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J 18, 4414–4423.

Yeh, D.C., Bula, D.V., Miller, J.W., Gragoudas, E.S., Arroyo, J.G., 2004. Expression of leukocyte adhesion molecules in human subfoveal choroidal neovascular membranes treated with and without photodynamic therapy. Invest. Ophthalmol. Vis. Sci. 45, 2368–2373.

Yu, Y., Moulton, K.S., Khan, M.K., Vineberg, S., Boye, E., Davis, V.M., O'Donnell, P.E., Bischoff, J., Milstone, D.S., 2004. E-selectin is required for the antiangiogenic activity of endostatin. Proc. Natl. Acad. Sci. USA 101, 8005–8010.

Zatterstrom, U.K., Felbor, U., Fukai, N., Olsen, B.R., 2000. Collagen XVIII/endostatin structure and functional role in angiogenesis. Cell Struct. Funct. 25, 97–101.

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.[1,2] 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.[3] Photodynamic therapy seems to be an ideal treatment approach for CNV, allowing selective photothrombosis 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.[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 acuity loss of 2 Early Treatment Diabetic Retinopathy Study lines within 6 months.[3,9]

Table. Clinical Characteristics of Patients Treated With Photodynamic Therapy Before Surgical Removal of the Choroidal Neovascular Membrane

Case No./ Sex/Age, y	Eye	CNV Type	Visual Acuity	PDT Treatments, No.	Time to Surgery From Each PDT Session, d
1/M/76	L	Classic	0.025	1	3
2/F/78	R	Classic	0.020	1	3
3/M/54	L	Predominantly classic	0.063	2	113, 3
4/M/84	L	Classic	0.025	1	3
5/M/83	L	Classic	0.030	1	34
6/F/85	L	Classic	0.030	1	37
7/F/73	R	Occult	0.100	3	208, 138, 40
8/M/79	L	Classic	0.100	1	55
9/F/80	R	Classic	0.100	2	172, 69
10/M/77	L	Minimally classic	0.250	1	84
11/M/93	R	Classic	0.160	2	154, 95
12/F/76	L	Occult	0.600	1	105
13/M/81	L	Classic	0.080	2	213, 131
14/F/70	R	Classic	0.050	2	151, 132
15/F/78	L	Classic	0.050	3	344, 222, 146
16/M/77	L	Classic	1/30 MV	3	329, 245, 147
17/M/72	L	Predominantly classic	0.080	2	232, 156
18/F/74	R	Hemorrhagic	0.300	1	246
19/F/73	L	Classic	HM	4	677, 558, 467, 383
20/F/77	L	Predominantly classic	1/15 MV	7	Unknown, 772, 655*

Abbreviations: CNV, choroidal neovascularization; HM, hand movement; L, left; MV, meter vision; PDT, photodynamic therapy; R, right.
*Time of first through fifth PDT sessions unknown.

Although many preclinical studies[3] 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[4-6,8] and Verteporfin in Photodynamic Therapy[7,8] studies. 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 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[4,5]) 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.[4,5] 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 cytokeratin 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 acid 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-S5; 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-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 (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. $P \leq .05$ was considered statistically significant.

RESULTS

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 1**A). 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 2**A). In CNV membranes devoid of PDT, all of the vessels stained positively for CD34 (**Figure 3**A) 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/mm^2; range, 0-514.08 nuclei/mm^2) than inflammatory-inactive specimens (n = 10; median proliferative activity, 6.81 nuclei/mm^2; range, 0-113.69 nuclei/mm^2) ($P = .001$). 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

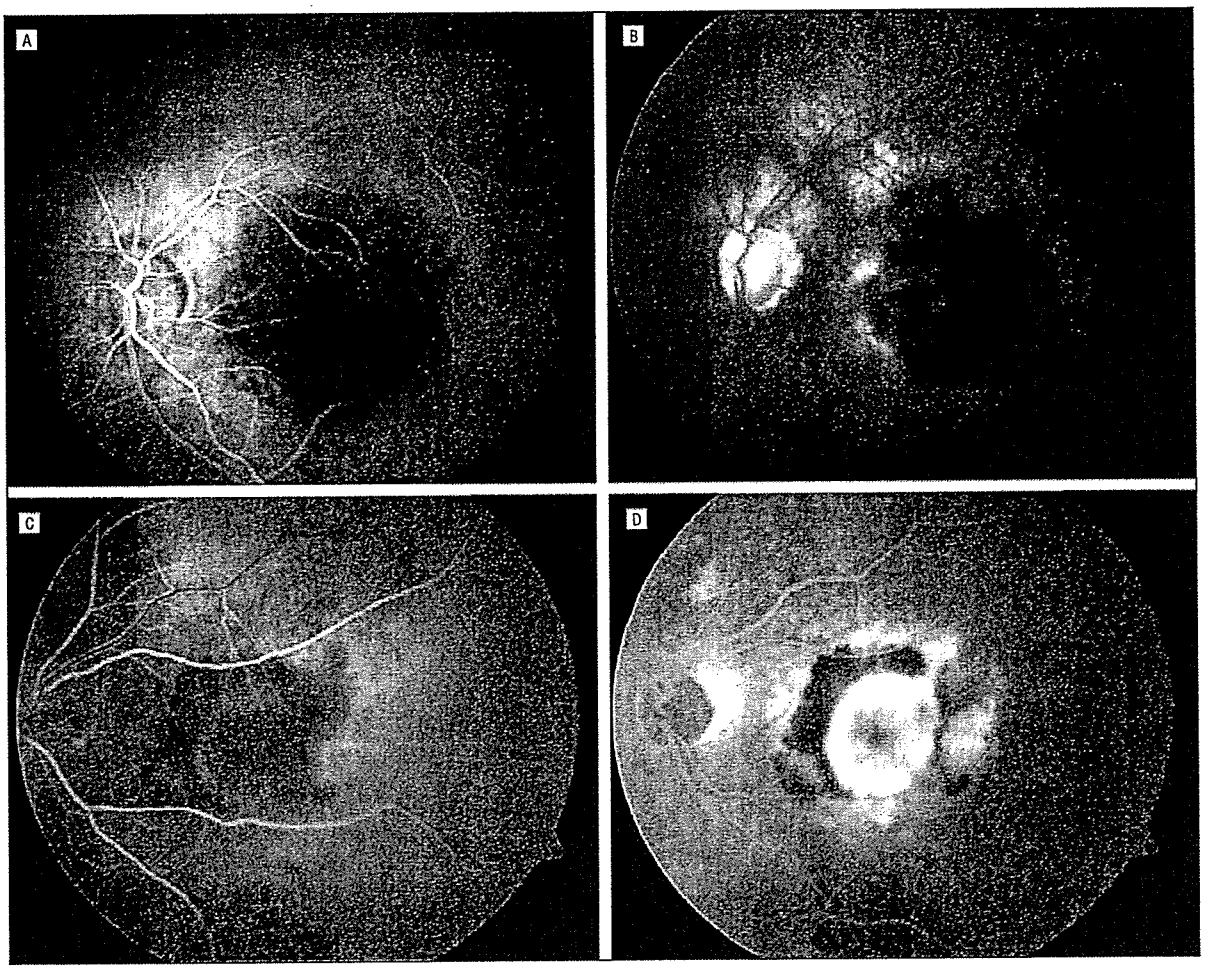

Figure 1. Fluorescein angiographs taken on the day of surgical extraction of the choroidal neovascular membrane. A, Early phase of fluorescein angiography 3 days after photodynamic therapy in case 4. B, Late phase of fluorescein angiography 3 days after photodynamic therapy in case 4. C, Early phase of fluorescein angiography 34 days after photodynamic therapy in case 5. D, Late phase of fluorescein angiography 34 days after photodynamic therapy in case 5.

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/mm^2; range, 0-9.71 nuclei/mm^2) was smaller than that in the CNV membranes without prior PDT (median proliferative activity, 53.20 nuclei/mm^2; range, 0-514.08 nuclei/mm^2), 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/mm^2; range, 0-829.29 nuclei/mm^2) (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 4**A 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 (66.7%) 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-

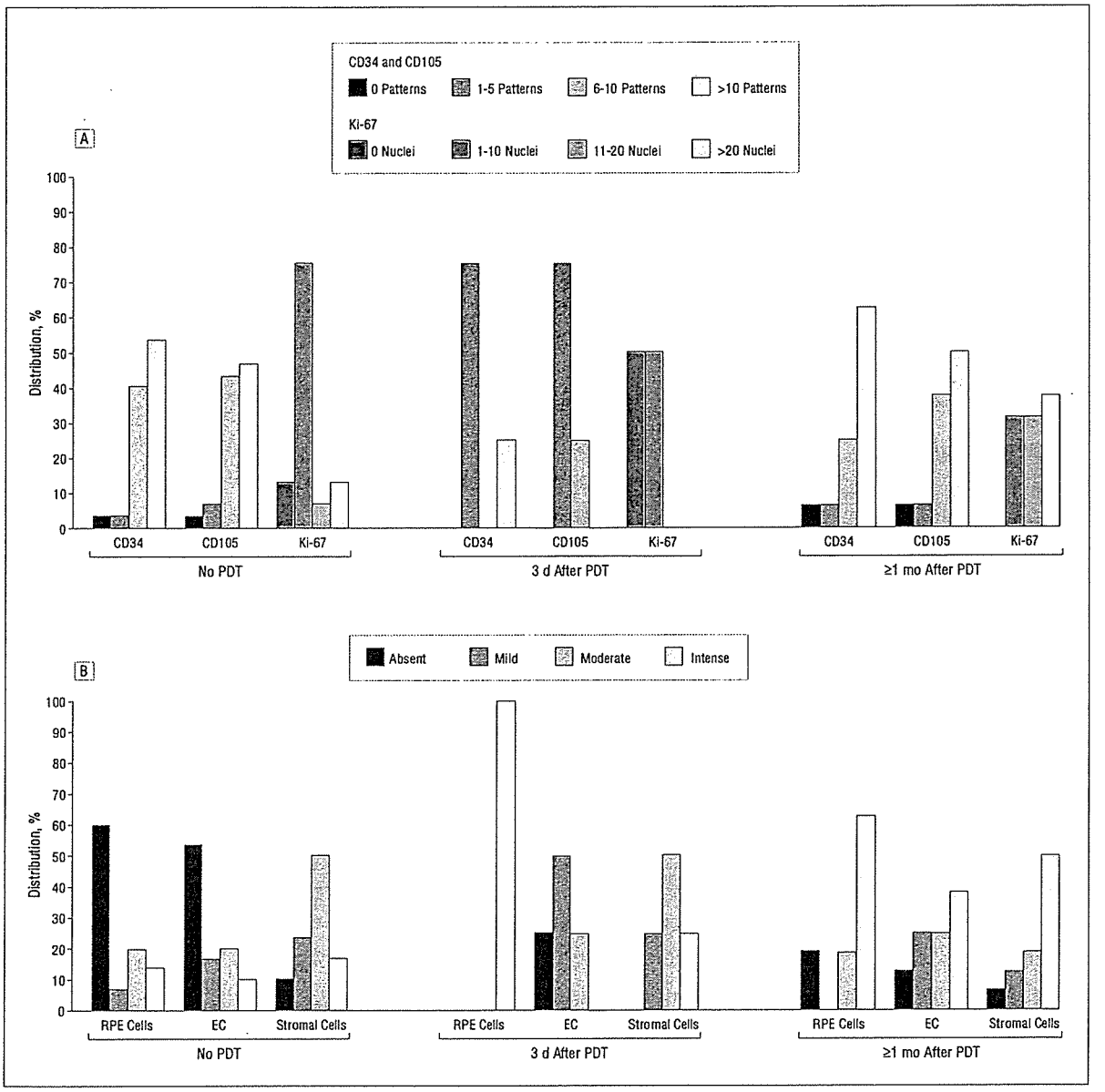

Figure 2. The distribution of endothelial cells (EC) (with vascularization calculated by counting the number of CD34- and CD105-positive vascular-like patterns in the most vascularized area under 200× magnification) and proliferation marker (with proliferative activity evaluated by counting the absolute 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.

tensity showed no predilection according to the localization in the CNV.

COMMENT

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.[9-15] 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

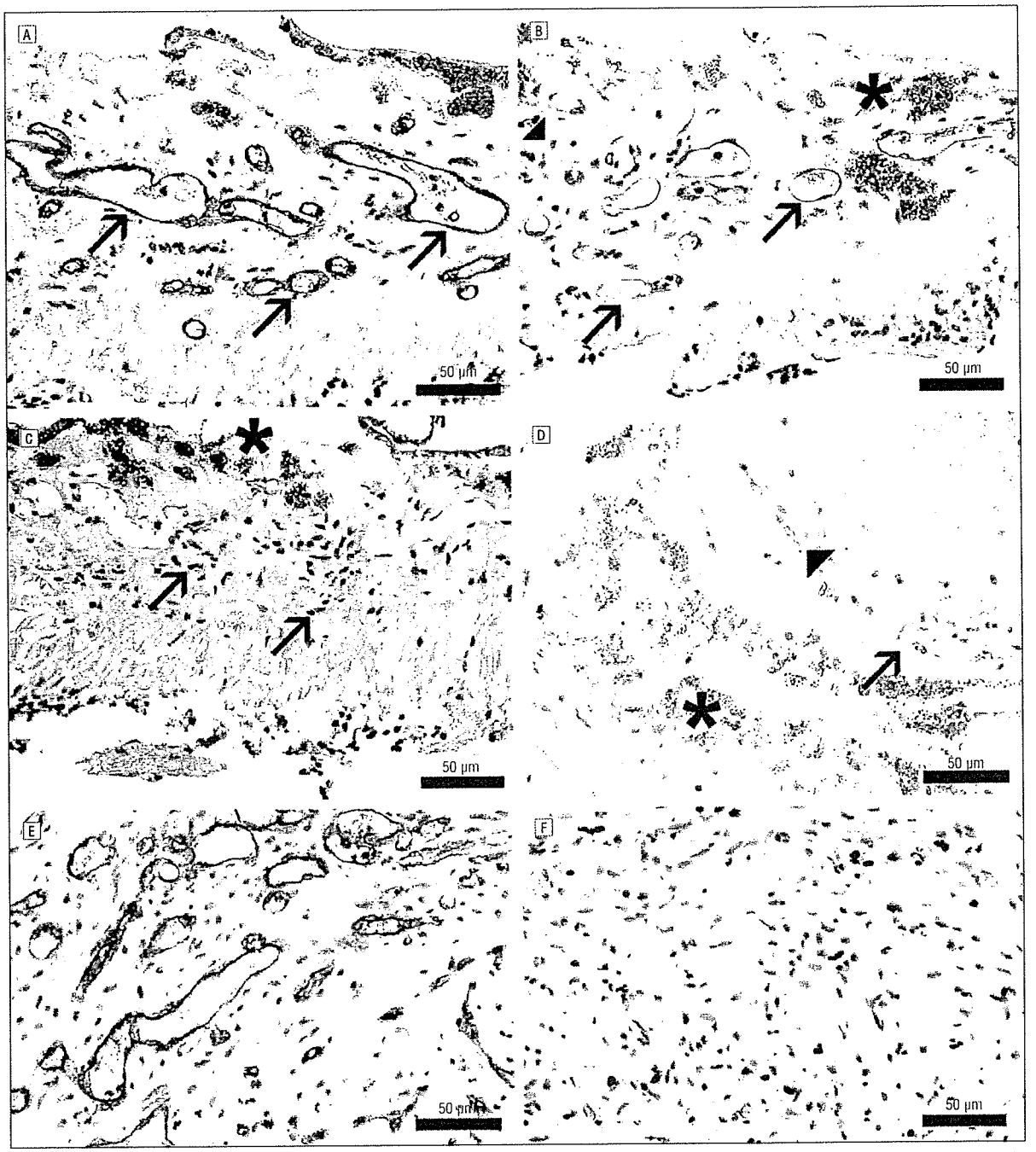

Figure 3. Photomicrographs of a surgically excised choroidal neovascular membrane. The specimen of a case that did not receive photodynamic therapy was probed with antibodies against CD34 (A) and CD105 (B), stained with 3-diaminobenzidine (resulting in a brown chromogen), and counterstained with hematoxylin. 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).

in animal models,[14] and CNV has been suppressed by anti-VEGF therapy in primates.[15]

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

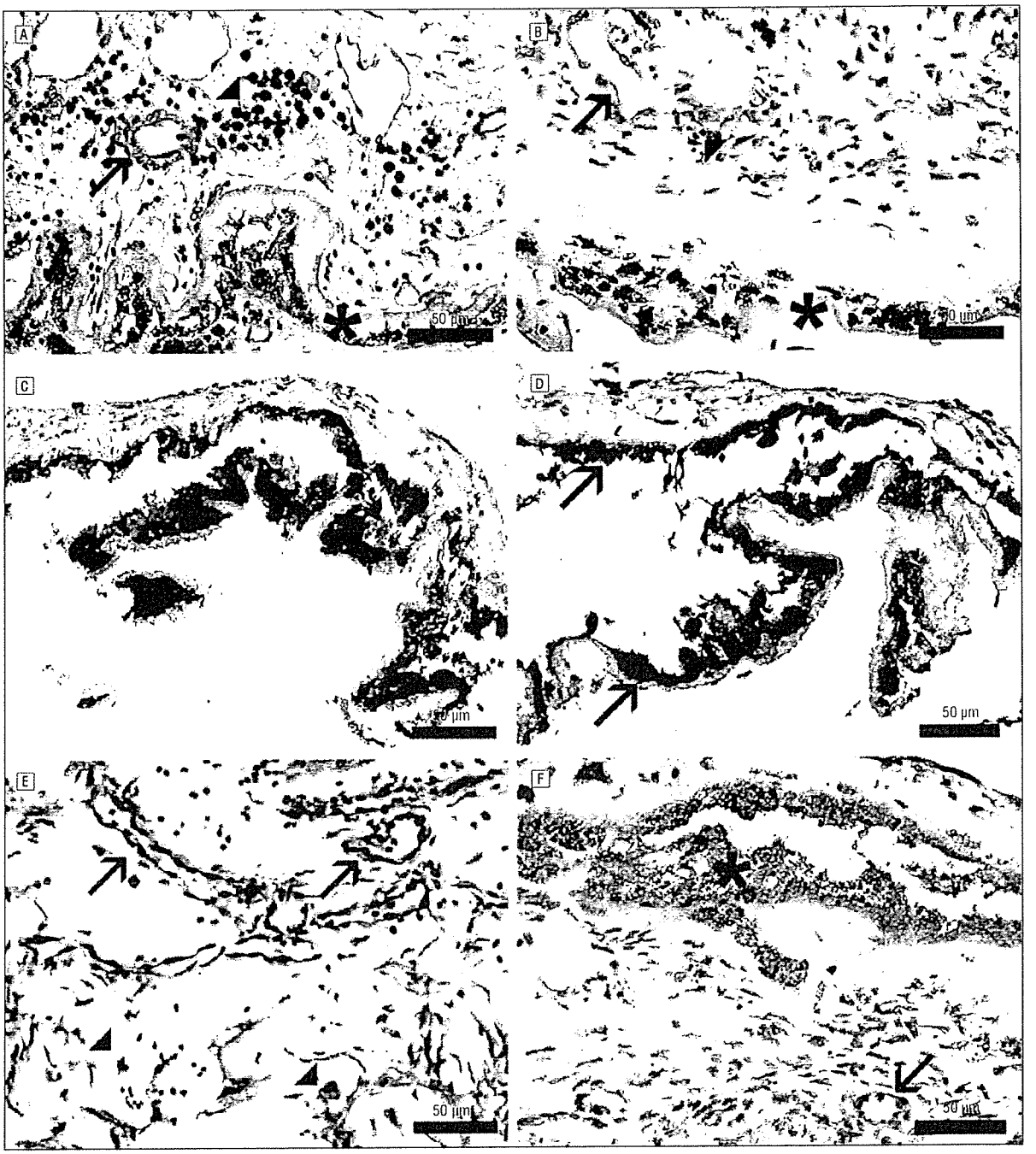

Figure 4. Photomicrographs of surgically excised choroidal neovascular membranes. A, A specimen that did not receive photodynamic therapy was stained for vascular endothelial growth factor (VEGF) (red chromogen) and showed a retinal pigment epithelial (RPE) cell layer (asterisk), vascularization (arrow), 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 (E) (asterisk).

found in 90% of the cases. These results confirm the findings by Kvanta et al,[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[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 stro-

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.[20,21] Histological analysis of PDT-treated human eyes supported these findings by identifying an occluded choriocapillary layer within the spot produced by the laser.[3,22,23] 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.[20] 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[24] as well as by reactive oxygen intermediates.[25] Either aggravated hypoperfusion of the choroid[21,26-28] 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.[29] 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[30] 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.[31] 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.[32,33] Intravitreal injections of anti-VEGF molecules should interrupt the vicious cycle induced by PDT. In a phase II study,[33] 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 reinitiation of the angiogenic cascade, the need for a counteracting adjunctive therapy started at the proper time becomes more and more obvious.[34]

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REFERENCES

1. Ghafour IM, Allan D, Foulds WS. Common causes of blindness and visual handicap in the west of Scotland. *Br J Ophthalmol.* 1983;67:209-213.
2. Hyman L. Epidemiology of eye disease in the elderly. *Eye.* 1987;1:330-341.
3. Schmidt-Erfurth U, Hasan T. Mechanisms of action of photodynamic therapy with verteporfin for the treatment of age-related macular degeneration. *Surv Ophthalmol.* 2000;45:195-214.
4. Treatment of Age-Related Macular Degeneration With Photodynamic Therapy (TAP) Study Group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials: TAP report 1. *Arch Ophthalmol.* 1999;117:1329-1345.
5. Treatment of Age-Related Macular Degeneration With Photodynamic Therapy (TAP) Study Group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials: TAP report 2. *Arch Ophthalmol.* 2001;119:198-207.
6. Treatment of Age-Related Macular Degeneration With Photodynamic Therapy (TAP) Study Group. Verteporfin therapy of subfoveal choroidal neovascularization in patients with age-related macular degeneration: additional information regarding baseline lesion composition's impact on vision outcomes: TAP report No. 3. *Arch Ophthalmol.* 2002;120:1443-1454.
7. Verteporfin in Photodynamic Therapy Study Group. Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: two-year results of a randomized clinical trial including lesions with occult with no classic choroidal neovascularization: verteporfin in photodynamic therapy report 2. *Am J Ophthalmol.* 2001;131:541-560.
8. Blinder KJ, Bradley S, Bressler NM, et al; Treatment of Age-related Macular Degeneration With Photodynamic Therapy Study Group; Verteporfin in Photodynamic Therapy Study Group. Effect of lesion size, visual acuity, and lesion composition on visual acuity change with and without verteporfin therapy for choroidal neovascularization secondary to age-related macular degeneration: TAP and VIP report No. 1. *Am J Ophthalmol.* 2003;136:407-418.

9. Grossniklaus HE, Ling JX, Wallace TM, et al. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. *Mol Vis.* 2002;8:119-126.
10. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci.* 1996;37:1929-1934.
11. Amin R, Puklin JE, Frank RN. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 1994;35:3178-3188.
12. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* 1996;37:855-868.
13. Wada M, Ogata N, Otsuji T, Uyama M. Expression of vascular endothelial growth factor and its receptor (KDR/fek-1) mRNA in experimental choroidal neovascularization. *Curr Eye Res.* 1999;18:203-213.
14. Kwak N, Okamoto N, Wood J, Campochiaro P. VEGF is major stimulator in model of choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2000;41:3158-3164.
15. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch Ophthalmol.* 2002;120:338-346.
16. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996;380:435-439.
17. Blaauwgeers HG, Holtkamp GM, Rutten H, et al. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris: evidence for a trophic paracrine relation. *Am J Pathol.* 1999;155:421-428.
18. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci U S A.* 1995;92:10457-10461.
19. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.
20. Grisanti S, Tatar O, Canbek S, et al. Immunohistopathologic evaluation of choroidal neovascular membranes following verteporfin photodynamic therapy. *Am J Ophthalmol.* 2004;137:914-923.
21. Michels S, Schmidt-Erfurth U. Sequence of early vascular events after photodynamic therapy. *Invest Ophthalmol Vis Sci.* 2003;44:2147-2154.
22. Schmidt-Erfurth U, Laqua H, Schlötzer-Schrehardt U, Vistenz A, Naumann GOH. Histopathological changes following photodynamic therapy in human eyes. *Arch Ophthalmol.* 2002;120:835-844.
23. Schlötzer-Schrehardt U, Vistenz A, Naumann GOH, Laqua H, Michels S, Schmidt-Erfurth U. Dose-dependent structural effects of photodynamic therapy on choroidal and retinal structures of human eyes. *Graefes Arch Clin Exp Ophthalmol.* 2002;240:748-757.
24. Shima DT, Adamis AP, Ferrara N, et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Mol Med.* 1995;1:182-193.
25. Kuroki M, Voest EE, Amano S, et al. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. *J Clin Invest.* 1996;98:1667-1675.
26. Schmidt-Erfurth UM, Michels S. Changes in confocal indocyanine green angiography through two years following photodynamic therapy with verteporfin *Ophthalmology.* 2003;110:1306-1314.
27. Schmidt-Erfurth U, Michels S, Barbazetto I, Laqua H. Photodynamic effects on choroidal neovascularization and physiological choroids. *Invest Ophthalmol Vis Sci.* 2002;43:830-841.
28. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol.* 2004;242:91-101.
29. Spilsbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. *Am J Pathol.* 2000;157:135-144.
30. Bula DV, Iliaki E, Gragoudas E, Miller JW, Arroyo J. Pigment epithelium–derived factor, angiopoietin-1, and VEGF expression in human choroidal neovascular membranes treated with photodynamic therapy [ARVO abstract]. *Invest Ophthalmol Vis Sci.* 2004;45:E-abstract 1787. http://www.iovs.org. Accessed February 15, 2005.
31. Schmidt-Erfurth U, Schlötzer-Schrehard U, Cursiefen C, Michels S, Beckendorf A, Naumann GOH. Influence of photodynamic therapy on expression of vascular endothelial growth factor (VEGF), VEGF receptor 3, and pigment epithelium–derived factor. *Invest Ophthalmol Vis Sci.* 2003;44:4473-4480.
32. Eyetech Study Group. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. *Retina.* 2002;22:143-152.
33. Eyetech Study Group. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. *Ophthalmology.* 2003;110:979-986.
34. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR; VEGF Inhibition Study in Ocular Neovascularization Clinical Trial Group. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med.* 2004;351:2805-2816.

the evaluation for CTX to support early definitive therapy. Moreover, the measurement of cholestanol levels when a subject is initially seen with presenile cataracts of questionable etiology could be a valuable clinical tool.

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1. Forsius H, Arentz-Grastvedt B, Eriksson AW. Juvenile cataract with autosomal recessive inheritance: a study from the Aland Islands, Finland. *Acta Ophthalmol (Copenh)*. 1992;70:26-32.
2. Verrips A, van Engelen BG, Wevers RA, et al. Presence of diarrhea and absence of tendon xanthomas in patients with cerebrotendinous xanthomatosis. *Arch Neurol*. 2000;57:520-524.
3. Lorincz MT, Rainier S, Thomas D, Fink JK. Cerebrotendinous xanthomatosis: possible higher prevalence than previously recognized. *Arch Neurol*. 2005;62:1459-1463.
4. Batta AK, Salen G, Tint GS. Hydrophilic 7 beta-hydroxy bile acids, lovastatin, and cholestyramine are ineffective in the treatment of cerebrotendinous xanthomatosis. *Metabolism*. 2004;53:556-562.
5. van Heijst AF, Verrips A, Wevers RA, Cruysberg JR, Renier WO, Tolboom JJ. Treatment and follow-up of children with cerebrotendinous xanthomatosis. *Eur J Pediatr*. 1998;157:313-316.

Familial Retinal Arterial Tortuosity Associated With Tortuosity in Nail Bed Capillaries

Familial retinal arterial tortuosity (fRAT) is characterized by marked tortuosity of second- and third-order retinal arteries with normal first-order arteries and venous system. Patients have variable transient vision loss owing to retinal hemorrhages after minor stress or trauma. Prognosis is usually excellent. Whether there is systemic involvement is controversial. We report 3 cases of fRAT associated with a high degree of tortuosity of capillaries at nailfold capillaroscopy as an indication of systemic vascular pathology.

Report of Cases. *Case 1*. A woman was first seen at age 19 years because of blurred vision after a minor car accident. Best-corrected visual acuity (BCVA) was 0.9 OD and 0.8 OS. Ophthalmologic examination revealed marked tortuosity of second- and third-order retinal arteries and multiple intraretinal and preretinal hemorrhages in both eyes (**Figure 1**A). The patient was observed. Four weeks later, BCVA was fully restored in both eyes and the hemorrhages had almost resolved.

Five years later, the patient reported frequent episodes of migraine. Tortuosity of the retinal vessels and BCVA remained unchanged, but the macular reflex appeared duller, and mild thickening of the inner limiting membrane was noted in both eyes (Figure 1B). No hemorrhages were observed.

Seven years after she was first seen, the patient had decreased visual acuity of 0.4 OD and 0.8 OS. She was in her 18th week of pregnancy and had undergone amniocentesis 2 days previously. Fundus examination showed several preretinal, foveal hemorrhages in both eyes. Four weeks later, BCVA returned to 1.0 OU and hemorrhages had resolved.

Twelve years after she was first seen, the patient reported episodes of blurred vision once per year, usually following minor exercise. Best-corrected visual acuity had always fully recovered. At this ophthalmologic examination, thickening of the inner limiting membrane was stable and there was 1 asymptomatic preretinal hemorrhage inferotemporal to the macula (Figure 1C). The patient still experienced 5 to 6 episodes of migraine per year but was otherwise healthy.

In visual field tests, scotomas were noted that corresponded to the locations of the hemorrhages. Fluorescein angiography demonstrated no leakage, staining, hypoperfusion, or capillary dropout. Neurologic examinations, including cranial magnetic resonance imaging, yielded normal findings. Extensive examinations in internal medicine, explicitly, tests for serologic factors including virus and bacteria antibody titers and for rheumatologic and autoimmune factors, coagulation tests, and serum electrophoresis, also yielded normal findings. The patient was not taking any systemic medication that would alter coagulation, and blood pressure was within normal limits.

At nailfold capillaroscopy, which was performed at the second and fourth visits, tortuosity of capillaries was highly increased in all fingers of both hands (Figure 1D and E). Minor rarefication and 1 avascular zone, but no microhemorrhages, were detected. Sodium fluorescein video nailfold capillaroscopy showed normal inflow and outflow, demonstrating absence of capillary spasm; normal transcapillary and interstitial diffusion of fluorescein; and normal halo. No other dermatologic disease, including Raynaud syndrome, was observed.

Case 2. The older sister of patient 1 reported a slight decrease in BCVA when first seen at age 28 years. However, BCVA was 1.0 OU. We found marked tortuosity of second- and third-order retinal arteries in both eyes but no hemorrhages. The patient had a history of 1 episode of transient microhematuria of unknown origin, but otherwise reported that she was healthy.

Seven years later, extensive ophthalmologic (**Figure 2**A), neurologic, and medical examinations were performed, analogous to those in patient 1. All findings were normal with the exception that antinuclear antibodies were 2-fold positive. Findings at dermatologic examination and nailfold capillaroscopy were identical to those in patient 1 (Figure 2B and C).

Case 3. The father of patients 1 and 2 was first seen at age 56 years and reported that he had never experienced any visual disturbances. He had a stroke with speech disturbance 8 years earlier, but reported no residual adverse effects. Best-corrected visual acuity was 1.0 OU. Marked tortuosity of second- and third-order retinal arteries was found in both eyes, without hemorrhages.

He was seen 7 years later, and extensive ophthalmologic (**Figure 3**A), neurologic, and medical examinations revealed atrial fibrillation, and

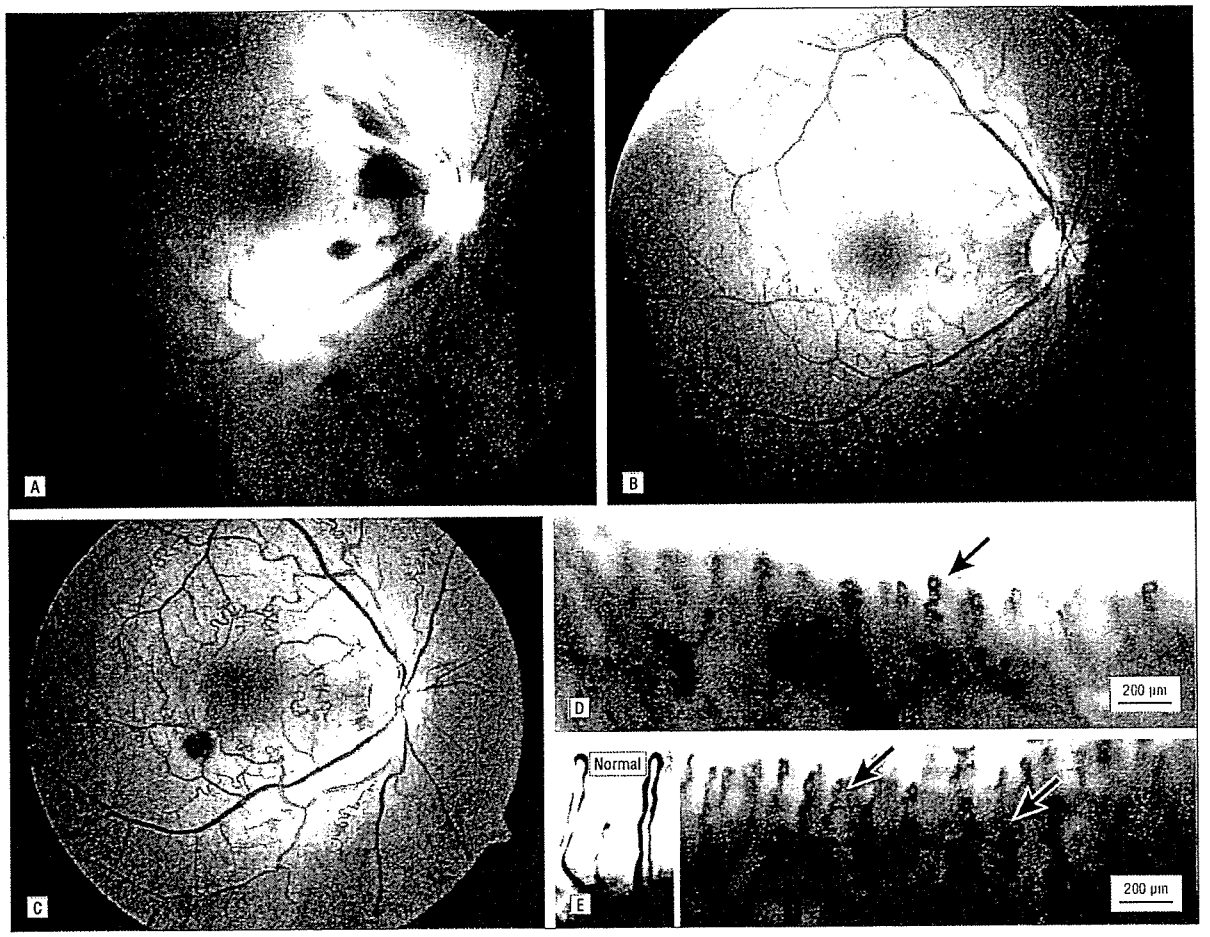

Figure 1. Patient 1. Views of the fundus and findings at nailfold capillaroscopy. A, When the patient was first seen after a minor car accident at age 19 years, multiple small hemorrhages were noted. She exhibited all of the typical features of familial retinal arterial tortuosity, with tortuosity in second- and third-order retinal arteries and normal large arteries and retinal veins. B, Five years later, no hemorrhages were seen and the retinal vasculature remained unchanged. C, Twelve years later, there was 1 preretinal hemorrhage inferotemporal to the macula; the retinal vasculature otherwise remained unchanged. D and E, Nailfold capillaroscopic findings in the right third and fourth fingers. Except for the high degree of tortuosity (present in approximately 30% of capillary loops; arrows depict typical examples), the capillaries were normal, a finding that was confirmed at sodium fluorescein video microscopy. For comparison, the inset in E shows enlargement of normal capillary loops at nailfold capillaroscopy in a healthy person. Note parallel arrangement of inflow and outflow arms.

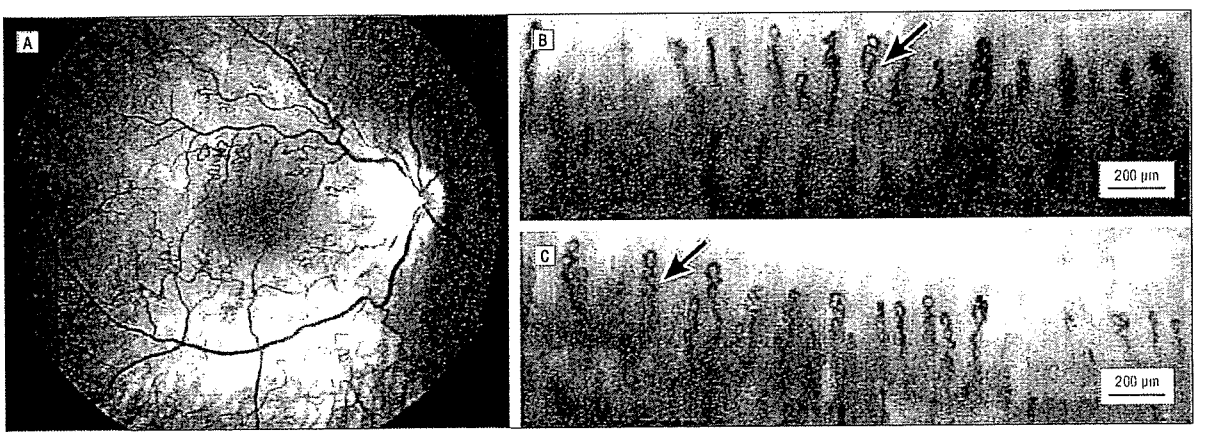

Figure 2. Patient 2. Views of the fundus and findings at nailfold capillaroscopy. A, At age 35 years, the patient demonstrated all of the typical findings of familial retinal arterial tortuosity. She had never experienced visual disturbances, although the degree of tortuosity was higher than in patient 1. B and C, Nailfold capillaroscopic findings in the left third and fourth fingers. Also in this patient, approximately 30% of capillary loops showed a high degree of tortuosity (arrows depict typical examples), while all other findings were normal, analogous to those in patient 1.

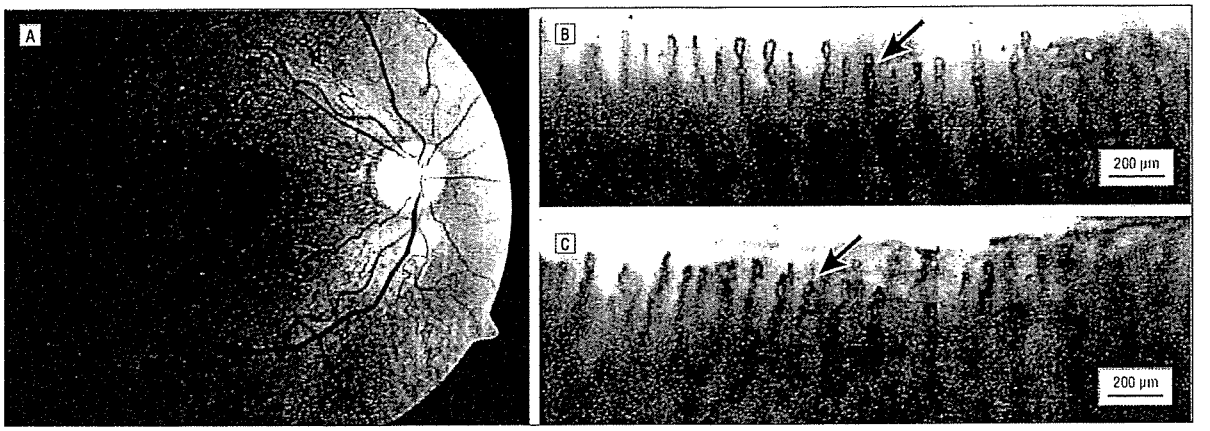

Figure 3. Patient 3. Views of the fundus and findings at nailfold capillaroscopy. A, When the patient was last seen at age 62 years, he demonstrated all of the typical findings of familial retinal arterial tortuosity. The patient had never experienced visual disturbances, although the degree of tortuosity was greater than in patients 1 and 2. B and C, Nailfold capillaroscopic findings in the patient's right second and fourth fingers. Approximately 30% of capillary loops showed a high degree of tortuosity (arrows depict typical examples), while all other findings were normal, analogous to those in patients 1 and 2.

warfarin sodium therapy was prescribed. As in patients 1 and 2, all other systemic findings were normal. Findngs at dermatologic examination and nailfold capillaroscopy were identical to those in patient 1 (Figure 2B and C).

Comment. All 3 patients had typical features of fRAT: only second- and third-order arteries were affected, while first-order arteries and the venous system were normal. The caliber, shape, and branchings of affected arteries were normal; no leakage or staining was observed at fluorescein angiography; symptomatic hemorrhages followed minor stress or trauma; visual prognosis was excellent; and no associated systemic disease was found in any of our 3 patients. These findings correspond well with the previously reported approximately 100 cases.[1,2] While in isolated cases systemic disease was found in patients with fRAT (malformation in the Kieselbach nasal septum, vascular mass in the spinal cord, sixth nerve palsy, simultaneous conjunctival hemorrhage, teleangiectasis of the bulbar conjunctiva), no consistent associated systemic disease has been reported and, thus, fRAT was generally believed to be an isolated retinal finding.[2,3] Our finding of marked capillary tortuosity at nailfold capillaroscopy favors systemic vascular disease in fRAT.

Recently, a syndrome has been reported consisting of features of fRAT with hematuria, muscular contractures, and sporadic other disorders such as cardiac arrhythmia.[4] This syndrome is distinct from the finding in our patients; only patient 2 had transient microhematuria and pronounced tortuosity in nail bed capillaries, in contrast to the unspecific findings at nailfold capillaroscopy found in patients with the newly reported syndrome, which is dominated by renal disease.

Nailfold capillaroscopy, as it was performed in our patients, is considered a mirror of systemic vascular processes, and a high degree of validity of correspondence and prognostic value is found, for example, in diabetes mellitus, systemic scleroderma, primary chronic polyarthritis, and systemic lupus erythematosus, and especially with ocular capillaries and in glaucoma.[5] Nailfold capillaroscopy demonstrated the identical features of capillary loops as retinal vessels in showing a high degree of tortuosity without any other pathologic findings such as leakage of dye, occlusion, or caliber abnormalities. A milder form of tortuosity at nailfold capillaroscopy has been described in patients with psoriatic arthritis,[6] but, to our knowledge, tortuosity of this magnitude has not been reported before. Considering that hemorrhages from retinal vessels are a hallmark of fRAT, manifestation in other tissues could be expected, although evidence for consistent systemic disease is thus far lacking in fRAT.

By demonstrating that capillary abnormalities are also found in nailfold capillaries of patients with fRAT, retinal vascular abnormalities can no longer be accepted as an isolated finding. We believe that, because of an increasing number of reports of this disease,[2] further investigation as to systemic involvement in patients with fRAT is warranted.

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1. Gass DM. Macular dysfunction caused by retinal vascular diseases. In: Gass DM, ed. *Stereoscopic Atlas of Macular Diseases*. 4th ed. St Louis, Mo: Mosby-Year Book Inc; 1997.
2. Sutter FK, Helbig H. Familial retinal arteriolar tortuosity: a review. *Surv Ophthalmol*. 2003; 48:245-255.
3. Scars J, Gilman J, Sternberg P Jr. Inherited retinal arteriolar tortuosity with retinal hemorrhages. *Arch Ophthalmol*. 1998;116:1185-1188.
4. Plaisier E, Alamowitch S, Gribouval O, et al. Autosomal-dominant familial hematuria with retinal arteriolar tortuosity and contractures: a novel syndrome. *Kidney Int*. 2005;67:2354-2360.
5. Flammer J, Pache M, Resink T. Vasospasm: its role in the pathogenesis of diseases with particular reference to the eye. *Prog Retin Eye Res*. 2001;20:319-349.
6. Grassi W, Core P, Carlino G, Cervini C. Nailfold capillary permeability in psoriatic arthritis. *Scand J Rheumatol*. 1992;21:226-230.

Impact of Verteporfin Photodynamic Therapy on Endostatin and Angiogenesis in Human Choroidal Neovascular Membranes

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IMPACT OF VERTEPORFIN PHOTODYNAMIC THERAPY ON ENDOSTATIN AND ANGIOGENESIS IN HUMAN CHOROIDAL NEOVASCULAR MEMBRANES

Running Title: IMPACT OF PDT ON ENDOSTATIN EXPRESSION IN CNV

Figures: 4
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ABSTRACT

Aim: To evaluate impact of verteporfin photodynamic therapy (PDT) on endostatin with regard to vascular endothelial growth factor (VEGF) expression in human choroidal neovascular membranes (CNV) secondary to age-related macular degeneration.
Methods: Retrospective review of an interventional case series of sixty-eight patients who underwent removal of CNV. Twenty-nine patients were treated with PDT 3 to 655 days prior to surgery. Thirty-nine CNV without previous treatment were used as controls. CNV were stained for CD34, CD105, Ki-67, cytokeratin18, endostatin, E-selectin and VEGF. "Predominance score of VEGF over endostatin" (PS) was defined calculating the difference between VEGF and endostatin staining scores.
Results: In four CNV treated by PDT 3 days previously, PS was significantly higher in retina pigment epithelium (RPE) (PS=2.5, p=0.0059) and stroma (PS=2, p=0.0152) than in the control group (PS=0). At longer post-PDT intervals, PS was significantly decreased in RPE (PS=0, p=0.0192) and stroma (PS=0, p=0.0152). Proliferative activity was high (p=0.0227) but mostly related to inflammatory cells. PDT did not influence E-selectin expression significantly.
Conclusions: VEGF predominance over endostatin early after PDT might contribute to enhanced angiogenic activity associated with recurrences. Strategies upregulating or replacing endostatin early after PDT might increase the effectiveness of PDT.