than resident macrophages which express CD68.[24,25] Thy-1 is a cell surface marker expressed on vascular EC which is up-regulated by inflammatory cytokines interleukin (IL)-1ß and tumor necrosis factor (TNF)-α but remains unaffected by growth factors such as VEGF.[26]

METHODS

Subjects and Treatments

We retrospectively reviewed 29 eyes of 29 consecutive AMD patients who had been treated with full macular translocation surgery with CNV removal **at ten distinct surgical sites between 1997 and 2005**. Except 14 patients without any preoperative therapy (control CNV), surgery was performed 3 to 9 days after verteporfin PDT (PDT CNV, n=4), TA monotherapy (TA CNV, n=5) or PDT+TA combination therapy (PDT+TA CNV, n=6). Clinical characteristics of patients treated with PDT, TA or PDT+TA preoperatively are summarized in Table 1.

Full macular translocation was offered when a) visual acuity was worse than 20/200 being the minimum visual acuity to recommend first PDT[2] b) visual deterioration progressed after initial PDT c) patient refused (re)-treatment with PDT or TA or PDT+TA due to continuous visual deterioration in the fellow eye in spite of therapy and d) (re)-treatment with PDT was impossible due to recurrent or massive submacular hemorrhage. Preoperative therapy with PDT, TA and PDT+TA was intended to decrease intraoperative hemorrhage, postoperative CNV recurrence and proliferative vitreoretinopathy rate.[5-7,27] **TA was prepared preservative free through sedimentation technique as previously described.**[28,29] Each patient gave written informed consent after experimental nature, risks and benefits of all therapy options had been explained. The study followed guidelines of declaration of Helsinki. The study and histological analysis of specimens were approved by local Institutional Review Board.

Table 1: Clinical characteristics of patients treated with intravitreal triamcinolone acetonide (TA) and/or verteporfin photodynamic therapy (PDT) before surgical removal of **subfoveal** choroidal neovascularization (CNV).

Case	Eye	Age/ Sex	CNV type	CNV Size (mm^2)	Preoperative VA	Time to surgery from	
						TA Injection	each PDT
1	R	90,m	hemorrhagic occult	0.12	10/200	3 days	
2	R	78,f	occult	0.21	10/100	3 days	
3	R	84,f	PED (RAP)	0.22	10/63	4 days	
4	R	70,f	PED, occult	0.13	10/40	7 days	
5	L	80,f	RAP	0.22	10/50	8 days	
6	L	76,m	classic	0.27	10/400		3 days
7	R	78,f	classic	0.10	10/500		3 days
8	L	54,m	predominantly classic	0.04	10/160		113/3 days
9	L	84,m	classic	0.46	10/400		3 days
10	R	83,m	classic	0.40	10/200	4 days	5 days
11	L	83,m	classic	0.39	10/400	4 days	5 days
12	L	82,f	occult	0.57	1/35 MV	5 days	5 days
13	R	85,f	occult	0.76	10/50	5 days	5 days
14	R	75,f	occult	0.16	10/50	6 days	7 days
15	L	74,m	occult	0.23	10/125	9 days	3 days

R: Right, L: Left, m: male, f: female;

PED: Pigment epithelium detachment, RAP: Retinal angiomatous proliferation

VA: Visual Acuity MV: Meter Vision

Immunohistology

The CNV specimens were fixed in formalin and embedded in paraffin. **Each specimen was sectioned into 5-micrometer sections and mounted on poly-L-lysine coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining.** After de-paraffinization antigen retrieval was through heat treatment in citrate buffer **(0.01 M, ph: 6.0) for 2 minutes** for CD34, ICAM-1, E-selectin, CD45, CD68 and Ki-67. For cytokeratin18 and thy-1, antigen retrieval was accomplished by proteolytic digestion with 0.5% protease XXIV (Sigma, **St Louis, Missouri**) **for 10 minutes** whereas **pre-treatment was with proteinase K (Dako) for 10 minutes** was used for VEGF. Due to inadequate pre-treatment stability of two sections, only 2 PDT CNV were stained for E-selectin, ICAM-1, CD68 and CD45.

Immunohistochemical staining with the primary mouse monoclonal antibodies specific for CD34 (**Clone QBEnd-10**, Immunotech, FRG), cytokeratin18 (Clone:Ks 18.04,Progen, **Heidelberg**, Germany), ICAM-1 (**Clone 23G12**, Novocastra, **Newcastle upon Tyne, England**), E-selectin (**Clone 16G4,** Novocastra), CD45 (**Clones 2B11+PD7/26**, Dako) and Ki-67 (**Clone MIB-1**, Dako) was performed using horseradish peroxidase method as previously described.[26] For E-selectin, cytokeratin 18 and ICAM-1 staining, the brown chromogen **3,3'-diaminobenzidine** was replaced with 3-Amino-9-ethylcarbazole (AEC) high sensitive substrate chromogen (Cytomation, Code K3461, Dako).

Immunohistochemical staining with the primary mouse antibodies specific for VEGF (sc-7269, Santa Cruz, CA), thy-1 (**clone 5E10**, BD **Biosciences**, Pharmingen, **San**

Jose, California) and CD68 (**Clone PG-M1**, Dako) was performed by alkaline-phosphatase method as previously described.[30]
Color was developed using chromogen red (ChemMate Detection Kit, Dako).

Hematoxylin (Chemmate, Code S2020, Dako) was used for counterstaining.

For negative controls, primary antibodies were substituted either by appropriate normal sera.

Analysis

Two serial sections from a specimen were analyzed for each specimen independently by two masked observers (OT, SG) by light microscopy.

Immunoreactivity for VEGF, ICAM-1 and E-selectin was analyzed separately in RPE, EC and stroma by light microscopy. 3,2,1,0 were assigned to indicate intense (70-100% positive cells), moderate (40-69% positive cells), weak labelling (1-39% positive cells) and absence of any staining, respectively. "Total score" for VEGF, ICAM and E-selectin (range=0-9) was described for each CNV by sum of staining scores in RPE, EC and stroma.

Thy-1 expression was determined with percentage of thy-1 expressing vessels in overall vascularization detected by number of CD34 positive vascular-like patterns in each membrane.

All Ki-67 positive nuclei, CD45 immunoreactive leukocytes and CD68 immunopositive macrophages were counted in each specimen. Area of each specimen was

measured using appropriate software (AxioVision, Version 3.1, Carl Zeiss, Göttingen, Germany). Density of leukocytes and macrophages and proliferative activity were determined separately for each CNV by ratio of total number of leukocytes, macrophages and Ki-67 positive nuclei to total area of membrane (mm^2), respectively.

ANOVA followed by Fisher's PLSD post hoc test was used for statistical analysis. $p<0.05$ was considered significant.

RESULTS

Immunohistopathological findings were summarized in Figure 1.

Expression of Cellular Adhesion Molecules ICAM-1 and E-Selectin

ICAM-1 immunoreactivity was detected in RPE, EC and stroma in 92.86% (13/14), 35.72% (5/14), and 71.43% (10/14) of control CNV, respectively (Figure 1A, 2A). Expression of ICAM-1 in EC (ANOVA $p=0.0008$) and stroma (ANOVA $p<0.0001$) as well as total ICAM-1 score (ANOVA $p<0.0001$) showed significant differences between subgroups.

ICAM-1 was intense in RPE in both PDT CNV and was weak in stroma of 1 PDT CNV (Figure 1A, 2B).

In all TA CNV (n=5), RPE, EC and stromal cells displayed ICAM-1 intensely. ICAM-1 was significantly stronger in EC, stroma and in total score than control CNV ($p=0.0008$, $p<0.0001$, $p<0.0001$, respectively) and PDT CNV ($p=0.0035$, $p=0.0005$, $p=0.001$, respectively) (Figure 1A, 2C).

Intense ICAM-1 expression was detected in RPE of all, in EC of 4 (66.67%) and stroma of 5 (83.3%) PDT+TA CNV (n=6) (Figure 1A, 2D). ICAM-1 was significantly stronger in EC, stroma and total score than both control CNV ($p=0.0041$, $p<0.001$, $p=0.0001$, respectively) and PDT CNV ($p=0.0106$, $p=0004$, $p=0.0018$, respectively). No significant change was detected in ICAM-1 expression between TA and PDT+TA CNV.

Control CNV disclosed E-selectin in RPE in all but in EC in 8 (57.14%) and in stroma in 9 (64.29%) specimens (Figure 1B). In PDT CNV, E-selectin was present in RPE

and EC of 1 CNV (Figure 1B). TA CNV disclosed E-selectin in RPE of 2 CNV (40%) and in EC and stroma of 1 CNV (20%) (Figure 1B, 2E). E-selectin was detected in RPE in 4 (66.67%), in EC in 3 (50.00%) and in stroma in 1 PDT+TA CNV (16.67%) (Figure 1B, 2F) **(Figure 2 is available on website: www.archophthalmol.com).** No significant difference in E-selectin expression was found between any of subgroups.

Leukocyte and Macrophage Density and Proliferative Activity in CNV

Mean density of macrophages in control CNV, PDT CNV and TA CNV were 1028.33±224.64, 235.95±185.63 and 1433.26±399.94, respectively (Figure 1C). Macrophage density in PDT+TA CNV **(2831.31± 481.29)** was significantly higher than control CNV (p=0.0005), PDT CNV (p=0.002) and TA CNV (p=0.019) (ANOVA p=0.002) (Figures 3A-D). Higher macrophage density in TA CNV than control CNV and PDT CNV did not show significance (p=0.41).

Leukocytes were present in all but one control CNV with a mean density of 180.92±52.34. Density of leukocytes in 2 PDT CNV ranged between 0 to 3.73 (Figure 1C). In TA CNV, mean density of leukocytes tended to decrease to 56.67±17.69 (Figure 1C, 3E). Leukocytes were found in 5 of 6 PDT+TA CNV with a mean density of 131.13± 41.57 (Figure 1C, 3F). No significant difference was found between any groups (ANOVA p=0.35)

Mean proliferative activity was 96.57±37.48 in control CNV and 22.18±19.02 in PDT CNV (Figure 1C). Mean proliferative activity in TA CNV (38.91± 21.29) (Figure 1C, 3G) and PDT+TA CNV (18.19± 4.96) (Figure 1C, 3H) was smaller than control CNV,

but without significance (ANOVA p=0.35) **(Figure 3 is available on website:** www.archophthalmol.com).

Angiographic findings, CD34 and thy-1 Immunoreactivity,

Following PDT and PDT+TA, a hypofluorescence suggesting nonperfusion of the irradiated area and CNV was detected in fluorescein angiography on the day of surgery (Figure 4A). Supportingly, CD34 immunohistochemistry demonstrated mostly occluded vessels with damaged EC (Figure 4B). In contrast, control CNV disclosed patent vessels lined with healthy EC.

All CNV were vascularized. Thy-1 immunoreactivity was detected in all but one control CNV with a mean percentage of 73.93±8.76 of vessels (Figure 1D, 4C). In PDT CNV, 10% of vessels of only one CNV displayed thy-1 immunoreactivity whereas other CNV were immunonegative (Figure 1D, 4D). All vessels in all TA CNV were stained for thy-1 (Figure 1D, 4E). Mean percentage of thy-1 expressing vessels was 98.33±1.67 in PDT+TA CNV (Figure 1D, 4F) **(Figure 4 is available on website:** www.archophthalmol.com). Percentage of thy-1 expressing vessels showed significant difference between subgroups (ANOVA $p<0.0001$), being significantly higher in TA CNV and PDT+TA CNV than both control CNV ($p=0.0454$, $p=0.0455$, respectively) and PDT CNV ($p<0.0001$, $p<0.0001$, respectively). Percentage of thy-1 expressing vessels in TA and PDT+TA CNV was comparable to each other ($p=0.9$).

Immunoreactivity of VEGF in CNV

VEGF immunoreactivity was detected in RPE, EC and stroma in 10 (71.43%), in 8 (57.14%) and in 13 (92.86%) control CNV, respectively. Intense VEGF expression was found in RPE in 4 (28.57%) CNV (Figure 1E, 5A). PDT CNV displayed VEGF in RPE intensely in all CNV, and either weakly or moderately in EC of 3 and stroma of all specimens (Figure 1E, 5B). RPE, EC and stroma displayed VEGF in all TA CNV and intensely in 3 CNV (60%) (Figure 1E, 5C). PDT+TA CNV displayed VEGF intensely in RPE and stromal cells in 5 (83.33%) CNV and in EC in 3 CNV (50%) (Figure 1E, 5D) **(Figure 5 is available on website: www.archophthalmol.com)**. VEGF was significantly more intense in RPE in PDT+TA CNV than in control CNV (ANOVA $p=0.0464$, $p=0.0243$). Total VEGF scores in TA ($p=0.047$) and PDT+TA ($p=0.013$) CNV were also higher than in control CNV (ANOVA $p=0.048$). VEGF expression in TA and PDT+TA CNV did not show any significant changes with respect to each other or PDT CNV.

DISCUSSION

Knowledge about inflammation in pathogenesis of neovascular AMD is increasing. Inflammatory constituents of drusen induce VEGF expression.[31] VEGF, in turn, stimulates expression of ICAM-1 and E-selectin on vascular EC and facilitates migration of inflammatory cells to neovascularization.[32-37] There is a closed but amplifying circuit between VEGF and inflammatory cells since leukocytes produce VEGF and also stimulate RPE and fibroblasts to produce VEGF.[36-40] Enhanced VEGF expression in RPE induces CNV.[41] New neovascular AMD treatment strategies therefore target complement system, ICAM-1 and macrophages.[39,40,42] They inhibit leukocyte infiltration and VEGF expression and, therefore, CNV development.[39,40] TA inhibits experimental CNV.[10-12] TA was suggested to alter inflammatory cell activity and/or numbers,[10,12] reduce VEGF expression[16-21] or downregulate ICAM expression.[13,14] TA is efficacious in AMD treatment.[3,4] Herein, we evaluated early effects of TA on cellular adhesion molecules, inflammatory cell infiltration and activity, thy-1, and proliferation in human CNV.

In our series, control CNV were mostly inflammatory active with varying densities of leukocytes, macrophages, thy-1, ICAM-1 and E-selectin expression. Leukocytes and macrophages were previously found to be present in CNV.[43-46] In concordance to Yeh et al.[47] we found ICAM-1 expression mainly and intensely in RPE. E-selectin was present in EC, stroma and RPE as previously reported.[47-49] Intense VEGF expression was detected in RPE, EC and stroma in less than 30% of the specimens.

In TA or PDT+TA CNV, density of CD45 immunopositive peripheral leukocytes tended to decrease in comparison to control CNV similar to observations of Ciulla et

al.[11] Surprisingly, density of CD68 immunoreactive resident macrophages was higher in both TA or PDT+TA CNV than control CNV, but significantly increased only in PDT+TA CNV. Macrophages synthesize IL-1 and TNF-α.[36,45,50,51] Hence, increased macrophage density was associated with significantly higher thy-1 expression reflecting enhanced IL-1 and TNF-α activity. IL-1 and TNF-α enhance ICAM-1 expression in EC and RPE further.[52-54] Additionally, macrophages induce VEGF production in RPE through IL-1 and TNF-α.[38] Density of infiltrating inflammatory cells is correlated with VEGF levels in CNV.[39,40,50,55] Increased density of macrophage infiltration and thy-1 expression, therefore, is associated with increased total VEGF expression score in TA and PDT+TA CNV.[42] Enhanced VEGF expression might re-stimulate the cascade through enhancing ICAM-1 expression and macrophage infiltration.[32-37]

Impact of TA on inflammation has been previously studied. Ishibashi et al. supposed that TA might inhibit experimental CNV through inhibiting infiltration of inflammatory cells, especially of macrophages.[10] Penfold et al. suggested that TA diminished numbers of dendritiform microglia but not macrophage-like population in neural retina overlying subretinal proliferation.[15] TA monotherapy reduced permeability and expression of ICAM-1 in choroidal EC.[13,14] However, E-selectin was unaffected.[14] Macrophage infiltration was increased after subconjunctival injection of TA.[56] Antoszky et al. suggested that angiostatic but not anti-inflammatory effects of TA was significant in preventing neovascularization.[8]

Age, maturity, pre-treatment inflammatory activity and VEGF expression of CNV cannot be predicted. In contrast, time of PDT application acts as an artificial time zero. PDT induces a significant decrease in inflammatory cell infiltration and

activity[46] but significant increase of VEGF expression by RPE.[25,49] Therefore, PDT CNV serves as an ideal control group for PDT+TA CNV. Their comparison revealed also increased macrophage infiltration, enhanced thy-1 and ICAM-1 expression and increased VEGF total score in PDT+TA CNV as well as persisting intense VEGF expression in RPE. Enhanced VEGF in ARPE-19 by cellular up-take of verteporfin was suppressed by TA in vitro.[20] However, TA reduced VEGF expression in RPE induced by either oxidative stress[18] or IL-1 but did not affect hypoxia stimulated VEGF expression.[21] It is still unknown which one is causative for increased VEGF expression after PDT. Furthermore, responses in vivo are different and influenced by cell types, vascularization and perfusion. Supporting, TA reduces VEGF in ARPE-19 cell line, vascular smooth cells, EC and Muller cells in-vitro,[17-19,21] but not in rat retina and hemangioma in vivo.[57,58]

Our work shows that TA and PDT+TA CNV are inflammatory active disclosing many macrophages. However, macrophages **are** not only proangiogenic. Firstly, macrophages control vessel growth and are required for cell death and tissue remodelling in eye.[59,60] During vascular regression, macrophages regulate EC apoptosis. In case of macrophage elimination, EC survive and capillaries persist.[59-62] **Similarly, in our specimens, macrophages might be recruited to remove the cellular debris early after the treatment.** Secondly, macrophages inhibit angiogenesis through release of proteolytic enzymes[36] which activate endogeneous angiogenesis inhibitors such as endostatin. Correspondingly, endostatin was enhanced in TA and PDT+TA CNV.[23] Thirdly, macrophages are involved in CNV inhibition. Mice deficient in monocyte chemotactic protein-1 develop CNV.[31] Inhibition of macrophage entry into eye promotes CNV whereas direct injection of macrophages inhibits CNV.[63] Previously, macrophage depletion with liposomes was

shown to inhibit CNV.[39,40] However, reduction of neovascularization was recently suggested to be due to direct toxicity of liposomes on EC.[63]
Proliferative activity is significantly higher in inflammatory active CNV.[30,46,48] TA CNV and PDT+TA CNV were highly infiltrated with macrophages, however, mean proliferative activity was lower than control CNV possibly due to anti-proliferative effect of TA.[64]

We are unaware of previous reports of clinicopathological evaluation of ICAM-1, inflammation and proliferation in human CNV treated with TA or PDT+TA combination therapy. Proper interpretation of the study is limited by the small number of specimens and **the possibility of selection bias. An absolute quantification of the mRNA and/or protein expression by real time PCR and/or western blot in further studies will surely supply additional valuable information.** Nevertheless, TA and PDT+TA CNV disclose infiltration with a significantly higher density of resident macrophages and intense VEGF expression **early after the therapy although its duration is unknown.** Proliferative activity and density of leukocytes tend to be lower in TA and PDT+TA CNV. In contrast, CNV treated with bevacizumab, a full length recombinant humanized monoclonal antibody against VEGF, discloses higher proliferative activity and leukocyte density than control CNV.[65] Whether these are sufficient rationales for a triple combination therapy including PDT, TA and anti-VEGF agents as recently introduced needs to be further evaluated.[66]

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