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Figure 1: Graphs showing intercellular adhesion molecule (ICAM)-1 (A) and E-selectin (B) immunostaining intensity, proliferative activity, density of leukocyte and macrophages (C), percentage of thy-1 immunopositive vessels (D) and vascular endothelial growth factor (VEGF) immunoreactivity intensity (E) in choroidal neovascularization (CNV) membranes without previous therapy (Control CNV), excised after verteporfin photodynamic therapy (PDT) (PDT CNV), triamcinolone acetonide (TA) monotherapy (TA CNV) and PDT+TA combination therapy (PDT+TA CNV). ICAM-1, E-selectin and VEGF immunostaining in retinal pigment epithelium (RPE), endothelial cells (EC) and stroma were evaluated separately and semiquantitatively as intense (70-100% positive cells), moderate (40-69% positive cells), mild (1-39% positive cells) or absent (A, B, E). All Ki-67 positive nuclei, CD45 immunoreactive leukocytes and CD68 immunopositive macrophages were counted in each specimen. Proliferative activity and density of leukocytes and macrophages were determined separately for each CNV by the ratio of total number of proliferating cells, leukocytes and macrophages to the total area of the membrane (mm^2), respectively; and their mean values with standard errors were shown in (C). Percentage of thy-1 expressing vessels in the overall vascularization of each membrane was calculated for each membrane (D).

Figure 2: Photomicrographs of choroidal neovascularization (CNV) membranes without previous therapy (A), excised 3 days after verteporfin photodynamic therapy (PDT) (B, case 6 in Table), 4 days after intravitreal triamcinolone (TA) injection (C, E, Case 3 in Table) and 4 days after intravitreal TA and 5 days after PDT (D, F, Case 10 in Table). Specimens were probed with antibodies against intercellular adhesion molecule (ICAM)-1 (A-D) or E-selectin (E, F) and stained with AEC red chromogen. Hematoxylin was used as counterstain. (A-D) Intense ICAM-1 expression was detected in retina pigment epithelium (asterisk). CNV treated with TA monotherapy (C) or PDT+TA combination therapy (D) display intense ICAM-1 expression also in endothelial cells (arrow) and stromal cells (black arrow head). (E, F) Some retina pigment epithelium cells display E-selectin (asterisk).

Figure 3: Photomicrographs of choroidal neovascularization (CNV) membranes probed for macrophages with CD68 antibody and stained with red chromogen (A-D), for leukocytes with CD45 antibody (E, F) and for proliferating cells with Ki-67 antibody (G, H) stained with the brown chromogen 3-Diaminobenzidine. Hematoxylin was used as counterstain. (A) In control CNV without previous therapy, some macrophages were detected in retina pigment epithelium (RPE) cell layer (asterisk) and stroma (arrow). Contrary to CNV excised 3 days after photodynamic therapy (PDT) (B, case 9 in table), CNV excised 3 days after triamcinolone acetonide (TA) monotherapy (C, TA CNV, case 1 in Table) and CNV extracted 5 days after PDT and TA combination therapy (D, PDT+TA CNV, case 12 in Table) displayed many macrophages in RPE cell layer (asterisk) and within stroma (arrows) but only a few leukocytes (E, F). Only a few proliferating cells were detected in TA CNV (G, arrows). PDT+TA CNV disclosed no proliferating cells (H). Scale bar: 50 µm.

Figure 4: (A, B, F) Photomicrographs of choroidal neovascularization (CNV) membrane extracted 5 days after verteporfin photodynamic therapy (PDT) and 4 days after intravitreal triamcinolone acetonide (TA) injection (Case 10 in Table). (A) Late phase of fluorescein angiography on the day of surgery displays non-perfusion of the CNV and laser spot area. (B) The specimen was probed with antibody against CD34 and stained with 3-Diaminobenzidine resulting in a brown chromogen. Most of the vessels are occluded and were lined with damaged endothelial cells (EC, arrows). (C-F) Photomicrographs of CNV membranes probed with antibody against thy-1 and stained with red chromogen. Hematoxylin was used as counterstain. In control CNV without previous therapy (C), some EC were immunopositive for thy-1 (arrow) whereas some EC were immunonegative (white arrowhead). (D) CNV excised 3 days after PDT (case 7 in Table) displayed no thy-1 immunoreactive EC. (E) CNV excised 3 days after intravitreal TA injection (case 2 in Table) and (F) CNV extracted after PDT+TA combination therapy disclosed strong thy-1 expression in all the vessels (arrows). Scale bar: 50 µm.

Figure 5: Photomicrographs of choroidal neovascularization (CNV) membranes probed with antibody against vascular endothelial growth factor (VEGF) stained with red chromogen. Hematoxylin was used as counterstain. In control CNV without previous therapy (A), retina pigment epithelium (RPE) did not display VEGF (asterisk). VEGF staining was detected within some cells in stroma (arrow). In a CNV excised 3 days after photodynamic therapy (PDT) (B, Case 7 in Table), strong VEGF expression was especially prominent in RPE (asterisk). In CNV excised 4 days after intravitreal triamcinolone acetonide (TA) injection (C, Case 1 in Table), strong VEGF expression was present in RPE (asterisk) and in stromal cells (arrow). In CNV excised 3 days after PDT and 4 days following intravitreal TA injection (D, Case 11 in Table), RPE (asterisk) and stromal cells (arrow) disclose VEGF strongly. Scale bar: 50 µm.

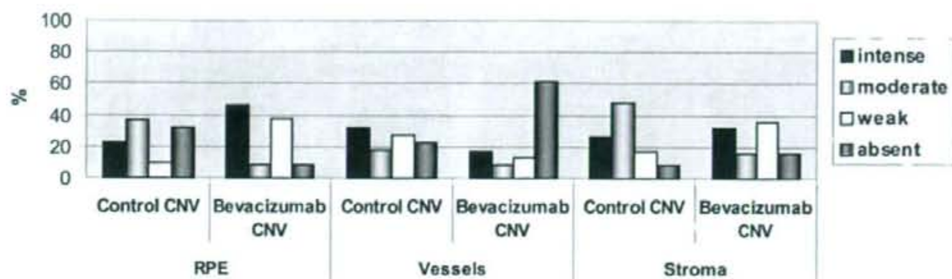
Table 1: Clinical characteristics of patients treated with intravitreal triamcinolone acetonide (TA) and/or verteporfin photodynamic therapy (PDT) before surgical removal of **subfoveal** choroidal neovascularization (CNV).

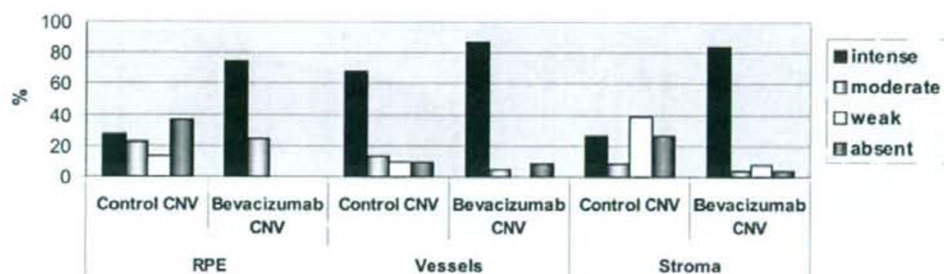
Case	Eye	Age/ Sex	CNV type	CNV Size (mm^2)	Preoperative VA	Time to surgery from	
						TA Injection	each PDT
1	R	90,m	hemorrhagic occult	0.12	10/200	3 days	
2	R	78,f	occult	0.21	10/100	3 days	
3	R	84,f	PED (RAP)	0.22	10/63	4 days	
4	R	70,f	PED, occult	0.13	10/40	7 days	
5	L	80,f	RAP	0.22	10/50	8 days	
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

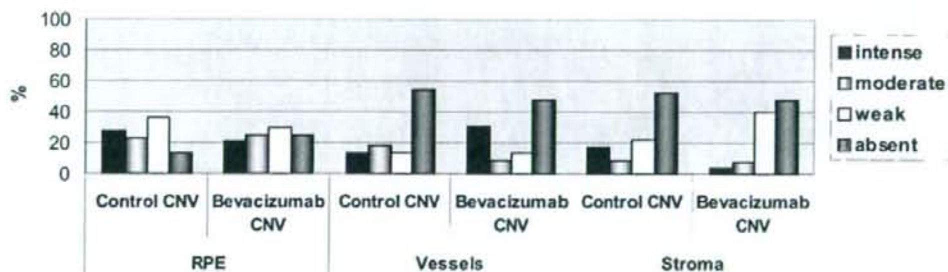
R: Right, L: Left, m: male, f: female;

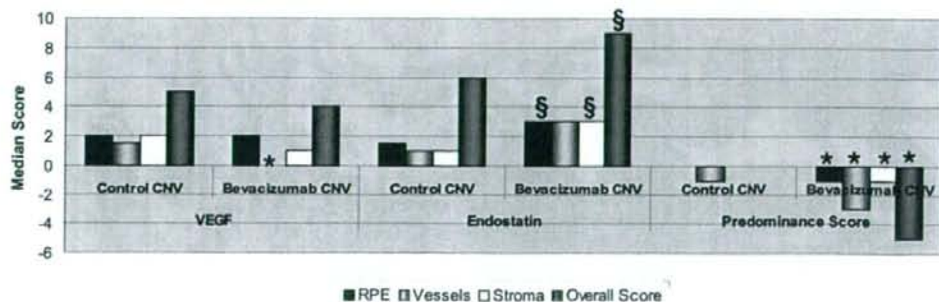
PED: Pigment epithelium detachment, RAP: Retinal angiomatous proliferation

VA: Visual Acuity MV: Meter Vision


Expression of VEGF in CNV
%
100
80
60
40
20
0
intense
moderate
weak
absent
Control CNV
Bevacizumab CNV
RPE
Vessels
Stroma


Expression of Endostatin in CNV
%
100
80
60
40
20
0
intense
moderate
weak
absent
Control CNV
Bevacizumab CNV
RPE
Vessels
Stroma


Expression of E-selectin in CNV
%
100
80
60
40
20
0
intense
moderate
weak
absent
Control CNV
Bevacizumab CNV
RPE
Vessels
Stroma


Median Staining Intensity and Predominance Scores in CNV
Median Score
10
8
6
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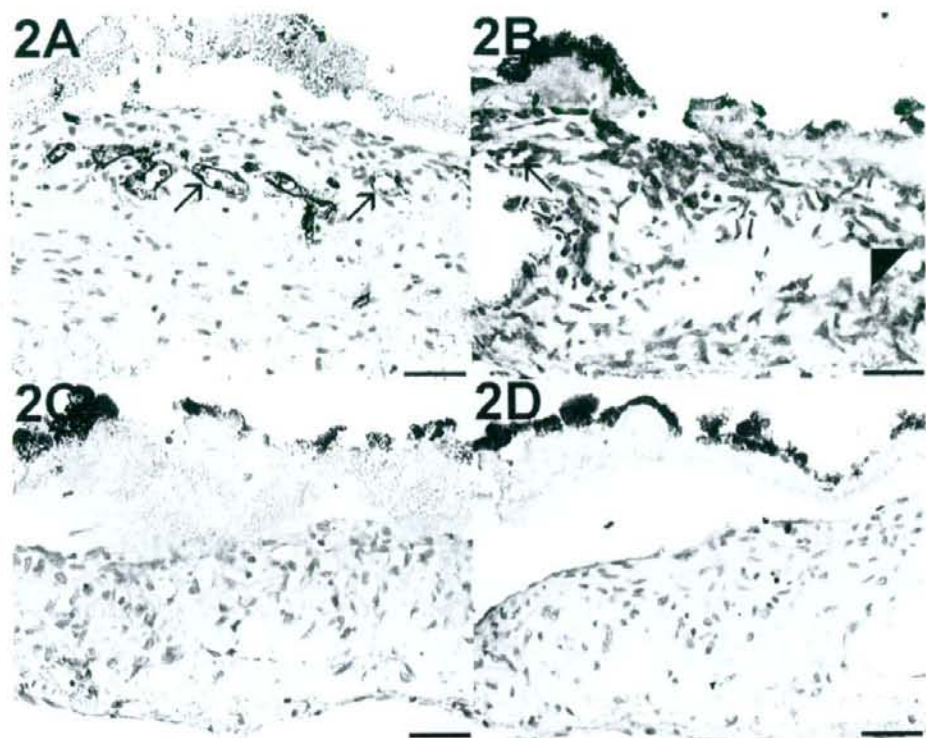
Control CNV
Bevacizumab CNV
VEGF
Endostatin
Predominance Score
RPE
Vessels
Stroma
Overall Score


2A
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2C
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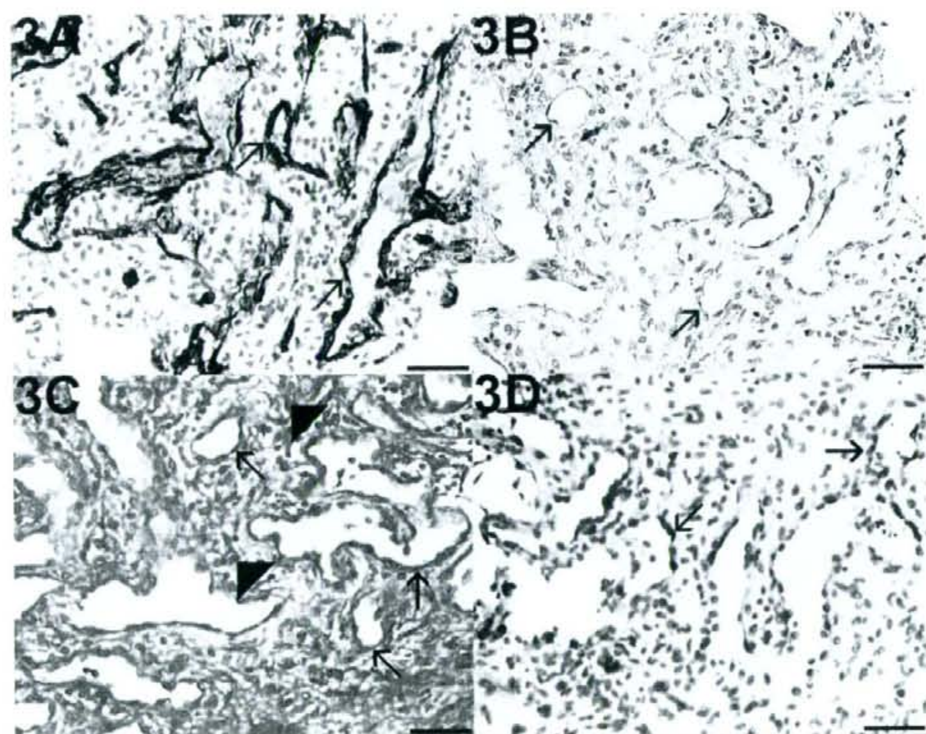
3A
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3D

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Implications of bevacizumab on vascular endothelial growth factor and endostatin in human choroidal neovascularisation

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ABSTRACT

Aim: To evaluate the implications of intravitreal bevacizumab on proangiogenic vascular endothelial growth factor (VEGF) with regard to the endogenous angiogenesis inhibitor endostatin in human choroidal neovascularisation (CNV) secondary to age-related macular degeneration.

Methods: Retrospective review of an interventional case series of 48 patients who underwent full macular translocation surgery with removal of CNV. Twenty-five patients were treated with intravitreal bevacizumab injection 1 to 154 days prior to surgery (bevacizumab CNV). Twenty-three CNV without any kind of previous treatment were used as controls (control CNV). CNV were stained for CD34, cytokeratin18, VEGF, endostatin and E-selectin. A "predominance score of VEGF over endostatin" (PS) was defined by the difference between VEGF and endostatin staining scores.

Results: Bevacizumab CNV revealed a weaker VEGF expression in endothelial cells ($p = 0.0245$) but significantly more intense endostatin in retina pigment epithelium (RPE) ($p = 0.0001$) and stroma ($p<0.0001$). Consequently, PS was significantly lower in RPE ($p = 0.02$), vessels ($p = 0.03$) and stroma ($p = 0.0004$) in bevacizumab CNV. The intensity of E-selectin expression in bevacizumab CNV was comparable with that in control CNV.

Conclusions: A shift within the angiogenic balance in terms of decreased VEGF predominance over endostatin is detected in human CNV treated with bevacizumab.

Neovascular age-related macular degeneration (AMD) is the leading cause of visual loss in elderly in the Western world.[1] Neovascularisation, as in choroidal neovascularisation (CNV), occurs due to local predominance of angiogenesis stimulators over inhibitors.[2] Vascular endothelial growth factor (VEGF) and endostatin are among these endogenous angiogenesis stimulators and inhibitors in human choroid, respectively.[3] Endostatin is a C-terminal fragment of Collagen XVIII. Endostatin/Collagen XVIII was found to be crucial for retina pigment epithelium (RPE) function and vision. Additionally, intraocular expression of endostatin reduces VEGF-induced retinal permeability and neovascularisation. Endostatin was found to be decreased in RPE and choriocapillaris of human eyes with AMD, and its deficiency was suggested to predispose to CNV formation.[6] Either external delivery of endostatin or endogenous endostatin inhibits experimental CNV.[7-10] However, E-selectin is required for the antiangiogenic effect of endostatin.[11] E-selectin is an intercellular adhesion molecule expressed in CNV but not in choroid of healthy human eyes without neovascularisation.[8,12]

Upcoming antiangiogenic agents[13,14] have changed the therapeutic approach for neovascular AMD considerably. The philosophy behind these antiangiogenic therapies is to rearrange the impaired angiogenic balance through either inhibiting the angiogenesis stimulators or strengthen the inhibitory factors. Pegaptanib (Macugen, Eyetech Pharmaceuticals, New York) and ranibizumab (Lucentis, Genentech, South San Francisco, California) are VEGF inhibitors which were shown to be effective for neovascular AMD treatment in large clinical trials[15,16] Recently, bevacizumab (Avastin, Genetech, South San Francisco, California), a full-length recombinant humanised monoclonal antibody against VEGF used for treatment of metastatic colorectal cancer,[19] was also reported to be beneficial in treatment of CNV and is widely used in an off-label fashion.[17,18]

In this histopathological study, we evaluated the effect of intravitreally applied bevacizumab on the local angiogenesis milieu in human CNV focussing on VEGF, VEGF predominance over endostatin and E-selectin. Vascularisation was determined by the expression of CD34, a panendothelial cell marker.[20] Cytokeratin 18 was used to identify RPE.[21] Specimens from 25 patients treated with intravitreal bevacizumab injection preoperatively (bevacizumab CNV) were compared with 23 CNV without any kind of previous treatment (control CNV).

METHODS

Subjects and treatments

We retrospectively reviewed 48 eyes of 48 consecutive AMD patients who were surgically treated with full macular translocation surgery with CNV extraction. Therapy options, including observation, conventional thermal laser photocoagulation, verteporfin photodynamic therapy (PDT), intravitreal injection of triamcinolone acetonide or antiVEGF agents, full macular translocation with 360° retinotomy and CNV extraction were discussed with the patients. Intravitreal bevacizumab injection and/or full macular translocation surgery was offered when (1) visual acuity was worse than 20/200 being the minimum visual acuity to

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recommend the first PDT according to the TAP Investigation,[22] (2) CNV was associated with RPE tear or massive subretinal/intravitreal haemorrhage, (3) visual deterioration progressed, subretinal haemorrhage and RPE tear appeared following treatment or (4) reading ability was recently lost despite improvement in far visual acuity after a previous treatment. Reduction of intraoperative bleeding was intended with the preoperative injection of bevacizumab as suggested in other different ocular pathologies.[23-25] The clinical characteristics of patients treated with intravitreal bevacizumab are summarised in table 1.

Each patient gave written informed consent after the off-label use and experimental nature of the treatment procedure as well as risks and benefits of all therapeutical options had been discussed in detail. The study followed the guidelines of the declaration of Helsinki. The histological analysis of the specimens was approved by the local Institutional Review Board.

Tissue preparation

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

Immunohistology

After paraffin sections had been deparaffinised and rehydrated with a graded series of alcohol, different techniques for antigen retrieval were applied. For cytokeratin18 and endostatin, antigen retrieval was accomplished by proteolytic digestion with 0.5% protease XXIV (Sigma, St Louis, Missouri) whereas Proteinase K (Dako) was used for VEGF. For CD34 and E-selectin, the method of antigen retrieval was heat treatment in citrate buffer (0.01 M, pH 6.0) in a pressure cooker under 120°C for 2 min.

Immunohistochemical staining with the primary antibodies specific for human CD34 (Mouse, Mab, Immunotech, Hamburg, Germany), cytokeratin18 (Mouse, Mab, Progen, Heidelberg, Germany) and E-selectin (mouse, Mab, Novocastra, UK) was performed using the ABC horseradish peroxidase as previously described.[7] For Cytokeratin18 staining and E-selectin, the diaminobenzidine tetrahydrochloride (DAB) chromogen was replaced with 3-amino-9-ethylcarbazole (AEC) highly sensitive substrate chromogen (Cytomation, Code K3461, Dako). Haematoxylin (Chemmate, Code S2020, Dako) was used for counterstaining.

Immunohistochemical staining for VEGF and endostatin was performed by the alkaline-phosphatase method according to the manufacturer's instructions (ChemMate Detection Kit, alkaline phosphatase/RED, rabbit/mouse, K5005; Dako) as previously described[8] using an antihuman VEGF-A antibody (mouse, Mab, clone C-1, Santa Cruz Biotechnology, Santa Cruz, California) and an antihuman endostatin antibody (rabbit, polyclonal, Dianova GmbH, Hamburg). For negative controls, the primary antibodies were substituted with the appropriate normal sera or omitted.

Analysis

Sections from a specimen were analysed by light microscopy. Immunoreactivity for VEGF, endostatin and E-selectin was analysed separately in RPE, vessels and stroma. A grading scheme indicating the degree of staining was used. 3, 2, 1 and 0 were assigned to indicate intense (70–100% of all the corresponding type of cells in the specimen are immunopositive), moderate

Table 1 Clinical characteristics of the patients treated with intravitreal bevacizumab before submacular surgery

Case	Eye	Age, sex	CNV type	No of bevacizumab injections	Time (days) to surgery from the	
					Photodynamic therapy	Intravitreal bevacizumab
1	L	80, f	Haemorrhagic	1	–	1
2	L	86, f	Haemorrhagic	1	–	3
3	R	75, m	Occult haemorrhagic with RPE tear	1	–	3
4	L	69, m	Occult	1	–	4
5	L	69, m	Occult	1	–	4
6	R	71, f	Haemorrhagic	1	–	6
7	R	83, m	Haemorrhagic PED	1	–	6
8	L	79, m	Predominantly classic with CRA	1	–	7
9	R	74, m	Haemorrhagic	1	–	9
10	R	69, f	Haemorrhagic with RPE tear	1	–	11
11	L	73, f	RPE tear	1	–	12
12	R	72, f	Haemorrhagic occult CNV	1	39	12
13	R	56, m	Haemorrhagic PED	2	–	89/15
14	L	76, f	Occult	1	–	19
15	L	73, m	RPE tear	1		26
16	R	58, m	Haemorrhagic with RPE tear	1		43
17*	R	79, f	Haemorrhagic with RPE tear	1	343	43
18	R	85, f	Occult with PED	1	–	44
19	R	81, f	Haemorrhagic with RPE tear	1	–	46
20	L	78, m	Occult haemorrhagic	5	249	209/167/125/92/50
21	L	76, f	Haemorrhagic	1	–	53
22	R	79, m	Occult with RPE tear	2	–	95/55
23	R	75, m	Occult	1	–	57
24	R	84, f	Haemorrhagic with PED	2		135/85
25	R	75, f	Predominantly classic	2	–	154

*Case 17 was treated with intravitreal triamcinolone acetonide injection 342 days before surgery.
CNV, choroidal neovascularisation; CRA, chorioretinal anastomosis; f, female; L, left; m, male; PED, pigment epithelial detachment; R, right; RPE, retina pigment epithelium.

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Table 2 Intensity of vascular endothelial growth factor (VEGF), endostatin and E-selectin immunostaining in choroidal neovascularisation (CNV) treated with bevazicumab, analysed based on timing or number of bevacizumab injections with a Spearmann correlation test and compared based on previous application of verteporfin photodynamic therapy (PDT) using the Mann–Whitney U test

	In bevacizumab CNV based on				In bevacizumab CNV based on previous treatment				
	Timing of injection		Frequency of injection		With PDT		Without PDT		
	p Value	ρ	p Value	ρ	Median	Range	Median	Range	p Value
VEGF									
RPE	0.22	0.26	0.12	0.33	1	0 to 2	3	0 to 3	0.12
EC	0.35	0.2	0.74	−0.07	0	0	0	0 to 3	0.16
Stroma	0.90	0.03	0.59	−0.11	1	0 to 2	1.5	0 to 3	0.29
Endostatin									
RPE	0.97	0.014	0.21	0.26	3	3 to 3	3	2 to 3	0.29
EC	0.46	0.16	0.34	0.20	3	2 to 3	3	0 to 3	0.35
Stroma	0.56	0.12	0.29	0.22	3	1 to 3	3	0 to 3	0.39
E-selectin									
RPE	0.08	0.37	0.76	−0.063	1	1 to 1	2	0 to 3	0.50
EC	0.44	0.16	0.98	0.005	3	1 to 3	0	0 to 3	0.08
Stroma	0.94	0.02	0.59	0.11	1	0 to 1	0.5	0 to 3	0.82

$p<0.05$ was considered significant.
EC, endothelial cells; RPE, retina pigment epithelium.

(40–69% positive cells) and weak labelling (1–39% positive cells), and absence of any staining, respectively. The "overall" VEGF and endostatin score (range = 0–9) has been described for each CNV by the sum of staining scores in RPE, vessels and stroma.

The "predominance score of VEGF over endostatin" (PS) was defined for RPE, vessels and stroma of each membrane separately calculating the difference between VEGF and endostatin staining scores.

The intensity of VEGF, endostatin and E-selectin immunostaining and PS were comparatively analysed between described groups of CNV using the Mann–Whitney U test. Immunohistological findings in bevacizumab CNV were analysed, based on timing or number of bevacizumab injections with the Spearmann correlation test. A p value of <0.05 was considered significant.

RESULTS

The frequency of VEGF, endostatin and E-selectin immunoreactivity intensity, median and overall staining intensity scores and PS in bevacizumab CNV and control CNV are summarised in fig 1.

Immunohistopathological findings in CNV without any kind of previous therapy

All but one membrane were vascularised as evidenced by CD34 positive endothelial cells (EC) (fig 2A). RPE cells were found in 22 of 23 specimens.

VEGF staining was present in RPE of 68.2% (15 of 22) of the specimens. In 22.7% (five of 22) of the CNV, VEGF was strongly expressed in RPE. VEGF expression was absent in EC of 22.7% (five of 22) of CNV. EC displayed strong to moderate VEGF staining in 50.0% (11 of 22) of the membranes (fig 2B). Cells within the stroma, such as fibroblasts and inflammatory cells, revealed VEGF in 91.3% (21 of 23) of CNV (figs 1A, 2B).

Positive staining for endostatin was detected in the RPE–Bruch membrane complex in 63.6% (14 of 22) of the membranes. Endostatin was absent in vessels in 9.1% (two of 22) of the specimens (fig 2C). Within the stroma, endostatin appeared to be present in fibroblast like and inflammatory cells in 73.9% (17 of 23) of the specimens (fig 1B).

E-selectin was found to be present in RPE of 86.4% (19 of 22) of the specimens. EC and stromal cells did not display E-selectin in 54.6% (12 of 22) and 52.2% (12 of 23) of the membranes, respectively (figs 1C, 2D).

Immunohistopathological findings in bevacizumab CNV

Immunohistology with CD34 disclosed vessels in 92.0% (23 of 25) of bevacizumab CNV (fig 3A). All but one CNV were extracted with RPE cells.

VEGF was found to be present in RPE of 91.7% (22 of 24) of the membranes and intensely in 11 CNV (45.8%). However, VEGF expression was absent in EC of 14 CNV (60.9%) (fig 3B). Intense to moderate VEGF expression was detected in EC of only four (17.4%) and two (8.7%) of the specimens, respectively. Stromal cells displayed VEGF in 84.0% (21 of 25) of CNV (fig 1A). VEGF expression in RPE and stroma of bevacizumab CNV was comparable with that in control CNV (fig 1D, $p = 0.21$, $p = 0.37$, respectively). However, VEGF was significantly weaker in EC of bevacizumab CNV than control CNV (fig 1D, $p = 0.025$).

The RPE–Bruch membrane complex displayed endostatin either strongly (75.0%, 18 of 24) or moderately (25.0%, six of 24). Endostatin was present in vessels of 91.3% (21 of 23) of CNV and intensely in 87.0% (20 of 23) of the specimens. Stromal cells revealed endostatin in 96.0% (24 of 25) and strongly in 84.0% (21 of 25) of the membranes (fig 1B, fig 3C). Endostatin was significantly stronger in the RPE–Bruch membrane complex ($p = 0.0001$) and stroma ($p<0.0001$), and the overall endostatin expression score was also significantly higher ($p = 0.0002$) in bevacizumab CNV than control CNV (fig 1D). Consequently, the PS in RPE, vessels and stroma as well as the overall PS was significantly lower in bevacizumab CNV than in control CNV (fig 1D, $p = 0.02$, 0.03, 0.0004 and 0.001, respectively).

E-selectin immunoreactivity was present in RPE of 18 of 24 (75.0%), EC of 12 of 23 (52.2%) and stromal cells of 13 of 25 CNV (52%). Intense E-selectin expression was detected in RPE of five (20.8%) CNV and EC of seven (30.4%) and stromal cells of one (4.0%) CNV (figs 1C, 3D). E-selectin expression in RPE, EC and stroma of bevacizumab CNV (median: 1 for RPE, EC and stroma; range: 0–3 for RPE, EC and stroma) was comparable with that in control CNV (median: 1.5, $p = 0.49$

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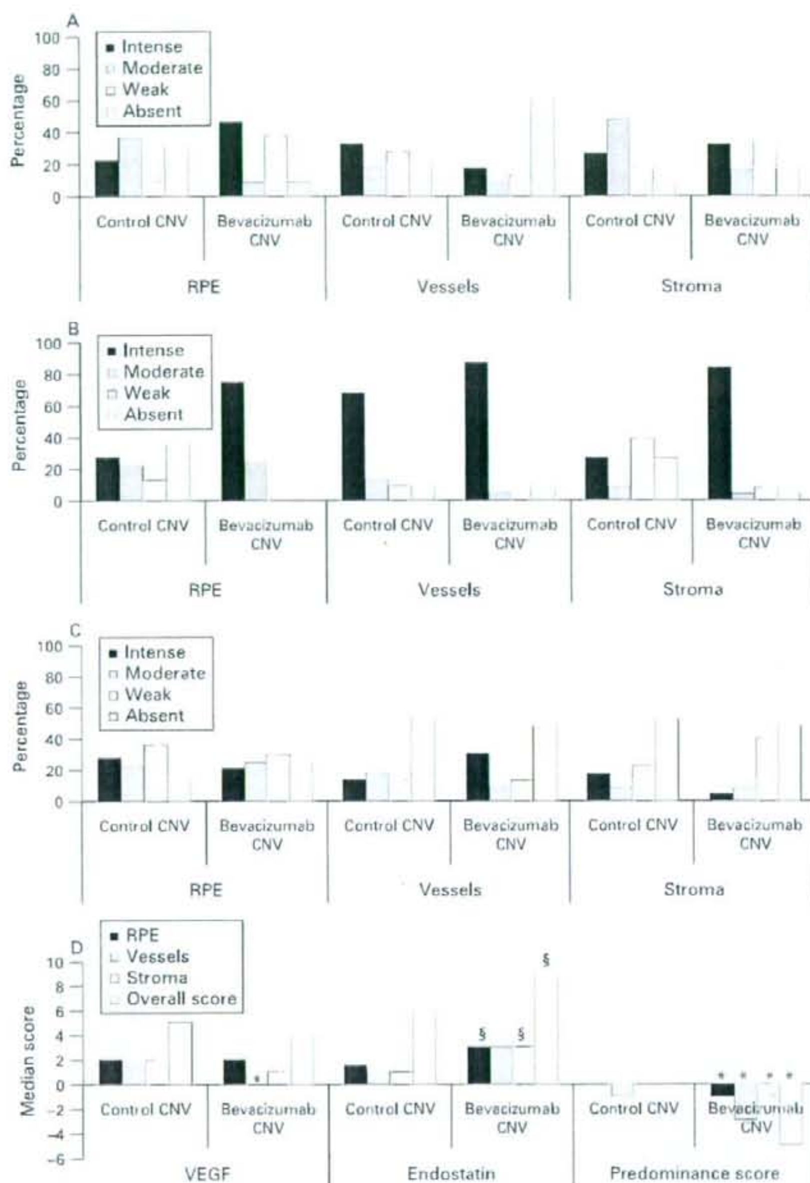

Figure 1 Graphs showing vascular endothelial growth factor (VEGF) (A), endostatin (B) and E-selectin (C) staining intensity and median staining intensity scores (D) in choroidal neovascularisation (CNV) excised without any pretreatment (control CNV) or following intravitreal bevacizumab injection (bevacizumab CNV). VEGF, endostatin and E-selectin immunostaining in retina pigment epithelium (RPE), vessels and stroma were evaluated separately and semiquantitatively as intense (70–100% positive cells), moderate (40–69% positive cells), mild (1–39% positive cells) or absent. Scores of 3, 2, 1 and 0 were assigned to "intense," "moderate," "mild" and "absent" intensity of staining, respectively. Median staining intensity scores of RPE, vessels and stroma were calculated for each group of CNV. The overall "VEGF" and "endostatin" score (range = 0–9) has been described for each CNV by the sum of staining scores in RPE, vessels and stroma. The "predominance score of VEGF over endostatin (PS)" was defined for RPE, endothelial cells and stroma of each membrane separately calculating the difference between VEGF and endostatin staining scores in each component. * and § denote significance lower and higher than the control CNV, respectively.

for RPE, median: 0, p = 0.51 for EC, median: 0, p = 0.76 for stroma; range: 0–3 for RPE, EC and stroma).

The immunohistological findings in bevacizumab CNV did not show any major differences based on timing or number of bevacizumab injections or previous application of PDT (table 2).

DISCUSSION

Anti-VEGF agents seem to be effective in preventing vision loss as well as improving visual acuity in patients with neovascular AMD.[18] Although experimental studies reveal inhibition of CNV by VEGF inhibitors,[9] insight into the impact of these

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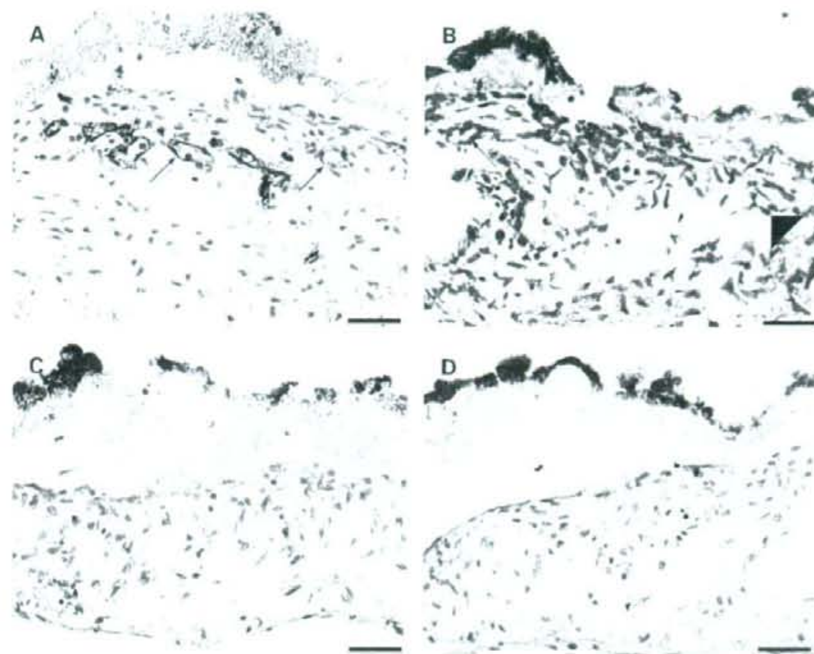

Figure 2 Photomicrographs of a surgically excised control choroidal neovascularisation without any previous therapy. The specimens were probed with antibodies against CD34 (A); vascular endothelial growth factor (VEGF) (B), endostatin (C) and E-selectin (D). Haematoxylin was used as a counterstain. (A) CD34 immunostaining with the brown chromogen 3-diaminobenzidine depicts selectively vascular structures (arrows). (B) VEGF stained with the red chromogen is detected intensely within endothelial cells (arrow) and stromal cells (black arrowhead). Neither endostatin (C) nor E-selectin (D) was detected in this membrane. Scale bar: 50 μm.

anti-VEGF drugs on the cellular and humoral level in human CNV is limited.[27 28] Since neovascularisation is regulated by the balance between angiogenesis stimulators and inhibitors, herein we investigated the expression of proangiogenic VEGF with regard to the antiangiogenic endostatin both in control CNV without any kind of previous therapy and in bevacizumab CNV.

In our series of control CNV, VEGF expression was found in RPE, EC and stroma as previously reported.[3 21 29 30] Fibroblastlike and inflammatory cells display VEGF in stroma. The density of macrophages and inflammatory activity of a CNV were found to be positively correlated with VEGF since inflammatory cells produce VEGF themselves and induce other cells such as RPE to express VEGF.[21 30–33] CNV treated with intravitreal bevacizumab are infiltrated with many macrophages and leucocytes.[28] An enhanced expression of VEGF in bevacizumab-treated CNV would, therefore, be an expected consequence. In our series of 25 bevacizumab CNV, however, VEGF expression in RPE and stroma was comparable with control CNV. In contrast, VEGF expression in EC was significantly weaker in bevacizumab CNV compared with control CNV. Speculative explanations for this finding may be reduced VEGF induction or masking by the antibody. Further analysis of the specific VEGF isoforms will contribute to our understanding considerably.

Macrophages and inflammatory cells are known to secrete proteolytic enzymes such as matrix metalloproteases.[34] Proteases cleave endostatin that is bound to collagen XVIII in vascular basement membranes and Bruch membrane. Through this cleavage, endostatin is released and activated.[35 36] Therefore, it was not surprising that endostatin expression was enhanced in inflammatory active bevacizumab CNV.[28] Correspondingly, in these series of 25 CNV, endostatin was significantly more intense in RPE and stroma in bevacizumab CNV than in control CNV. The expression of its cofactor E-selectin was comparable with that in control CNV. Enhanced endostatin expression in bevacizumab CNV would have significant consequences, since endostatin inhibits CNV and vascular permeability.[6 7 18] This inhibitory effect may occur through different pathways: at the transcriptional level, endostatin downregulates some angiogenic genes, including VEGF, and upregulates several antiangiogenic genes.[37] Endostatin is known to downregulate the VEGF expression directly and to block the VEGF/Flk-1 pathway.[38] Furthermore, it inhibits VEGF induced EC migration and stabilises newly formed endothelial tubes by inducing early junctions between EC.[40 41]

Major concerns with regard to non-selective antiangiogenic therapy are potential side-effects on physiological angiogenesis. The antiangiogenic action of endostatin, however, is restricted to angiogenic cells and does not affect quiescent cells.[17] A clinical trial has revealed that its use is not limited due to dose-dependent toxicity.[3] Additionally, the antiangiogenic effect of endostatin was enhanced in vitro and in vivo when it was used in combination with an anti-VEGF therapy.[42–45]

In CNV excised 5 days after verteporfin PDT, VEGF has been shown to be enhanced,[30 46] and endostatin to be significantly reduced.[46] An increased VEGF predominance over endostatin[47] is possibly responsible for recurrences after PDT. Intravitreal application of triamcinolone acetonide as adjuvant to PDT or monotherapy does not suppress VEGF but enhances endostatin expression in CNV. Therefore, in CNV treated with triamcinolone acetonide monotherapy or PDT triamcinolone acetonide combination therapy, the VEGF predominance score over

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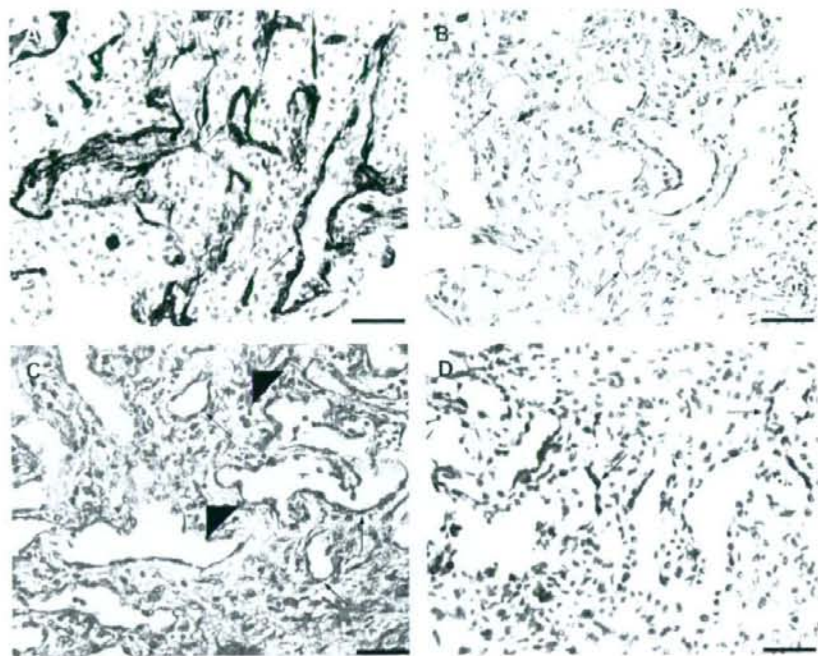

Figure 3 Photomicrographs of choroidal neovascularisation membranes extracted 4 days after intravitreal bevacizumab injection (case 5 in table 1). The sections were probed for CD34 (A), vascular endothelial growth factor (B), endostatin (C) and E-selectin (D). Haematoxylin was used as a counterstain. (A) CD34 immunostaining with brown 3-diaminobenzidine chromogen depicts vascularisation (arrows). (B) Endothelial cells (arrows) are immunonegative for vascular endothelial growth factor (VEGF) coloured with red chromogen. (C) Intense endostatin immunoreactivity is seen in vessels (arrow) and stromal cells (arrowhead) with the red chromogen. (D) E-selectin expression coloured with AEC chromogen was intense in endothelial cells (arrows). Scale bar: 50 µm.

endostatin is reduced to its level in control CNV.[40] However, bevacizumab seems to have a dual effect in angiogenesis inhibition by reducing intensity of VEGF expression besides enhancing endostatin. Consequently, VEGF predominance over endostatin was significantly lower in RPE, vessels and stroma of bevacizumab CNV than the control group. Bevacizumab, therefore, might be suggested as an ideal agent as adjuvant to verteporfin PDT. This seems to be clinically relevant, since decreased vascular leakage as well as a lower recurrence and retreatment rate have recently been reported after PDT–bevacizumab combination therapy.[44,45]

The authors are unaware of any previous histopathological reports investigating VEGF and VEGF predominance over endostatin in human CNV treated by intravitreal bevacizumab. The proper interpretation of this study is limited by the fact that our cases may represent a negative selection. Control and bevacizumab CNV groups with active and progressive disease activity were comparable in their clinics. However, the maturity and angiogenic activity of the specimens cannot be unified accurately, since the histopathology of the specimens reflects only a point within a dynamic process. Still, the results of this study give an important and clinically relevant insight into the effects of bevacizumab on human CNV. Besides decreased VEGF expression, an enhanced endostatin expression was detected in CNV treated with bevacizumab. Whether bevacizumab itself has a direct enhancing effect on endostatin expression needs to be further investigated in in vitro studies evaluating expression of endostatin in isolated human RPE, choroidal EC and stromal cells treated with bevacizumab. Nevertheless, bevacizumab therapy seems to enable VEGF predominance over endostatin to be significantly lower than control CNV, which could not be achieved with intravitreal triamcinolone acetonide injection and/or PDT.[46,47]

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CLINICAL SCIENCES

Effect of Bevacizumab on Inflammation and Proliferation in Human Choroidal Neovascularization

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Objective: To evaluate the effect of bevacizumab (Avastin; Genentech, Inc, South San Francisco, California) on inflammation and proliferation in human choroidal neovascularization (CNV) secondary to age-related macular degeneration.

Methods: Retrospective review of interventional series of 38 patients who underwent choroidal neaovascular membrane (CNVM) extraction. Twenty-four patients received intravitreal bevacizumab 1 to 154 days preoperatively (bevacizumab CNV group). Fourteen patients received no preoperative therapy (control CNV group). The CNVM were stained for cytokeratin 18, CD68, CD45, intercellular adhesion molecule (ICAM)–1, E-selectin, Ki-67, Thy-1, and endostatin.

Results: No significant difference was detected in ICAM-1 and E-selectin expression between groups. The density of leukocytes in the bevacizumab CNV group (median, 271.61 cells/mm^2) was higher than in the control CNV group (median, 116.87 cells/mm^2; $P = .07$), but without significance. Density of macrophages (median, 4661.95 cells/mm^2), proliferative activity (median, 160.19 cells/mm^2), and percentage of Thy-1–expressing vessels (median, 100%) were significantly higher in the bevacizumab CNV group than in the control CNV group (median, 882.66 cells/mm^2, $P < .001$, median, 34.34 cells/mm^2, $P < .001$; and median, 80%, $P < .001$, respectively). Endostatin immunoreactivity was considerably stronger in the retina pigment epithelium (RPE)–Bruch membrane complex (median, 3; range, 2-3; $P < .001$), and stroma (median, 3; range, 1-3; $P < .001$) of the bevacizumab CNV group than control CNV group (median, 1.5; range, 0-3 and median, 1; range, 0-3, respectively).

Conclusions: Unexpectedly, CNVM from patients treated by bevacizumab are characterized by significantly high inflammatory and proliferative activity and enhanced endostatin expression. These characteristics need to be considered when protocols for combination therapies are established.

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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] Neovascular AMD with the development of a choroidal neovascularization (CNV) accounts for 80% of the severe loss of visual acuity due to AMD.[2,3]

Introduction of angiogenesis inhibitory drugs, especially of vascular endothelial growth factor (VEGF) inhibitors, has been an important milestone in neovascular AMD treatment and has limited the indications for other treatment modalities considerably.

Of the VEGF inhibitors, pegaptanib[4] (Macugen; Eyetech Pharmaceuticals, New York, New York) and ranibizumab[5] (Lucentis; Genentech, Inc, South San Francisco, California) were shown to be effective in the treatment of neovascular AMD in large clinical trials.[6] Recently, bevacizumab (Avastin; Genentech, Inc), a full-length recombinant humanized monoclonal antibody against VEGF used previously for the treatment of metastatic colorectal cancer,[7] has been introduced to ophthalmology.[8-10] Use of bevacizumab either as a monotherapy or as an adjuvant to photodynamic therapy (PDT)[11] seems promising in CNV treatment. However, insight into the effect of these anti-VEGF drugs on human CNV is still limited.

Herein, we present our results of a clinicopathological evaluation of surgically excised choroidal neovascular membranes (CNVM) treated with an intravitreal bevacizumab injection prior to surgery. Our study focuses on inflammation and proliferative activity. Inflammatory infiltration was evaluated using antibodies against CD68 and CD45 (common leukocyte antigen) as markers for macrophages and leukocytes, respectively.[12-14] Considering their involvement in the recruitment of inflammatory cells, expression of intercellular adhesion molecule (ICAM)–1 and E-selectin were also evaluated.[15-17] The

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