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Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization

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Purpose: To determine the expression of angiogenic cytokines in macrophages and retinal pigment epithelium cells in choroidal neovascularization (CNV).
Methods: Ten surgically-excised subfoveal CNV specimens and ten eye bank eyes with subfoveal CNV were routinely processed, serially sectioned, and immunostained for factor VIII (F8), CD68 (KP1), cytokeratin 18 (CK18), vascular endothelial growth factor (VEGF), tissue factor (TF), and monocyte chemotactic protein (MCP). The CNV was classified as "inflammatory active" (more inflammation than fibrosis) or "inflammatory inactive" (morefibrosis than inflammation). The immunostaining was graded as none, mild (+), moderate (++), or heavy (+++). Five additional surgically-excised CNV specimens were dual labeled with CK18/MCP or CD68/TF and confocal scanning laser microscopy was performed.
Results: Vascular endothelium, macrophages, and RPE expressed F8, KP1, and CK18 respectively. Macrophages expressed + to ++ VEGF and ++ to +++ TF; RPE expressed ++ to +++ VEGF and ++ to +++ MCP. Staining for angiogenic cytokines was stronger in inflammatory active versus inflammatory inactive CNV. RPE dual labeled for CK18/MCP and macrophages dual labeled for CD68/TF.
Conclusions: This study shows that RPE cells express MCP, a cytokine involved with macrophage recruitment, and that macrophages express TF in CNV. Macrophages and RPE express VEGF, thus perpetuating angiogenesis. TF is involved with fibrin formation and provides a scaffold effect for growth of the CNV complex. CNV likely represents a dynamic process with inflammatory active and inflammatory inactive (involutional) stages.

The neovascular form of age-related macular degeneration (AMD), choroidal neovascularization (CNV), occurs in at least 0.6% to 0.7% of the population in some studies [1,2]. CNV that extends under the foveal avascular zone (subfoveal CNV) usually leads to loss of central vision and functional blindness. Subfoveal CNV also may occur in patients with the ocular histoplasmosis syndrome (OHS) and other diseases [3]. Histologic studies of post-mortem eyes with CNV have shown that the neovascularization arises in the choroid and invades through Bruch's membrane into the subretinal pigment epithelium and subretinal space [3-5]. CNV observed in post-mortem eyes is generally late in the course of the disease, showing involutional changes.

Submacular surgery has allowed for the study of CNV specimens earlier in the course of the disease than in post-mortem eyes. Histologic, immunohistochemical, and ultrastructural studies of surgically-excised CNV have shown that the cellular and extracellular constituents of CNV are the same regardless of the underlying disease, with the exception of the amount of basal laminar deposit and the presence of basal linear deposit, which is virtually exclusively found in CNV specimens from patients with AMD [6-11]. This leads to the concept that CNV represents granulation tissue proliferation as seen in a wound repair response. CNV is characterized by a stereotypic, non-specific response to a specific stimulus [9].

Retinal pigment epithelium (RPE) is a major component of CNV both in post-mortem eyes [3-5] and surgical specimens [6-10]. Additionally, surgically-excised CNV contains macrophages in approximately 60% of cases, regardless of the underlying disease [6-10]. The frequent presence of macrophages in surgically-excised CNV was not reported in post-mortem eyes [3-5], and indicates that CNV is a dynamic process associated with an influx of macrophages and later involutional changes.

Several studies have shown the presence of growth factors, including αFGF, βFGF, TGFβ and vascular endothelial growth factor (VEGF) in surgically-excised CNV [12-14]. Macrophages in CNV express tumor necrosis factor (TNFα) [15] and in vitro studies have shown that TNFα stimulates RPE production of interleukin-8 (IL-8) and monocyte chemotactic protein (MCP-1), the latter cytokine involved with the recruitment of macrophages [16-18]. It has been suggested that the macrophages expressing TNFα in CNV stimulate RPE expression of VEGF [15,19]. Additionally, TNFα produced by macrophages stimulates expression of integrins α3 and α5 on the RPE [20]. This tyrosinase kinase-mediated signal trans-

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Table 1. Immunohistochemical staining for cytokines in choroidal neovascularization

Case	Age	Sex	Disease	Type	VEGF	TF	MCP
Surgically-excised CNV:							
1	27	F	OHS	IA	+	+++	ND
2	27	F	OHS	IA	+	++	+
3	28	M	OHS	IA	+++	+++	ND
4	78	F	AMD	IA	+++	+++	+
5	34	M	OHS	II	+	++	ND
6	28	F	OHS	II	-	-	+
7	76	M	AMD	II	-	++	ND
8	75	F	AMD	II	++	++	ND
9	84	F	AMD	II	++	+	+
10	54	F	IDIO	II	-	-	-
Autopsy eyes:							
11	86	F	AMD	IA	++	++	ND
12	75	F	AMD	IA	++	++	ND
13	85	M	AMD	IA	+	++	+
14	99	F	AMD	II	-	+	+
15	74	M	AMD	II	+	+	+
16	82	M	AMD	II	+	-	++
17	87	M	AMD	II	+	-	-
18	91	M	AMD	II	+	-	+
19	79	M	AMD	II	+	-	ND
20	86	F	AMD	II	+	-	ND

Table shows the degree of immunostaining seen in each case. Key: VEGF=vascular endothelial growth factor, TF=tissue factor, MCP=macrophage colonization protein; F=female, M=male; OHS=ocular histoplasmosis syndrome, AMD=age-related macular degeneration, IDIO=idiopathic; IA=inflammatory active, II=inflammatory inactive cases; +=mild, ++=moderate, +++=heavy,ND=not done

duction pathway leads to cell migration in fibronectin [20], which is in the extracellular matrix of CNV [8]. Thus, macrophage production of the cytokine TNFα is involved with angiogenesis and cellular migration in CNV.

In this study, we immunostained post-mortem eyes with CNV and surgically-excised CNV for vascular endothelium (factor VIII; F8), macrophages (CD68), RPE (cytokeratin 18), and cytokines involved with recruitment of macrophages (MCP) and angiogenesis (VEGF), tissue factor (TF) in order to determine if there is in situ evidence of macrophage recruitment by RPE and macrophage production of angiogenic cytokines in CNV.

METHODS

Specimens: The methods conform to the Declaration of Helsinki for research involving human subjects. For immunohistochemistry, ten surgically-excised subfoveal CNV and ten eye bank eyes with AMD and subfoveal CNV from the L. F. Montgomery Ophthalmic Pathology Laboratory, Emory University, were studied. The surgically-excised CNV was re-

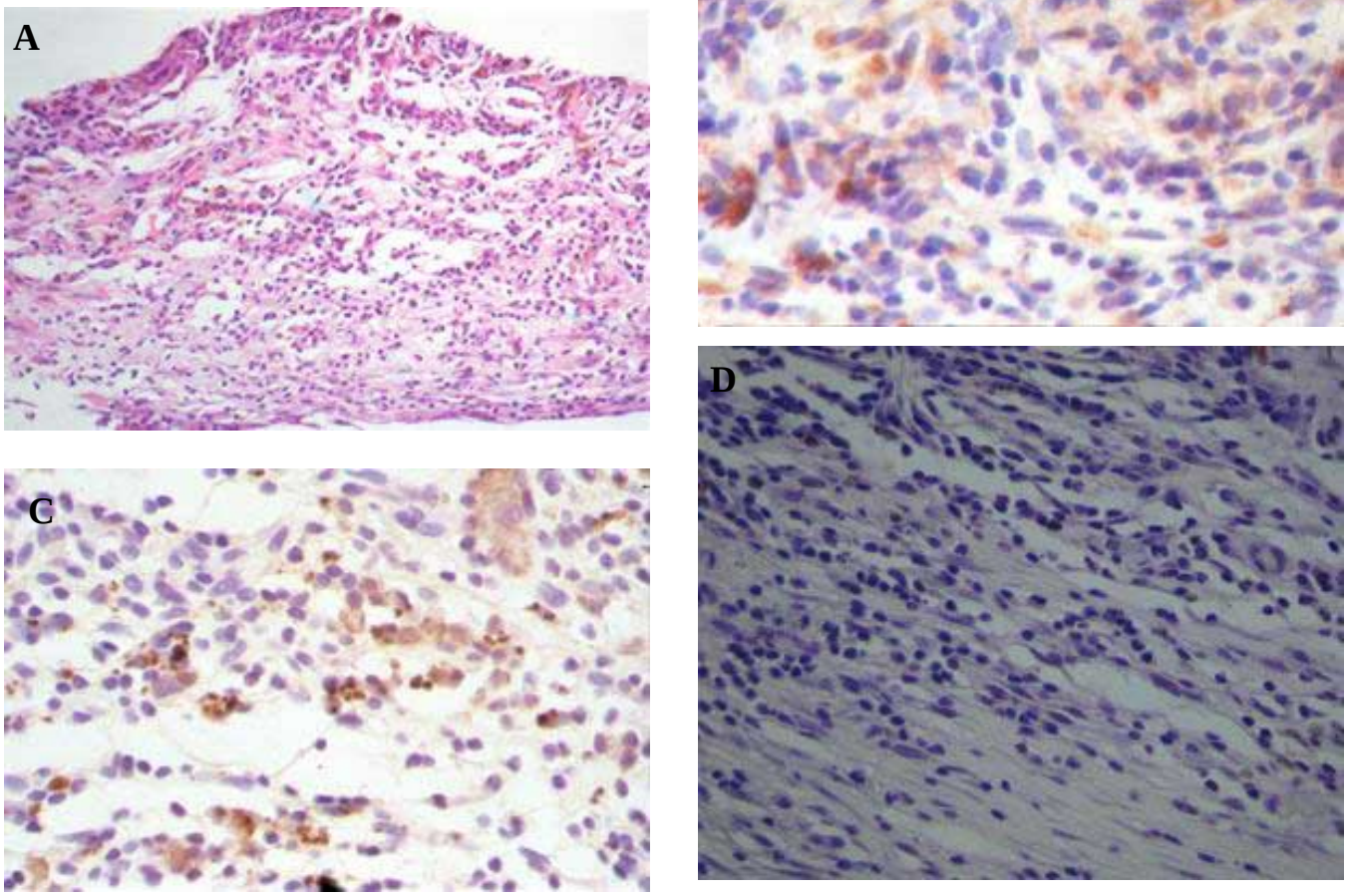

Figure 1. Immunostained surgically-excised inflammatory active CNV. **A**: Inflammatory active CNV with more inflammatory cells than fibrosis. (hematoxylin and eosin, 25x) **B**: There is VEGF expression in cells in the stroma, mainly macrophages and fibroblasts and some endothelial cells, as determined by cytologic characteristics [9]. (peroxidase anti-peroxidase, 63x) **C**: There are numerous cells in the stroma, mainly macrophages, expressing TF. (peroxidase anti-peroxidase, 63x) **D**: Negative control. (peroxidase anti-peroxidase, 63x)

moved as previously described [21]. All patients had visual acuities of 20/200 or less, exudative retinal detachments, and angiographically classic CNV. The eye bank eyes were grossly determined to have age-related macular degeneration by the Alabama age-related macular degeneration grading system for donor eyes [22] and histologically found to exhibit AMD with subfoveal CNV [3,4]. There was no clinical information available for these patients. For confocal microscopy, five additional surgically-excised CNV specimens with the same clinical features as described above were studied. All specimens were fixed in 10% neutral buffered formalin and routinely processed through increasing concentrations of alcohol, cleared in xylene, and embedded in paraffin. The surgically-excised CNV was characterized as "inflammatory active" if there were more inflammatory cells than fibrosis (i.e., >50% inflammatory cells) identified or "inflammatory inactive" if there was more fibrosis than inflammatory cells present (i.e., <50% inflammatory cells). For eye bank eyes, the CNV was classified as "active" if inflammatory cells occupied >25% of the CNV

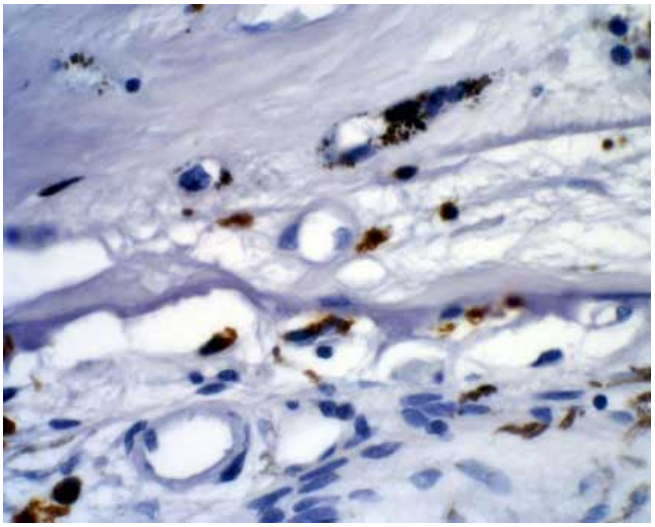

Figure 3. Macrophages along Bruch's membrane in CNV. There are CD68-positive macrophages along the outer side of Bruch's membrane and along the inner side in the choroidal neovascularization. (peroxidase anti-peroxidase, 100x)

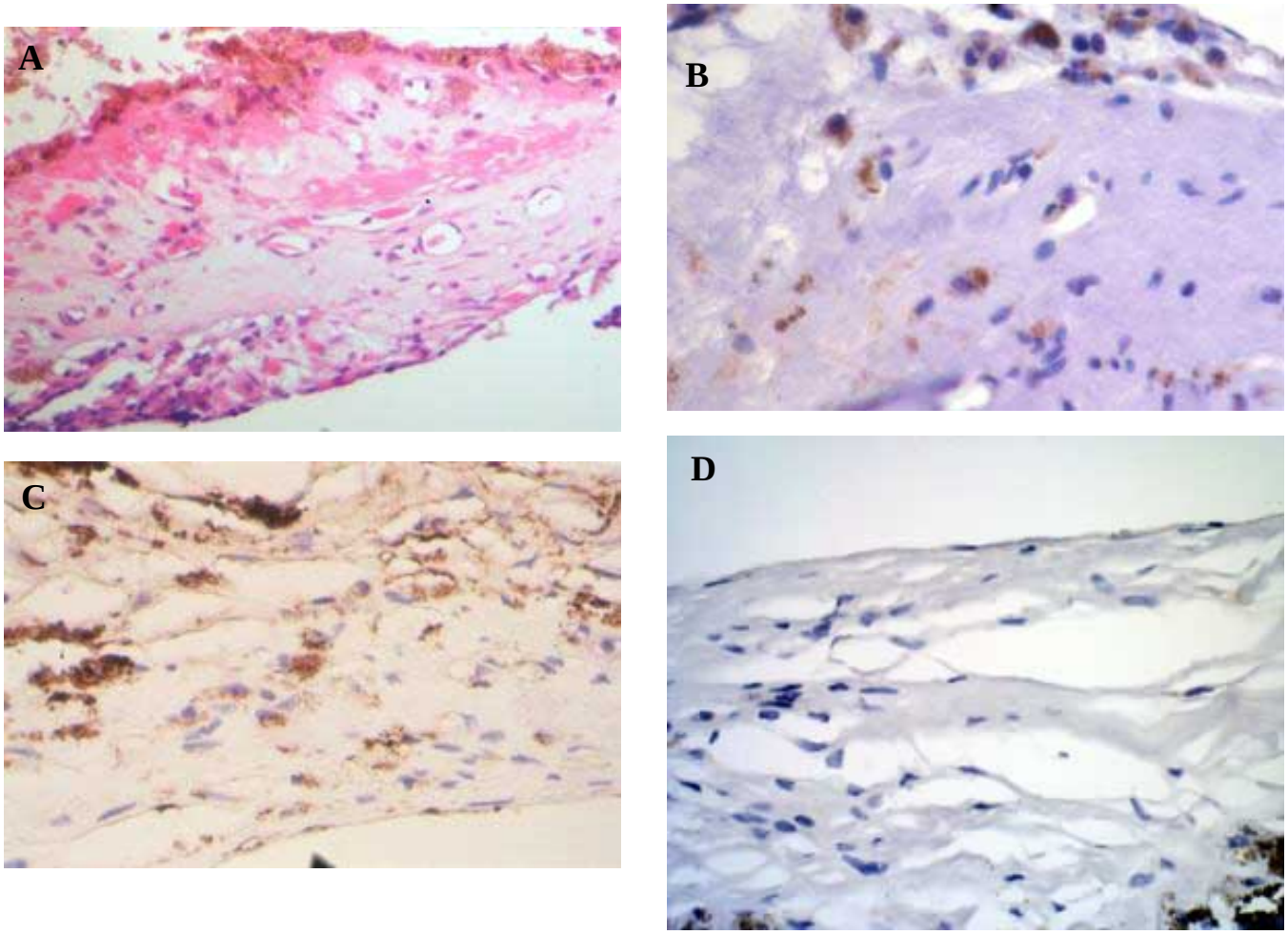

Figure 2. Immunostained surgically-excised inflammatory inactive CNV. **A**: Inflammatory inactive CNV with more fibrosis than inflammatory cells. (hematoxylin and eosin, 25x) **B**: There are rare macrophages and fibrocytes in the stroma expressing VEGF. (peroxidase anti-peroxidase, 63x) **C**: There are rare cells in the stroma, mainly macrophages, expressing TF. (peroxidase anti-peroxidase, 63x) **D**: Negative control. (peroxidase anti-peroxidase, 63x)

and "inactive" if inflammatory cells occupied <25% of the CNV.

Immunohistochemistry: Serial sections of formalin-fixed, paraffin embedded tissue were obtained through the centers of the CNV in the surgical and eye bank specimens. The sections were placed on poly-l-lysine coated slides, one slide was stained with hematoxylin and eosin, and the remaining slides were immunostained using the standard avidin-biotin-complex technique [23]. The primary antibodies were for F8 (1:100, Dako, Carpinteria, CA) to identify vascular endothelium; CD68 (KP1, 1:100, Dako) to identify macrophages; cytokeratin 18 clone CY90 (CK18, 1:10, Sigma, St. Louis, MO) to identify RPE [24], the angiogenic cytokine vascular endothelial growth factor [25] (VEGF, 1:120, NeoMakers, Union City, Ca); TF (1:160, American Diagnostica, Greenwhich, Conn), a primary cellular initiator of blood coagulation [26]; and macrophage chemotactic protein (MCP, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA), a chemoattractant and activator of macrophage infiltration into tissue [27]. The chromogen was diaminobenzidine (DAB) for all antibodies except MCP, with which amino-ethyl carbazole (AEC) was utilized. Due to limited amount of tissue, it was not possible to stain all specimens for MCP.

The intensity of the immunostaining was graded as none (-), mild (+, up to 1/3 cells present staining), moderate (++, 1/3-2/3 cells present staining), or heavy (+++, 2/3-all cells present staining). The cell type that exhibited positive immunostaining was determined by morphologic and cytolologic criteria [3,9] and immunostaining for F8, KP1, or CK18 in adjacent serial sections. Positive controls were spleen and normal eye; negative controls used pre-immune serum to replace the specific antibodies and were otherwise processed in the same manner as the test slides.

Confocal Scanning Laser Microscopy: Five surgically-excised choroidal neovascular (CNV) membranes from patients with ocular histoplasmosis syndrome (OHS) and age-related macular degeneration (AMD) were evaluated by confocal scanning laser microscopy. The CNV specimens were deparaffinized, blocked with horse serum washed with PBS, and incubated with CD68 IgG or MCP (1:100) at 4 °C. The tissue was washed, incubated in donkey anti-mouse IgG-Rhodamine Red conjugate (1:200), washed and incubated in goat anti-mouse Fab fragments (1:50) for two hours. The tissue was washed, incubated overnight with TF or CK18 (10µg/ml), washed, incubated with donkey anti-mouse IgG-FITC (1:200) conjugate at 2∞for 24 h, washed and incubated in clearing solution, and coverslipped. A Biorad confocal scanning laser microscope was used to obtain digitized images.

RESULTS

The results of the immunohistchemical staining are summarized in Table 1. There were ten surgically-excised CNV speci-

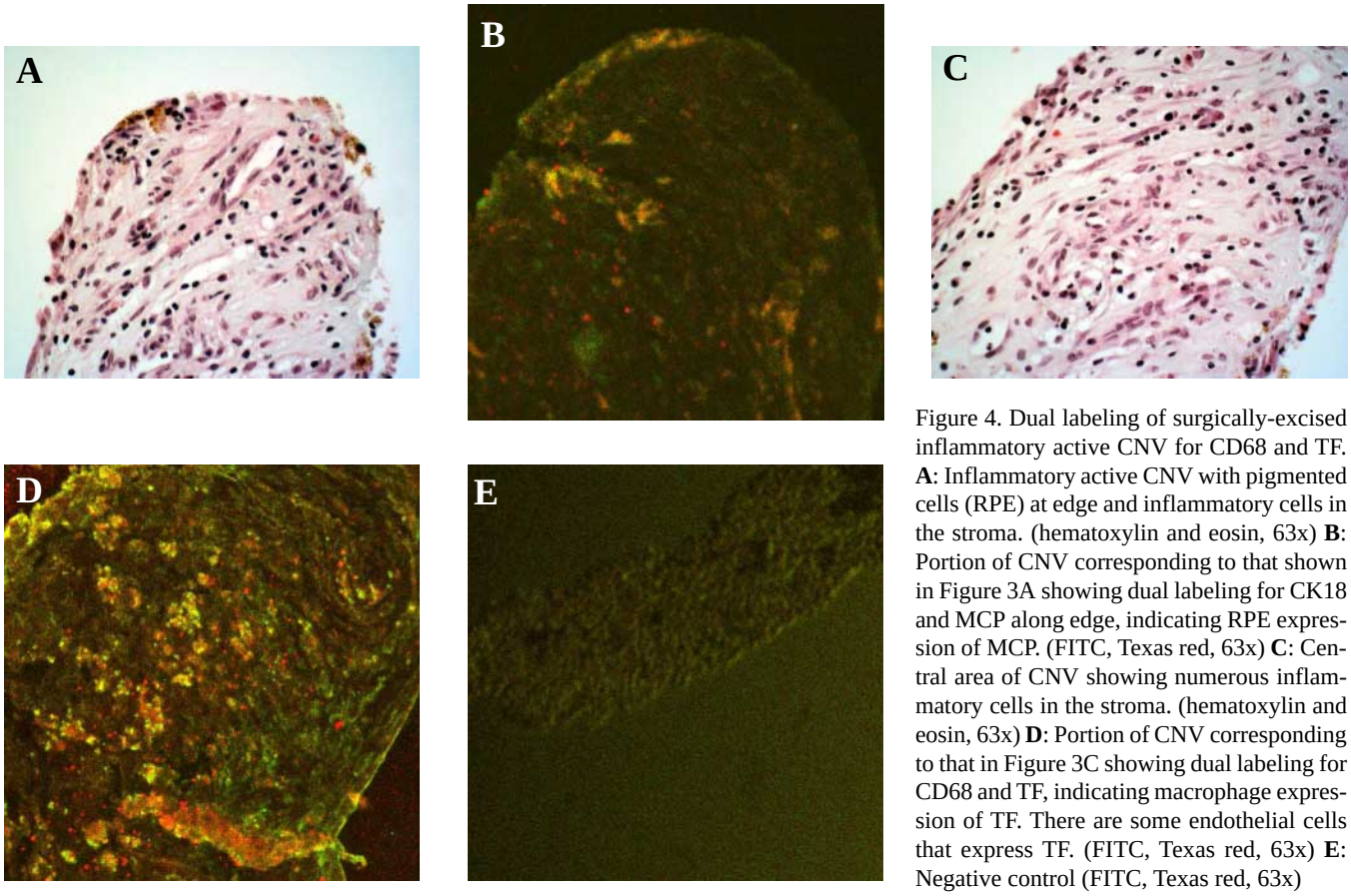

Figure 4. Dual labeling of surgically-excised inflammatory active CNV for CD68 and TF. **A**: Inflammatory active CNV with pigmented cells (RPE) at edge and inflammatory cells in the stroma. (hematoxylin and eosin, 63x) **B**: Portion of CNV corresponding to that shown in Figure 3A showing dual labeling for CK18 and MCP along edge, indicating RPE expression of MCP. (FITC, Texas red, 63x) **C**: Central area of CNV showing numerous inflammatory cells in the stroma. (hematoxylin and eosin, 63x) **D**: Portion of CNV corresponding to that in Figure 3C showing dual labeling for CD68 and TF, indicating macrophage expression of TF. There are some endothelial cells that express TF. (FITC, Texas red, 63x) **E**: Negative control (FITC, Texas red, 63x)

mens obtained from seven women and three men ranging in age from 27 to 84 years. Five of the patients had ocular histoplasmosis syndrome (OHS), four had AMD, and one had idiopathic disease. There were ten eye bank specimens from four women and six men ranging in age form 74 to 99 years. All of the eye bank specimens were from patients with AMD. There were both inflammatory active and inflammatory inactive CNV in both the surgical and eye bank groups.

CNV with F8 positive vascular endothelium was present in eight surgical and eight eye bank specimens. KP1 staining was positive in macrophages and CK18 was positive in RPE in all specimens. VEGF was variably expressed in macrophages and strongly expressed in RPE. VEGF was also expressed in fibrocytes and photoreceptors. TF was strongly expressed in macrophages, and variably expressed in RPE. There was stronger staining for VEGF and TF in inflammatory active (Figure 1) versus inflammatory inactive (Figure 2) surgically-excised CNV. MCP was expressed in RPE and fibrocytes. Macrophages were present in the choriocapillaris and exhibited migration along the outer (choroidal) side of Bruch's membrane areas of CNV was present (Figure 3). Results of the confocal scanning laser microscopy showed that approximately 50% of the RPE dual labeled for CK18/MCP and approximately 25% of macrophages dual labeled for CD68/TF, both in inflammatory active and inflammatory inactive CNV (Figure 4 and Figure 5). The RPE was concentrated around the edges of the CNV and macrophages were in the stroma of the CNV. The inflammatory active surgically-excised CNV (Figure 1 and Figure 4) was more cellular than the inflammatory inactive CNV (Figure 2 and Figure 5). The inflammatory active eye bank CNV (Figure 6) was more cellular than the inflammatory inactive eye bank CNV (Figure 7). The inflammatory active CNV contained more cytokine (TF, MCP) positive cells than the inflammatory inactive CNV in both surgically-excised CNV and eye bank CNV.

DISCUSSION

Evaluation of surgically-excised CNV has demonstrated that the majority of specimens contain macrophages, regardless of underlying disease [6-10], a finding not appreciated in postmortem eyes with CNV [3-5]. Initial studies of surgically-excised CNV showed that the cellular and extracellular components are consistent with a wound repair response (granulation tissue proliferation) [6-10]. Additionally, CNV in post mortem eyes appears to be more fibrotic than surgically-excised CNV, supporting the concept that CNV is a dynamic process that once initiated, contains an inflammatory cell component, and later undergoes involution.

Investigators have studied cytokine expression in surgically-excised CNV specimens and demonstrated that the RPE produces aFGF, bFGF, TGFβ, and VEGF [12-14]. Experimentally-produced CNV has also exhibited VEGF production by the RPE [28,29]. Angiopoietins and their vascular endothelial

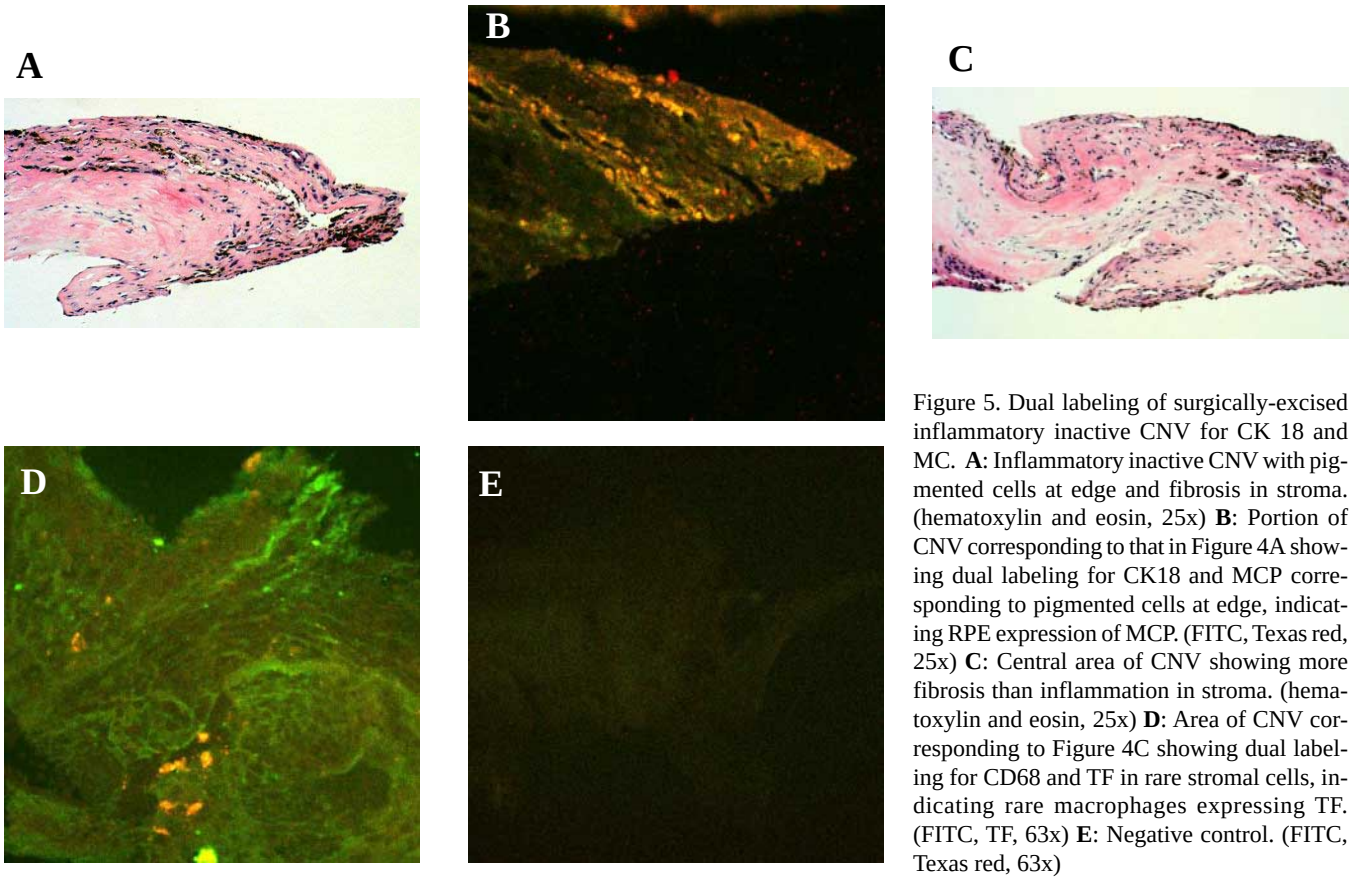

Figure 5. Dual labeling of surgically-excised inflammatory inactive CNV for CK 18 and MC. **A**: Inflammatory inactive CNV with pigmented cells at edge and fibrosis in stroma. (hematoxylin and eosin, 25x) **B**: Portion of CNV corresponding to that in Figure 4A showing dual labeling for CK18 and MCP corresponding to pigmented cells at edge, indicating RPE expression of MCP. (FITC, Texas red, 25x) **C**: Central area of CNV showing more fibrosis than inflammation in stroma. (hematoxylin and eosin, 25x) **D**: Area of CNV corresponding to Figure 4C showing dual labeling for CD68 and TF in rare stromal cells, indicating rare macrophages expressing TF. (FITC, TF, 63x) **E**: Negative control. (FITC, Texas red, 63x)

receptors (Tie1, Tie2) have been demonstrated in CNV [17]. Photoreceptors and M¸ller cells have been shown to express VEGF and TGFβ in association with CNV [28-30]. There is vascular endothelial production of αFGF, βFGF, TGFβ and VEGF in CNV [12,30]. Additionally, metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are produced by vascular endothelium and RPE in CNV [31]. Not surprisingly, macrophages and the above-mentioned cytokines, MMPs, and TIMPs found in CNV are present in granulation tissue proliferation [8,32,33]. The stage of CNV characterized by cytokine production and the presence of inflammatory cells [6-10] may be considered an inflammatory active stage, where autocrine and paracrine production of cytokines maintains the CNV, and CNV growth is contained, for instance, by TIMP production [31].

Prior to the inflammatory active stage, inflammatory cells are likely recruited during an initiation stage. There is experimental evidence of monocyte chemotactic protein (MCP) production by the RPE after stimulation with various cytokines (IL-1β, TNFα, IL4) [16-18] and macrophage adherence to vascular endothelium under flow conditons [34]. One study has shown in situ expression of TNFα by macrophages in CNV [15]. There is experimental evidence that TNFαactivation of integrins α3 and α5 cause RPE migration in fibronectin [20]. TNFα produced by macrophages upregulates RPE production of VEGF [15].

In our study, we demonstrated in situ expression of MCP by the RPE in CNV (Figure 3 and Figure 4). We observed

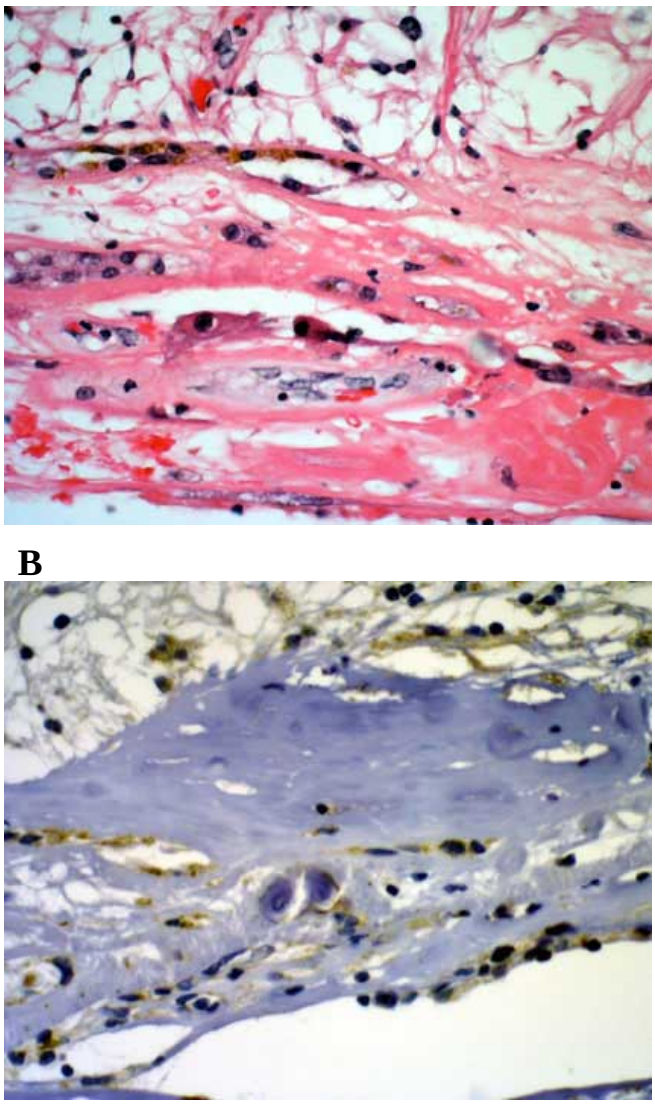

Figure 6. Immunostained autopsy eye inflammatory active CNV. **A**: Inflammatory active eye bank CNV showing inflammatory cells including macrophages and lymphocytes in fibrovascular tissue. (hematoxylin and eosin, 100x) **B**: Immunohistochemical stains are positive for TF in scattered fibrocytes and macrophages in the membrane. (peroxidase anti-peroxidase, 100x)

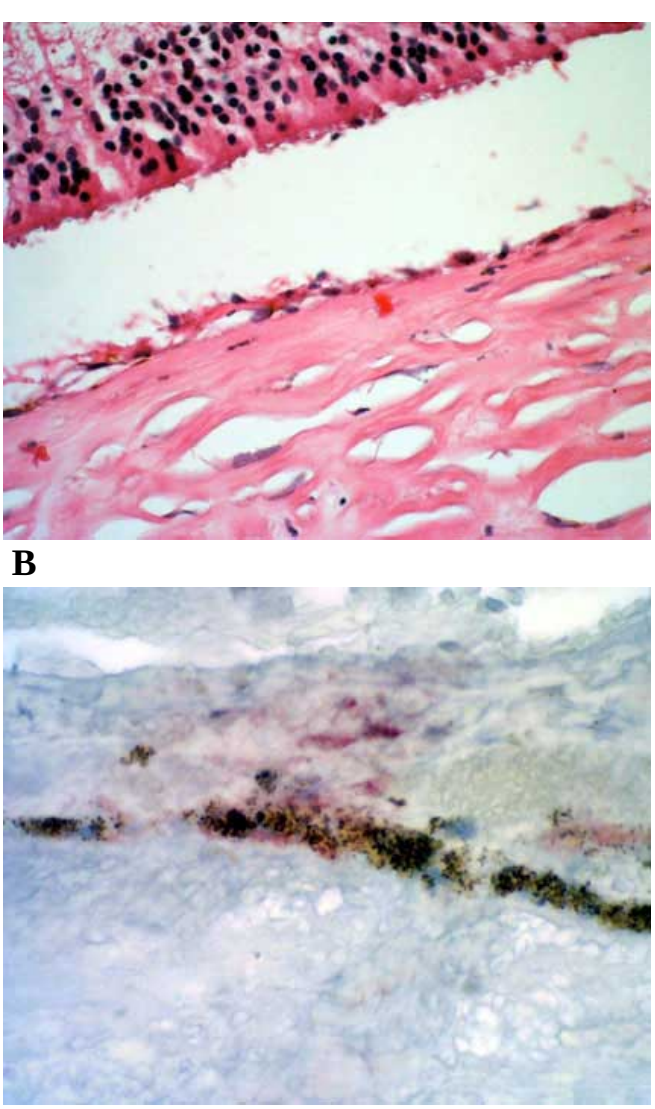

Figure 7. Immunostained autopsy eye inflammatory inactive CNV. **A**: Inflammatory inactive eye bank CNV showing lack of inflammatory cells in fibrovascular tissue. (hematoxylin and eosin, 100x) **B**: Immunohistochemical stains are positive for MCP in scattered RPE cells. (peroxidase anti-peroxidase, 100x)

KP1 positive macrophages aggregated in a linear arrangement along the outer (choroidal) side of Bruch's membrane in areas of CNV and in areas of drusen in eye bank eyes. This leads to the concept that during the initiation stage of CNV, there is an interaction between macrophages and the RPE, either causing RPE production of MCP or the result of MCP production by the RPE. We demonstrated TF production by the macrophages in the CNV (Figure 3 and Figure 4). TF is a 46-Kd glycoprotein that is the receptor and essential cofactor for coagulation factor VII/VIIa and is involved with fibrinogenesis [26]. This fibrin deposition potentially serves as a scaffold for vascularization in CNV and RPE migration, as in granulation tissue proliferation [8,32,33]. It is known that fibrin and fibronectin are major components of surgically-excised inflammatory active CNV [6-10], although it is not usually present in inflammatory inactive CNV in post-mortem eyes [3-5].

Thus far, we have discussed the initiation and active stages of CNV. There is both clinical [3] and experimental [29] evidence of spontaneous involution of CNV. This involution is accompanied by decreased cytokine production, in particular, VEGF [29]. Our study showed decreased staining for TF and VEGF in inflammatory inactive versus inflammatory active CNV, indicating shut-down of cytokine production and fibrosis as the CNV matures. In essence, CNV is a dynamic wound repair response, characterized by active and inactive (involutional) stages. Macrophages play an important role in CNV, with influx as a response to MCP production by RPE. Macrophages secrete TF and VEGF during the active stage of CNV. Further studies regarding the dynamics of CNV formation may lead to understanding the topographic appearance of CNV [35] and possible therapeutic interventions.

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