A R T I C L E S

The Potential Angiogenic Role of Macrophages in the Formation of Choroidal Neovascular Membranes

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Purpose. To investigate the distribution of inflammatory mediators such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α and angiogenic cytokines such as vascular endothelial growth factor (VEGF) and to identify their cellular source in surgically excised choroidal neovascular membranes (CNVMs) of various origins.

Methods. Immunoperoxidase staining was performed on paraffin-embedded sections of 11 surgically excised CNVMs to identify cellular distribution and localization of cytokines. Immunofluorescent double staining was performed to detect the cellular source of cytokines.

Results. Cytokeratin-positive cells were detected in the RPE layer, in stromal cells, and around neovascular vessels. Macrophages identified by their cellular marker CD68 showed almost the same distribution as cytokeratin-positive cells, although they were most prominent in the stroma. A substantial number of neovascular vessels were also immunoreactive to IL-1β and TNF-α. Immunofluorescent double staining revealed that the RPE layers immunopositive for cytokeratin were also immunopositive for all cytokines, whereas stromal cells immunostained for CD68 were positive for IL-1β and TNF-α, but not for VEGF.

Conclusions. These results indicate that IL-1β and TNF-α secreted by macrophages may promote, at least in part, angiogenesis in CNVMs by stimulating VEGF production in RPE cells. (*Invest Ophthalmol Vis Sci.* 1999;40:1891–1898)

Subfoveal neovascularization is a severe sight-threatening complication of various macular diseases, including age-related macular degeneration (ARMD). Recent immunohistochemical studies on surgically removed choroidal neovascular membranes (CNVMs) have implicated a variety of angiogenic factors.[1–3] One of the candidate agents for angiogenesis is vascular endothelial growth factor (VEGF), which has been shown to have remarkable potency to induce specific proliferation of endothelial cells[4,5] and to increase vascular permeability.[6] Moreover, this cytokine has been implicated also in pathologic angiogenesis, including tumor growth,[7,8] and in a variety of ocular disorders, such as proliferative diabetic retinopathy, central retinal vein occlusion, and retinoblastoma.[9,10] Cell types that have been reported to be immunopositive for VEGF in ocular tissue are retinal pigment epithelial (RPE) cells under physiological conditions[11] and glial cells, vascular endothelial cells, fibroblasts, and RPE cells under pathophysiological conditions.[2,12] Hypoxia has been shown to be the major stimulator of VEGF induction in various cell types.[7,11,13–15] Although ischemia has been suggested to be the pathogenic feature responsible in part for VEGF production in ARMD,[16,17] stimuli for induction of VEGF in CNVMs remains to be elucidated, because ischemia alone cannot account for all the ocular diseases that lead to the formation of CNVMs. Furthermore, a recent study indicated that VEGF expression can precede retinal nonperfusion and neovascularization.[18]

Macrophages produce various angiogenic cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, basic fibroblast growth factor (bFGF), VEGF, and transforming growth factor (TGF)-β,[19–21] and thus play a key role in angiogenesis. This concept is further supported by recent studies showing that chemoattractant of monocytes promotes angiogenesis[22] and that accumulation of macrophages significantly correlates with the number of newly formed vessels after vascular occlusion.[23] In spite of their paucity in normal retina, macrophages are thought to interact intimately with RPE cells under pathologic conditions, because they are seen in proximity to RPE cells in various ocular diseases, including proliferative vitreoretinopathy and the retinopathy of acquired immune deficiency syndrome and in animal models of experimental autoimmune uveitis.[24–26] Although the involvement of macrophages and other inflammatory cells has been identified in CNVM,[1,27,28] little is known about their roles other than mediation of inflammation.

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Table 1. Clinical Characteristics

Eye	Diagnosis	Age	Sex	Side	MPS Classifcation	CNVM Size*
1	ARMD	68	M	L	Well defined	2.2
2	ARMD	68	M	R	Occult	1
3	ARMD	65	M	L	Well defined	1.3
4	ARMD	74	M	L	Well defined	0.5
5	ARMD	67	F	L	Well defined	2
6	Myopia	34	M	L	Occult	0.7
7	Myopia	42	F	R	Well defined	0.5
8	Myopia	68	F	L	Well defined	1.2
9	AS	69	F	R	Well defined	1
10†	AS	69	F	L	Well defined	1.5
11	Idiopathic	43	F	L	Well defined	0.6

AS, angioid streaks; MPS, Macular Photocoagulation Study.
* In disc diameter.
† Fellow to eye 9.

We hypothesized that macrophages in CNVMs may exert some effects on production of VEGF by RPE cells and thereby initiate or contribute, at least in part, to choroidal neovascularization. Accordingly, we first performed immunohistochemical studies on the cellular distribution of RPE cells and macrophages and also on the localization of VEGF as an angiogenic factor and on IL-1β and TNF-α as presumably macrophage-derived cytokines. To determine the contribution to angiogenesis by macrophages, immunofluorescent double staining was used to confirm the overlap between cellular components and cytokines.

Materials and Methods

Clinical Characteristics

Specimens were obtained from 11 eyes of 10 patients. Their ages ranged from 34 to 74 years (59.8 ± 4.3 years, mean ± SD), at the time of vitrectomy. CNVMs of several origins were obtained: five ARMD, three myopia, two angioid streaks, and one idiopathic choroidal neovascularization (Table 1). Informed consent for the surgical procedure and for the use of excised tissue was obtained from all patients. All procedures followed the tenets of the Declaration of Helsinki, and institutional human experimentation committee approval was obtained for the study. Vitrectomy was performed according to the technique described previously.[29] Clinical characteristics of all patients are presented in Table 1. The size of the CNVM in relation to optic disc diameter was determined by measurements made on the preoperative fluorescein angiogram. None of the patients had undergone foveal laser photocoagulation.

Tissue Preparation

Each specimen of CNVM was fixed in 3.7% formalin with phosphate-buffered saline (PBS) at pH 7.4 for 4 hours at 4°C, dehydrated with a graded alcohol series, and then embedded in paraffin. Subfoveal membranes were serially sectioned at 5-μm thick and placed on aminopropyltriethoxysilane-coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining.

Immunohistochemistry

For immunohistochemical studies, all incubation steps were performed in a moist chamber, and rinses were performed by immersing the slides in a PBS bath. Sections were rehydrated with a graded series of alcohol and rinsed with PBS. Hydrogen peroxide-methanol (0.3%) was applied to each specimen for 10 minutes to block endogenous peroxide activity. After incubating with blocking serum for 20 minutes, the slides were incubated with primary antibodies for 30 minutes, washed again with PBS for 10 minutes, and then incubated with biotinylated secondary antibody for 30 minutes. Sections were rinsed in PBS for 10 minutes, incubated with avidin-conjugated alkaline phosphatase for 30 minutes, and then washed again with PBS. A standard indirect immunoperoxidase protocol using a kit (Elite ABC; Vector Laboratories, Burlingame, CA) were performed with diaminobenzidine tetrahydrochloride (DAB; Dako) as the substrate. Finally, the slides were rinsed with tap water for 5 minutes, dehydrated through a graded series of alcohol, and then coverslipped with xylene-based permanent mounting medium. Hematoxylin and eosin staining was also performed on adjacent sections to evaluate general pathologic changes.

The procedure of double immunofluorescence staining was similar for peroxidase immunostaining except that sections were incubated with primary antibodies overnight at 4°C, followed by 4 hours of incubation with fluorescent dye–conjugated secondary antibodies at room temperature. Bleaching to remove endogenous peroxide was not performed on any slides. A commercial mounting medium (Vecta Shield; Vector Laboratories) was used. Slides were viewed and photographed in an inverted high-resolution laser-scanning microscope (model LSM 410; Carl Zeiss, Oberkochen, Germany).

To study cell distribution, monoclonal mouse antibodies against cytokeratin (anti-pancytokeratin, 1:100 dilution; Sigma, St. Louis, MO) and CD68 (1:50 dilution; Elm, Rome, Italy) were used to visualize RPE cells and macrophages, respectively. Rabbit polyclonal antibodies against VEGF (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), IL-1β (1:300 dilution; Endogen, Woburn, MA), and TNF-α (1:200 dilution; Endogen) were used to detect cytokines. Fluorescein-isothiocyanate (FITC)-coupled rabbit anti-mouse antibody and tetramethylrhodamine isothiocyanate isomer R (TRITC)-coupled goat anti-rabbit antibody (both 1:40 dilution; Dako) were used as the detection system in immunofluorescent staining. All antibody concentrations were determined individually on ap-

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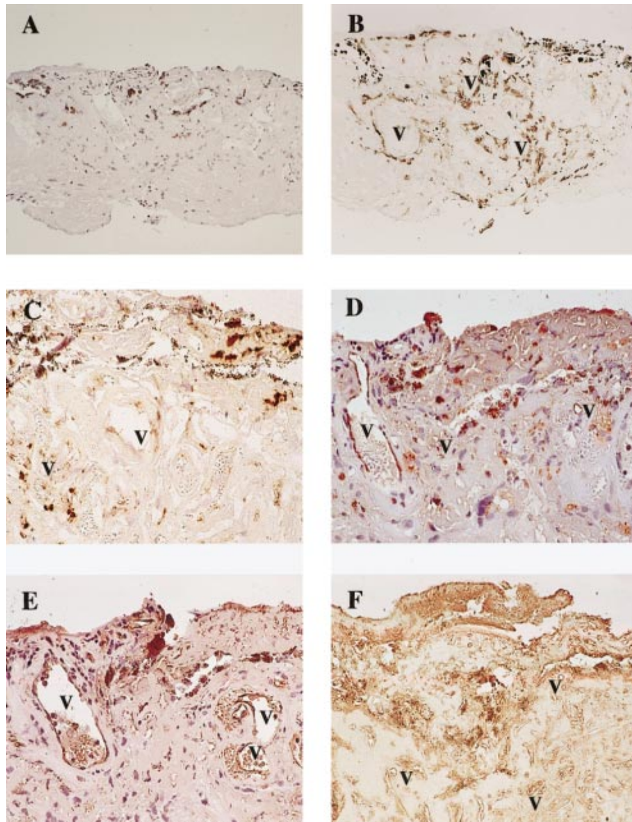

FIGURE 1. Immunohistochemistry of a CNVM caused by ARMD (specimen 4). (A) Negative control. (B) Immunohistochemistry for cytokeratin revealed that numerous cytokeratin-positive cells were located in the RPE monolayer, the stroma, and around the neovascular vessels. (C) CD68 immunoreactive cells were detected in the similar locations. (D) Immunohistochemistry for VEGF revealed that some of the cells in the RPE monolayer and many of the stromal cells were positive for VEGF. Of note, only some of the neovascular endothelial cells were positive for VEGF. (E) Immunohistochemistry for IL-1β showed that most of the neovascular endothelial cells and the RPE monolayer and fewer stromal cells were immunopositive for IL-1β. (F) Immunohistochemistry for TNF-α revealed uniform positive immunostaining in the neovascular endothelial cells and the RPE monolayer. Some stromal cells were also positive for TNF-α. V, neovascular vessels that had positive endothelial cells or positive perivascular immunostaining. Magnification, (A, B) $\times$200; (C through F) $\times$400.

propriate positive control tissues. Negative control slides were made by substituting primary antibody with an irrelevant primary antibody of the same isotype.

RESULTS

Immunohistochemical Study

All specimens contained a partially intact monolayer of RPE cells located on one side of the CNVMs (Figs. 1A, 2A). Other cell types, morphologically presumed to be fibroblasts and macrophages, were also observed in the stroma, in varying numbers. The extent of neovascularization and of fibrosis differed among specimens (Table 2). Neovascularization was more prominent than fibrosis in 6 of the 11 membranes.

Staining of Cellular Components

To identify the cellular origin of stromal cells, an immunohistochemical study was performed using cell type-specific antibodies. Immunostaining of pancytokeratin revealed many positive cells in the RPE monolayer. Positive immunostaining was also observed in stromal cells in some sections, and some of these cells were located in perivascular areas (Figs. 1B, 2B; Table 2). In most sections that contained abundant immunoreactive stromal cells or numerous neovascular vessels with immunopositive cells in the perivascular areas, neovascularization appeared to be predominant in comparison with fibrosis (specimens 4, 5, 6, 8). Macrophages, identified by immunostaining of CD68, were detected abundantly in all CNVMs and were located mainly in the stroma, with fewer seen in the RPE monolayer and in perivascular areas (Figs. 1C, 2C). Unlike

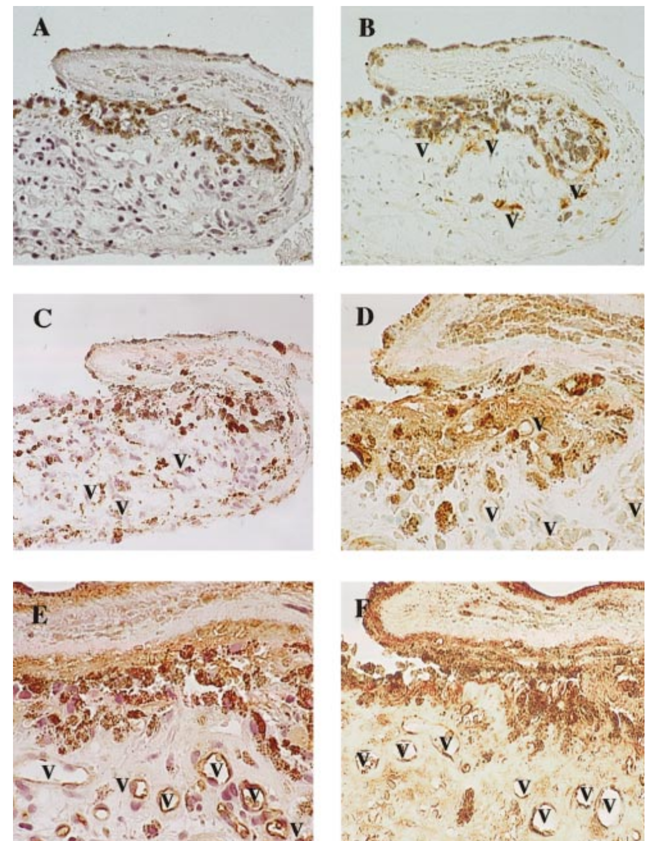

FIGURE 2. Immunohistochemistry of a cellular, angioid streaks–related CNVM (specimen 9). (A) Negative control. The outer surface shows an intact RPE monolayer. The membrane contains numerous neovascular vessels. (B) Immunohistochemistry for cytokeratin revealed scattered positive immunostaining in the RPE monolayer, the stromal cells, and around the neovascular vessels. (C) Numerous CD68-immunoreactive cells were detected in these locations. (D) Immunohistochemistry for VEGF revealed that the RPE monolayer, stromal cells, and neovascular endothelial cells were positive for VEGF. (E) Immunohistochemistry for IL-1β revealed prominent positive immunostaining in the neovascular endothelial cells and the RPE monolayer and patchy immunostaining in the stromal cells. (F) Immunohistochemistry for TNF-α revealed uniform positive immunostaining in the neovascular endothelial cells and the RPE monolayer. Some stromal cells were also positive for TNF-α. V, neovascular vessels that had positive endothelial cells or positive perivascular immunostaining. Magnification, (A, B, C) $\times$200; (D, E, F) $\times$400.

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Table 2. Histopathologic Characteristics

		Pancytokeratin			CD68		
Eye	Fibrosis*/ NV†	RPE Monolayer‡	Perivascular Area‡	Stroma‡	RPE Monolayer‡	Perivascular Area‡	Stroma‡
1	+/++	++	+	+	+	+	++
2	+++/+	++	++	+	+	+	++
3	+++/+	++	+	+	+	+	++
4	+/+++	++	++	++	+	++	++
5	+/++	++	++	++	+	+	++
6	+/+++	++	++	+	+	++	++
7	++/++	++	+	+	+	+	++
8	+/++	++	++	++	+	+	++
9	++/+++	++	+	+	+	++	++
10	+++/++	++	+	+	+	+	++
11	++/++	++	++	+	+	++	++

* Percent area on histologic section. +, 10–40%; ++, 40–70%; +++, 70–100%.

† By analysis of hematoxylin–eosin stain and immunoperoxidase stain for human von Willbrand factor. +, few vessels; ++, moderate number of vessels; +++, frequent vessels.

‡ +, Patchy staining; ++, uniform staining.

immunoreactivity to cytokeratin, immunostaining for CD68 was uniform in the stroma. In most sections that contained numerous neovascular vessels with immunopositive cells in the perivascular areas, as in the case of immunoreactivity for cytokeratin, neovascularization was more predominant in comparison with fibrosis (specimens 4, 6, 9). No cause-specific distribution was observed in the immunostaining for either cytokeratin or CD68.

Staining of Cytokines

Immunoperoxidase staining for cytokines was detected to some extent in all specimens. Regardless of the type of cytokine, positive immunostaining was distributed in the RPE monolayer, stromal cells, and neovascular vessels, although the intensity of immunostaining showed a cytokine-specific difference. In most sections, uniform immunostaining for VEGF was observed in the RPE monolayer (Figs. 1D, 2D; Table 2). In contrast, only patchy immunostaining was detected in neovascular vessels and in stromal cells; however, uniform immunostaining of these two components seemed to correlate with the extent of neovascularization (specimens 4, 5, 6, 8, 9). Staining for IL-1β revealed marked immunoreactivity in neovascular vessels and focal immunoreactivity in the RPE monolayer and in some stromal cells (Figs. 1E, 2E). Positive immunostaining for TNF-α in the RPE monolayer was more prominent than that of IL-1β. Uniform immunostaining was also observed in the neovascular vessels (Figs. 1F, 2F). Again, no cause-specific distribution was observed in the immunostaining for these cytokines.

Immunofluorescent Double Staining

Immunofluorescent double staining was performed to confirm further the cellular source of cytokines. Most of the cytokeratin-positive cells in the RPE monolayer were also immunoreactive to VEGF (Figs. 3A, 3B). A similar pattern of staining between cytokeratin-positive cells and cytokine-positive cells was also observed with both IL-1β and TNF-α (Figs. 3C through 3F). Some stromal cells immunopositive for CD68 were found to be stained for IL-1β and TNF-α (Figs. 4A through 4D). However, no overlap was observed by double staining for CD68 and VEGF (Fig. 4E).

Discussion

The stroma of ARMD-related CNVMs is composed of various cell types, including RPE cells, glial cells, fibroblasts, myofibroblasts, vascular endothelial cells, pericytes, macrophages, and lymphocytes.[2,3,27,28,30–33] Our results on cellular distribution and VEGF localization, revealed by immunohistochemical techniques, are in agreement with these previous reports. Because RPE cells in CNVMs have been shown to be immunoreactive to various types of angiogenic cytokines, including VEGF, acidic or bFGF, and TGF-β, they may be one of the most potent cell components for angiogenesis in CNVM.[1–3] bFGF has substantial immunoreactivity in both normal retina[34] and CNVMs, and has a remarkable potency to promote proliferation of endothelial cells[35]; however, it is not likely to be the primary inducer of neovascularization, because it has no signal sequence for secretion, and its mitogenic activity is not endothelial cell specific.[36,37] It is thought to be only after tissue damage progresses to some extent, which facilitates intracellular cytokines to act in a paracrine manner, that FGF can exert its angiogenic effects.[38–40] Although TGF-β, similar to bFGF, also shows detectable immunopositivity in normal ocular tissue and CNVMs[3,41] and is angiogenic in vivo,[42] possible roles for this cytokine in CNVMs remain to be elucidated, because it has a direct inhibitory effect on endothelial proliferation in vitro.[43]

In contrast to bFGF and TGF-β, VEGF is an endothelial cell–specific mitogen and is considered to be one of the major inducers of angiogenesis both in vitro and in vivo.[4,5,8,9] VEGF has been shown to be secreted by various cell types under hypoxic conditions,[7,10,11,13,14,44,45] and four isotypes are produced by alternative splicing of mRNA from one gene.[46] Previous studies have shown that RPE cells, endothelial cells, and fibroblast-like cells are VEGF-positive in ARMD-related CNVMs.[1,2,12] Because only minimal VEGF immunostaining has been reported to be observed in normal RPE cells, our data demonstrating extensive overlap of staining for VEGF and RPE

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VEGF			IL-1β			TNF-α		
RPE Monolayer‡	Neovascular Vessels‡	Stroma‡	RPE Monolayer‡	Neovascular Vessels‡	Stroma‡	RPE Monolayer‡	Neovascular Vessels‡	Stroma‡
++	+	+	++	+	+	++	+	+
++	+	+	+	++	+	+	++	+
+	+	+	+	++	++	++	++	+
+	+	++	++	++	+	++	++	+
++	++	++	+	++	+	++	++	++
++	++	++	+	++	+	++	++	+
++	+	+	+	++	+	++	++	+
++	++	++	+	++	+	++	++	++
++	++	++	++	++	+	++	++	+
++	++	+	++	++	+	++	++	+
++	+	+	+	+	+	+	++	++

cells may suggest that VEGF is a candidate for angiogenesis in CNVMs, at least for cytokines associated with RPE cells. Moreover, recent studies showing that VEGF promotes proliferation of RPE cells in an autocrine manner may indicate that RPE cells in CNVMs can initiate a self-amplifying circuit through VEGF production, and the considerable amount of RPE cell–derived VEGF may eventually lead to pathologic angiogenesis.[47]

Monocytes produce a variety of cytokines and are one of the most common cell types in CNVMs.[28] Little is known, however, about their roles other than as inflammatory mediators. Monocyte-conditioned medium can induce morphologic changes in RPE cells and, when prestimulated with lipopolysaccharide, can also elicit a marked increase in mRNA of cytokines such as IL-1β, IL-6, IL-8, and macrophage colony-stimulating factor.[48] Moreover, experimental animal models of uveitis[49,50] can be created using some of these peptides. Because the promotion of cytokine mRNA expression induced by monocyte-conditioned medium is completely suppressed by using the neutralizing antibodies to IL-1 and TNF-α in combination, macrophages in ocular diseases may affect RPE cells primarily through production of these two cytokines.[48] A previous immunohistochemical study suggested that there was IL-1 secretion by macrophages in the experimental model of laser photocoagulation-induced CNVM.[51] Because recent studies have shown that both IL-1β and TNF-α stimulate VEGF production,[52,53] our results of immunofluorescent double staining, which demonstrated that some macrophages in CNVMs were immuoreactive to both IL-1β and TNF-α, may suggest an indirect angiogenic role of macrophages in CNVMs. By using human RPE cells, we also confirmed that both IL-1β and TNF-α increased VEGF mRNA expression, which is consistent with these studies (data not shown). Accordingly, the abundance of VEGF in the RPE monolayer demonstrated by both immunoperoxidase staining and immunofluorescence double staining may indicate the production of VEGF by RPE cells, induced at least partly by macrophages. In regard to VEGF production by macrophages, although we did not observe any colocalization of this cell type with VEGF by immunofluorescent double staining in CNVMs, because macrophages have been reported to produce VEGF in pigs and mice,[19] we cannot entirely exclude the possibility. A possible

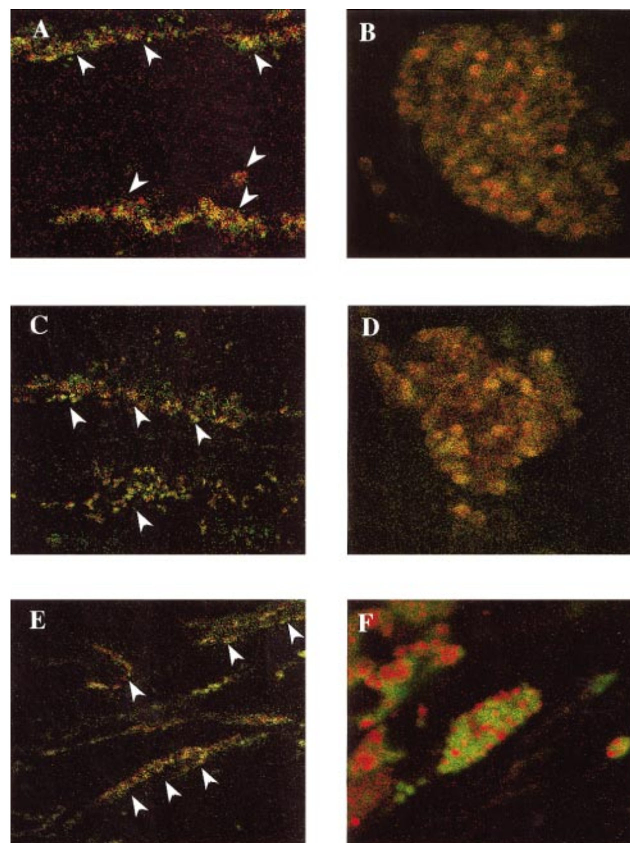

FIGURE 3. Double immunofluorescence immunohistochemistry to study the relationship between RPE cells and cytokines in a cellular, ARMD-related CNVM (specimen 1). (A) Extensive overlap is observed between the RPE layers that were positive for cytokeratin (green) and cells that were positive for VEGF (red). (B) Higher magnification. (C) Extensive overlap is observed between the RPE layers that are positive for cytokeratin (green) and cells that are positive for IL-1β (red). (D) Higher magnification. (E) Extensive overlap is observed between the RPE layers that are positive for cytokeratin (green) and cells that are positive for TNF-α (red). Arrowheads, representative cells immunoreactive for both markers. (F) Higher magnification. Magnification, (A) ×300; (C) ×200; (E) ×100; (B, D, F) ×3200.

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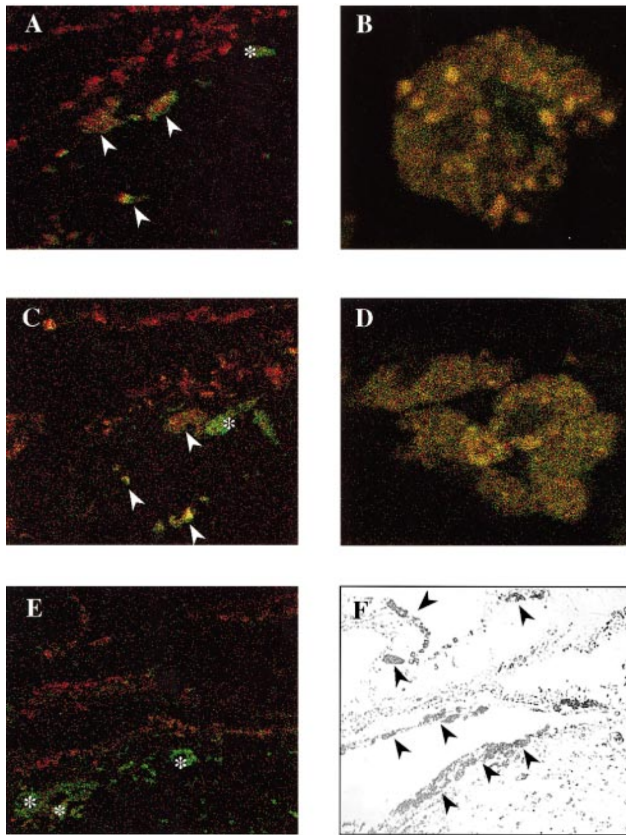

Figure 4. Double immunofluorescence immunohistochemistry to study the relationship between macrophages and cytokines in a cellular, ARMD-related CNVM (specimen 1). (A) Extensive overlap is observed between macrophages that were positive for CD68 (green) and cells that were positive for TNF-α (red). (B) Higher magnification. (C) Extensive overlap is observed between macrophages that were positive for CD68 (green) and cells that were positive for IL-1β (red). (D) Higher magnification. (E) No detectable overlap is observed between macrophages that were positive for CD68 (green) and cells that are positive for VEGF (red). (F) Image observed with translucent illumination for orientation of the section. White arrowheads, representative cells immunoreactive for both markers; *macrophages that are not positive for cytokines; black arrowheads, the RPE layer. Magnification, (A, C) ×200; (B, D) ×3200; (E, F) ×100.

explanation for this may be that macrophages in CNVMs are not sufficiently activated for VEGF production, because activation is prerequisite for VEGF production by this cell type.[19] However, further in vitro studies using human macrophages are required to clarify this point.

The present study also showed definite immunoreactivity of RPE cells to both IL-1β and TNF-α, in addition to VEGF. A previous study has shown that only IL-1β mRNA expression but not protein production by RPE cells increases in response to exogenous IL-1β and TNF-α.[54] This apparent paradox may be explained by the possibility that a second signal, not found in the in vitro study, was required for IL-1β secretion in vivo. Otherwise, because protein secretion of IL-1β and TNF-α has not been demonstrated in RPE cells in vitro[54] and the method in the present study detected protein itself but not mRNA, the immunoreactivity detected in our study may not necessarily mean synthesis by this cell type and may only reflect binding of these cytokines to RPE cells.

The uniform immunostaining of IL-1β and TNF-α was also observed in neovascular vessels. Because both IL-1β and TNF-α have been reported to be angiogenic in vivo,[55–57] this may indicate a more direct pathway, not only through upregulation of VEGF in RPE cells, for these two cytokines to exert effects on angiogenesis in CNVMs. Interleukin-1 is secreted by several types of cells, including endothelial cells, smooth muscle cells, and macrophages[58] and is capable of inducing marked ocular neovascularization in vivo.[55] TNF-α is definitely an angiogenic factor in vivo[57] and mediates adhesion and activation of additional monocytes through upregulation of adhesive molecules on both endothelial cells and monocytes, and by upregulation of granulocyte–macrophage colony-stimulating factor.[59,60] However, both cytokines also have an inhibitory effect on proliferation of endothelial cells in vitro.[61,62] Consequently, because both IL-1β and TNF-α have complex angiogenic effects, additional study is required to elucidate their exact roles in relation to angiogenesis in CNVMs.

Recent studies of ARMD, presumed ocular histoplasmosis, and myopia suggest that CNVMs represent a stereotypic and nonspecific response, regardless of underlying diseases.[63,64] Little is known at present about the pathologic features of CNVMs derived from diseases other than ARMD. Our results showed that both cellular localization and cytokine distribution did not correlate significantly with the cause of CNVMs. This may further support the concept that CNVMs represent a common pathologic condition irrespective of the underlying diseases. However, an interesting correlation was observed between the extent of staining for cytokeratin and neovascularization, which may provide evidence of an angiogenic role of RPE cells in CNVMs, as suggested by previous studies.[1,2] Moreover, we also demonstrated the same tendency in the case of macrophages.

In summary, the present study provides evidence that macrophages in CNVMs secrete both IL-1β and TNF-α and thereby contribute greatly to the development of neovascularization through triggering VEGF production by RPE cells. Our data also suggest the stereotypic pathologic conditions underlying the formation of CNVMs, regardless of cause.

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ANNOUNCEMENT

JOACHIM KUHLMANN FELLOWSHIP FOR OPHTHALMOLOGISTS 2000: In honour of its founder's memory the Joachim Kuhlmann AIDS-Stiftung, Essen, Germany, is sponsoring two fellowships for qualified ophthalmologists at a well-known institute in Boston, who want to train in CMV-retinis and other HIV-related ophthalmological diseases. The fellowships each include 5000 $ US. Deadlines for applications are 31st January 2000 and the 31st July 2000. Detailed applications including CV and publication list should be sent to the:

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