Transdifferentiated Retinal Pigment Epithelial Cells Are Immunoreactive for Vascular Endothelial Growth Factor in Surgically Excised Age-Related Macular Degeneration-Related Choroidal Neovascular Membranes

Pedro F. Lopez,* Brian D. Sippy,† H. Michael Lambert,‡ Allen B. Thach,* and David R. Hinton†

Purpose. To determine the cellular origin and the vascular endothelial growth factor (VEGF) immunoreactivity of the nonvascular stromal cells in surgically excised age-related macular degeneration (ARMD)-associated choroidal neovascular membranes (CNVMs).

Methods. Immunohistochemical analysis was performed on frozen sections of eight surgically excised ARMD-related CNVMs.

Results. Cytokeratin-positive, smooth muscle actin-positive polygonal or fibroblastic (transdifferentiated RPE) cells were the principal nonvascular stromal cells detected. The polygonal cells were more commonly found in active (highly vascularized) regions and were strongly immunoreactive for VEGF. The fibroblastic cells were predominantly found in fibrotic (hypo-vascular) regions and were minimally immunoreactive for VEGF.

Conclusions. Transdifferentiated RPE cells are the principal nonvascular stromal cells of both vascular and fibrotic ARMD-related CNVMs. Preferential localization of VEGF immunoreactivity within the cytoplasm of the polygonal transdifferentiated RPE cells in the highly vascularized regions of the surgically excised CNVMs suggests an important angiogenic role of these cells and this growth factor in the progression of ARMD-related choroidal neovascularization. Invest Ophthalmol Vis Sci. 1996; 37:855-868.
eral potential sources, including ingrowth of choroidal pericytes or fibroblasts across a defect in Bruch’s membrane,
17,19,21,27–29 diapedesis of blood-borne monocytes between neovascular choroidal endothelial cells with differentiation into macrophages,4,12–16,30,31 ingrowth of elongated glial cells from the adjacent retina,12–17,28,29 or metaplasia of migrating RPE cells.1,14–17,20,21,28,29,32 Because each of these cell types may be distinguished by its immunohistochemical phenotype,2,3,7,27,33–35 we performed an immunohistochemical study on frozen sections of surgically excised ARMD-related CNVMs to determine the phenotype and cellular origin of these stromal cells. Because the stromal cells are critically located around the proliferating new vessels,1,5,9 these cells are a likely source of angiogenic factors.29,29 Recently, basic fibroblast growth factor has been identified within RPE cells in surgically excised ARMD-related CNVMs1; however, this growth factor lacks a signal sequence and, therefore, normally is not secreted from the cells that produce it.40 Moreover, basic fibroblast growth factor is not a specific mitogen for endothelial cells.22–26,40 Vascular endothelial growth factor (VEGF)41–60 may be a better candidate for a critical angiogenic factor in CNVM development17,22–26,56–60 because it is a secreted product with greater selectivity for endothelial cells. Characterization of the origin of the stromal cells and their capacity for the release of angiogenic factors may allow a better understanding of the nature of CNVM and may lead to the development of more specific therapeutic strategies for this disease process.

MATERIALS AND METHODS

Surgical excision of subfoveal choroidal neovascular membranes was performed in eight eyes of seven patients with ARMD who had not undergone previous foveal laser photocoagulation. Tenets of the Declaration of Helsinki were followed, informed consent was obtained, and institutional human experimentation committee approval was granted for this study. The surgical indications and the techniques used have been described.31,62 Clinical characteristics of the patients and the study eyes are summarized in Table 1. The presence or absence of preoperative nonfoveal laser photocoagulation for choroidal neovascularization also was recorded.

The Macular Photocoagulation Study (MPS) classification63 of each CNL as either a classic and/or an occult CNVM (<25% white fibrous tissue) or as a CNVM–scar (>25% white fibrous tissue) was ascertained. The size in disc areas of each CNL on the preoperative MPS protocol fluorescein angiogram65 was determined as follows: An optimal preoperative angiographic frame of the CNL was enlarged and photographed, and the margins of each CNL were outlined on the photograph using the guidelines suggested by the MPS group.65 Each photograph was then digitized, and the scored CNL area was quantitated and normalized to the corresponding optic disc area.64 The presence and extent (% of total CNL area) of subretinal hemorrhage associated with the CNL was estimated similarly on the preoperative stereoscopic color fundus photographs.

Each of the fresh, surgically excised CNL specimens was immediately placed in balanced salt solution at 4°C, then snap frozen in OCT (Ames/Miles, Elk-hart, IN) within 1 hour of surgical extraction. Chorioretinal specimens from fresh age-matched postmortem donor eyes, without evidence of chorioretinal disease, were obtained within 12 hours of death and were processed similarly as normal human control specimens. Each specimen was then serially sectioned on a cryostat into 6-μm frozen sections on poly-L-lysine ( Sigma, St. Louis, MO)-coated glass slides for immunohistochemical and immunofluorescent staining. The sections were fixed in reagent grade acetone for 5 minutes at room temperature and stored at −80°C. Hematoxylin and eosin-stained adjacent sections were evaluated to determine histologic diagnosis and to ensure tissue quality and orientation. Thawed tissue sections were air dried, rehydrated with phosphate-buffered saline (pH 7.4), and incubated for 15 minutes with blocking serum. The specimens were incubated for 30 minutes with the primary antibody, then washed for 15 minutes with phosphate-buffered saline. Immunoperoxidase detection was performed using the ABC Elite kit (Vector, Burlingame, CA) with aminoethylcarbazole as the red chromogen. Finally, the slides were rinsed with tap water, counterstained with hematoxylin, and mounted with glycerin-gelatin mounting medium. To allow comparison between the levels of protein expression in the different samples, the staining for each antigen was performed at the same time.

Double immunofluorescence staining was performed by first incubating the tissue section for 1 hour with either mouse monoclonal or rabbit polyclonal antibodies, followed by a second incubation for 30 minutes with the corresponding fluorescent dye-conjugated secondary antibodies. Slides were rinsed for 20 minutes in phosphate-buffered saline after both primary and secondary antibody incubation, and then mounted with VectaShield mounting medium (Vector). Epifluorescence was examined and photographed with a high-resolution Zeiss laser-scanning microscope (model LSM 10 BioMedical; Carl Zeiss, Oberkochen, Germany).

Monoclonal mouse antibodies against cytokeratin (pancytokeratin cocktail, 1:400 dilution; Enzo Diagnostics, New York, NY), smooth muscle actin (SMA, 1:500 dilution; Accurate Chemical and Scientific, Westbury, NY), desmin (1:100 dilution; Dako, Carpentry, CA), glial fibrillary acidic protein (GFAP, 1:500 dilution; Biogenex Laboratories, San Ramon, CA) and
Transdifferentiated RPE and VEGF in ARMD-Related CNVMs

TABLE 1. Clinical Characteristics

<table>
<thead>
<tr>
<th>Eye</th>
<th>Age (years)/Race/Sex</th>
<th>Previous Laser</th>
<th>MPS Classification</th>
<th>Preoperative CNL Size*</th>
<th>Preoperative Subretinal Hemorrhage* (% of CNL Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNV/Scar Without Subretinal Hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>83/W/F</td>
<td>Right</td>
<td>No</td>
<td>CNV/scar</td>
<td>6–9</td>
</tr>
<tr>
<td>2</td>
<td>70/W/M</td>
<td>Left</td>
<td>No</td>
<td>CNV/scar</td>
<td>10.5</td>
</tr>
<tr>
<td>CNV/Scar With Subretinal Hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>71/W/M</td>
<td>Right</td>
<td>No</td>
<td>CNV/scar</td>
<td>7.2</td>
</tr>
<tr>
<td>4</td>
<td>77/W/F</td>
<td>Left</td>
<td>No</td>
<td>CNV/scar</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>72/W/F</td>
<td>Left</td>
<td>No</td>
<td>CNV/scar</td>
<td>4.8</td>
</tr>
<tr>
<td>Recurrent CNV Without Subretinal Hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>84/W/M</td>
<td>Right</td>
<td>Yes†</td>
<td>Recurrent classic and occult CNV‡</td>
<td>4.3</td>
</tr>
<tr>
<td>Recurrent CNV/Scar Without Subretinal Hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>71/W/F</td>
<td>Right</td>
<td>Yes†</td>
<td>Recurrent CNV/scar</td>
<td>14.6</td>
</tr>
<tr>
<td>8§</td>
<td>71/W/F</td>
<td>Left</td>
<td>Yes†</td>
<td>Recurrent CNV/scar</td>
<td>13.9</td>
</tr>
</tbody>
</table>

* In disc areas; †juxtapfoveal macular photocoagulation; ‡occult CNV = fibrovascular pigment epithelial detachment, in this case; §fellow to eye 7.

MPS = Macular Photocoagulation Study; CNL = choroidal neovascular lesion; CNV = choroidal neovascular membrane.

CD11c (1:200 dilution; Becton Dickinson, San Jose, CA) were used. Polyclonal rabbit antibodies against cytokeratin (1:500 dilution; Accurate Chemical and Scientific), factor VIII (1:100 dilution; Dako), and VEGF (1:100 dilution; Peptotech, Rocky Hill, NJ) also were used. Biotinylated secondary antibodies (1:400 dilution) and Texas Red conjugated secondary antibodies (1:100 dilution) were obtained from Vector. Conjugated secondary antibodies were obtained from Chemicon (Temecula, CA) for DTAF (green, 1:100 dilution) and Cy3 (red, 1:70 dilution). All antibody concentrations were determined individually on appropriate positive control tissues. Negative controls included omission of primary antibody and use of an irrelevant primary antibody of the same isotype.

RESULTS

Histologic examination of the eight surgically excised ARMD-related CNVMs revealed a spectrum of changes, ranging from moderately cellular membranes with prominent neovascularization (Fig. 1A) to paucicellular fibrotic membranes (Fig. 2A) with no demonstrable vascular channels (Table 2). A partially intact, normal RPE monolayer was found in six of the eight CNVMs (Figs. 2A, 2B). Three of the membranes were present in the subretinal space (internal to the normal RPE monolayer), and three were present in both the subretinal and the subRPE spaces (both internal and external to the normal RPE monolayer). In the remaining two cases, the location of the CNVM was indeterminate because of the lack of an intact RPE monolayer. Other cells with the morphologic appearance of lymphocytes and macrophages also were seen in variable numbers. In three cases, a plaque of adherent atrophic retina was found attached to the CNVM (Fig. 1B). Stromal cells were common in all lesions and varied in shape from polygonal cells with multiple processes to elongated fibroblast-like cells. Small numbers of pigment-containing cells were seen adjacent to the normal RPE monolayer in the stroma.

To determine the cellular origin of the stromal cells, the tissue sections were stained with a series of cell type-specific antibodies. Immunoperoxidase staining for SMA (Figs. 1C, 1D, 2E) revealed many positive stromal cells. These cells ranged in morphology from large polygonal cells most prominent in active (highly vascularized) CNLs (Figs. 1C, 1D) to spindle cells, which were seen most commonly in the fibrotic (poorly vascularized) lesions (Fig. 2E). The cells of the normal RPE monolayer were clearly SMA negative. Desmin stain revealed that only rare perivascular cells were positive, whereas none of the large stromal cells were positive for desmin (Fig. 1H). Factor VIII stained the endothelial cells of the new vessels prominently; however, none of the stromal cells were positive for this marker (data not shown).

Immunoperoxidase staining for cytokeratin (Figs. 1E, 1F, 2C, 2D) revealed that many of the stromal cells were also cytokeratin positive. The cytokeratin-positive cells were similar in distribution to the SMA-positive cells and were identified as large polygonal stromal cells and elongated spindle-shaped cells; however, the number of cytokeratin-positive cells was generally less than that of the SMA-positive cells. The large stromal, cytokeratin-positive polygonal cells often encircled the

Downloaded From: https://iovs.arvojournals.org/pdaccess.ashx?url=/data/journals/iovs/933416/ on 10/11/2018
neovascular channels in the CNVMs (Figs. 1E, 1F). In the fibrotic lesions, almost all the spindle-shaped cells were cytokeratin positive (Figs. 2C, 1D). The intact normal RPE monolayer was cytokeratin positive, as were many of the polygonal pigmented cells adjacent to the normal RPE monolayer.

Staining for GFAP (Fig. 1B) revealed prominent staining in the adherent plaques of retinal tissue found in some surgically excised CNVMs. In none of these cases did the GFAP-positive glial cells infiltrate into the CNVM. None of the stromal cells in the CNLs were GFAP positive. Variable numbers of macrophages, identified by their immunoreactivity to CD11c, were seen in the stroma of every CNVM; however, these macrophages
VEGF immunoreactivity was localized to individual transdifferentiated RPE and VEGF in ARMD-related CNVMs. Very focal, weak VEGF immunoreactivity in the fibroblastic stromal cells was found surrounding blood vessels (Figs. 3B, 3C). The RPE monolayer in normal human control chorioretinal specimens was not immunoreactive for VEGF (Fig. 3G).

DISCUSSION

The stroma of ARMD-related CNVMs contains a mixed population of cells; however, many of the stromal cells, which comprise a large fraction of the cells in these membranes, are of uncertain origin and cannot be defined by routine histology. Electron microscopic studies have shown that many of these cells contain microfilaments, leading to the conclusion that these are myofibroblasts or fibroblasts; however, definitive evaluation requires immunohistochemistry. In the current study, we found that a large number of the stromal cells in both highly vascularized (active) and poorly vascularized (fibrotic) CNVMs are immunoreactive for SMA. Positivity for SMA is found in smooth muscle cells, cardiac muscle cells, myofibroblasts, and some pericytes. The presence of only rare desmin-positive cells does not support the notion that SMA-positive cells are muscle derived. Pericytes are variably reactive for desmin, so lack of this marker alone cannot definitively rule out the presence of this cell type. When these same CNVMs were stained for cytokeratin, the pattern of immunoreactivity with prominent staining of a large number of polygonal and fibroblastic stromal cells was similar to that seen with SMA. Double staining confirmed extensive overlap, with many cells positive for both SMA and cytokeratin in both highly vascularized (active) and poorly vascularized (fibrotic) CNVMs. Within the retina, only RPE cells are immunoreactive for cytokeratin and SMA-positive stromal cells, and finally to cytokeratin-negative, SMA-positive cells—exists in the stroma. Interestingly, the induction of myofibroblastic trans-
FIGURE 2. Histology and immunohistochemistry of a fibrotic, age-related macular degeneration-related choroidal neovascular membrane (CNVM) (specimen 2). (A) A paucicellular fibrotic CNVM is shown stained with hematoxylin and eosin. (B) At higher magnification, the remnants of the retinal pigment epithelial (RPE) monolayer are seen at the outer edge (arrowheads). (C) At higher magnification in (D), immunohistochemistry for cytokeratin demonstrates that most of the fibroblast-like stromal cells are cytokeratin positive, as are the normal RPE cells. (E) A similar population of cells is positive for smooth muscle actin (SMA). (F) Double immunofluorescent immunohistochemistry reveals extensive overlap between cells that are positive for SMA (red) and those that are positive for cytokeratin (green). Many of the double-stained cells appear yellow. Representative cells immunoreactive for both markers are shown with arrows. Magnifications, ×67 (A,C,E) and ×269 (B,D,F).
TABLE 2. Histopathologic Characteristics

<table>
<thead>
<tr>
<th>Eye</th>
<th>Fibrosis*</th>
<th>Neovascularization†</th>
<th>Intact RPE</th>
<th>Location of Membrane§</th>
<th>Glial Plaque¶</th>
<th>Desmin §</th>
<th>CD11c §</th>
<th>Keratin+ / Actin+ Fibroblastic¶</th>
<th>Keratin+ / Actin+ Polygonal¶</th>
<th>Actin+ / Keratin+ Cells¶</th>
<th>VEGF¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>Subretinal and SubRPE</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>++*</td>
<td>+</td>
<td>Subretinal SubRPE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++*</td>
<td>+</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>Subretinal SubRPE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>4</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>Subretinal SubRPE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>#</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>Subretinal SubRPE</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>6</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>Indeterminate SubRPE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>Indeterminate SubRPE</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>8</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>Subretinal SubRPE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>#</td>
</tr>
</tbody>
</table>

* By percentage of area on histologic section: + = 10% to 40%; ++ = 40% to 70%; +++ = 70% to 100%.
† By analysis of hematoxylin and eosin stain and immunoperoxidase stain for factor VIII: + = infrequent individual vessels; ++ = moderate numbers of vessels arranged in groups; +++ = confluent groups of vessels.
‡ By hematoxylin and eosin section: + = <1/3 length of biopsy; ++ = 1/3 to 2/3 of biopsy; +++ = >2/3 of biopsy.
§ Relative to intact RPE monolayer and Bruch’s membrane; indeterminate = no intact RPE layer seen.
¶ By analysis of immunoperoxidase stain for GFAP: + = >25% of the area of the biopsy specimen.
‖ Quantified by counting the number of positive cells using immunoperoxidase stains in an average of three ×40 objective fields: ± = <1; + = 1.5; ++ = 5-20; +++ = >20. Adjacent sections stained with SMA and keratin were compared to estimate the population showing staining for both markers or SMA alone.
# Present along one edge of the specimen.
** Present in a perivascular distribution.

RPE = retinal pigment epithelium; VEGF = vascular endothelial growth factor; GFAP = glial fibrillary acidic protein; SMA = smooth muscle actin.
differentiation (SMA immunoreactivity) of quiescent cultured fibroblasts by transforming growth factor-β (TGF-β) has been described recently.68 Because TGF-β1 also has been shown recently to be expressed in surgically excised ARMD-related CNVMs,11 perhaps this growth factor might induce similar myofibroblastic transdifferentiation and SMA positivity in the CNVM stromal RPE cells.

Transdifferentiation of RPE cells is well described both in vitro and in vivo. Retinal pigment epithelial cells in culture may change in shape from hexagonal to elongated fibroblastic-appearing cells when grown on laminin-free, fibronectin-containing fibrin matrices.71'72 This type of matrix is observed commonly in
FIGURE 3. Vascular endothelial growth factor (VEGF) immunohistochemistry of a cellular and fibrotic age-related macular degeneration-related choroidal neovascular membrane (CNVM) (specimens 2 and 5). (A) Prominent immunoreactivity for VEGF is seen in the stroma and in the region of the adherent retinal plaque. (B) VEGF-positive stromal cells are present (arrowheads) surrounding a central vessel (V). Focal VEGF immunoreactivity is present in the adjacent RPE monolayer (arrow). (C) Clusters of VEGF-positive cells (arrowheads) are seen in association with tangentially sectioned new vessels. Many of the immunoreactive cells contain melanin pigment. (D) Double immunofluorescent immunohistochemistry reveals extensive overlap between the cells positive for VEGF (red) and those positive for cytokeratin (green). Representative cells immunoreactive for both markers are shown with arrows. (E) VEGF-positive cells with the appearance of reactive Müller cells are seen in the adherent retinal plaque. Occasional pigmented cells are seen at the interface with the subretinal membrane. (F) Minimal immunoreactivity for VEGF is detected in the paucicellular fibrotic CNVM (arrowhead). (G) No VEGF immunoreactivity is detected in the RPE monolayer of normal control postmortem eyes. Magnifications, ×67 (A) and ×269 (B to G).

ARMD-related CNVMs in the subRPE space, secondary to neovascular exudation, as well as in the subretinal space after breakdown of the outer blood-retinal barrier over the CNVM. Migration of RPE cells along these matrices may explain the presence of metaplastic RPE in the stroma on both the retinal (internal) and the choroidal (external) surfaces of the normal RPE monolayer. Recently, the immunophenotypic changes in normal RPE cells grown on fibronectin and type 1 collagen substrata have been described. The RPE cells acquired a mesenchyme cell-like phenotype, showed decreased expression of cytokeratin, and began to express SMA.

Morphologic transdifferentiation of RPE cells has been suggested in several systems, including epiretinal membrane formation, either spontaneous (idiopathic) or after vitrectomy, or after periretinal proliferation after RPE autotransplantation, and proliferative vitreoretinopathy after retinal detachment. These conditions are characterized by prominent RPE proliferation and transdifferentiation with RPE cell morphologic features similar to those seen in the stromal cells in surgically excised ARMD-related CNVMs. It has been suggested that the expression of SMA in transdifferentiated RPE cells in proliferative vitreoretinopathy (PVR) membranes may result in the development of contractile properties, which may be critical for the evolution of the periretinal membrane contraction that leads to traction retinal detachment. Smooth muscle actin is an actin isoform normally present in smooth and cardiac muscle cells; thus, its expression in other tissues implies a potential for cellular contraction. In vitro, the ability of porcine RPE cells to generate tractional forces correlated with both increased cell-matrix adhesion and SMA expression. Similarly, contraction of transdifferentiated RPE cells within the CNVM stroma may explain certain clinical phenomena, such as binocular diplopia secondary to CNVM-associated foveal displacement or ectopia, CNM-associated spontaneous RPE tears, and retraction of the edge of subfoveal CNVMs after initial blunt dissection during submacular surgery.

The finding that transdifferentiated RPE cells with the potential for contraction are the principal stromal cells in active (highly vascularized) and fibrotic (poorly vascularized) ARMD-related CNVMs suggests the importance of the RPE cell in the progression and possibly the initiation of these lesions. Retinal pigment epithelial cells are known to produce many cytokines, growth factors, and potent angiogenic factors that could influence the course of CNVM development in ARMD. Vascular endothelial growth factor may not only stimulate vascular endothelial cell proliferation, it may act as an autocrine growth factor for RPE cells in the CNVM stroma. In this study, we found a relationship between the morphology of the transdifferentiated RPE cells in the CNVM stroma and the degree of VEGF immunoreactivity. Polygonal RPE cells were strongly immunoreactive for VEGF and were primarily present in the active (highly vascularized) regions of the CNVMs, particularly in the cells surrounding the neovascular channels, whereas fibroblastic transdifferentiated RPE cells showed little VEGF immunoreactivity and were found primarily in the fibrotic (poorly vascularized) regions of the CNVMs.

Vascular endothelial growth factor is a secreted endothelial-specific mitogen that is induced in a variety of cell types by hypoxia and appears to be a principal biochemical mediator of neovascularization in vivo. It is a highly conserved homodimeric protein with an approximate molecular weight of 35 to 45 kDa. The gene for VEGF, which has been cloned and sequenced, exhibits limited but significant (18% to 20%) homology with the A and B chains of platelet-derived growth factor. Four molecular species of the human VEGF family are produced by alternative splicing of the same messenger RNA (mRNA), generating polypeptides of 121, 165, 189, and 206 amino acids.
The smaller two species (121 and 165) are freely diffusible, whereas the larger two (189 and 206) are bound to heparin-containing proteoglycans on the cell surface or basement membrane.  

In our study, VEGF immunoreactivity was not detected in the RPE monolayer or choroid fromagematched control eyes. Biochemical studies have demonstrated small amounts of VEGF protein in the normal RPE monolayer, however, these levels may be below the detection threshold of our immunohistochemical assay. Vascular endothelial growth factor may be produced by several cell types normally present in ARMD-related CNVMs, including RPE cells, endothelial cells, fibroblasts, macrophages, and monocytes. Although the mitogenic activity of VEGF originally was presumed to be limited to vascular endothelial cells, other cell types, such as monocytes, corneal endothelial and lens epithelial cells, also may express VEGF receptors. Recently, RPE cells have been shown to express VEGF receptors and respond to VEGF by proliferation, suggesting that VEGF may act as an autocrine growth factor in RPE cells.  

Recent studies have detected VEGF expression in vitreous aspirates and surgically excised preretinal neovascular tissue in patients with proliferative diabetic retinopathy, as well as in animal models of experimental preretinal neovascularization. In the animal models, a direct temporal correlation was established between VEGF mRNA and protein expression and the formation and regression of the preretinal neovascularization. In our specimens, the degree of staining for VEGF appeared to correspond to the evolutionary stage of the choroidal neovascularization. Vascular endothelial growth factor immunoreactivity was highest in the most active (highly vascularized) CNVMs and was minimal in the least active (poorly vascularized), most fibrotic CNVMs. In the surgically excised ARMD-related CNVMs, VEGF immunoreactivity was most prominent within the cytoplasm of the polygonal transdifferentiated RPE cells that surrounded the neovascular channels within the CNVM stroma. In addition, expression of different numbers of VEGF receptors on the endothelial cell surface in different vascular beds may affect the susceptibility of different vascular beds to the proliferative effects of VEGF. Retinal vascular endothelial cells, for example, have particularly high levels of VEGF receptors compared with aortic vascular endothelial cells. It is possible that a similar VEGF sensitivity exists, particularly in patients with ARMD, and predisposes the choroidal vascular endothelium to the formation of choroidal neovascularization.  

The high levels of VEGF immunoreactivity found in association with retinal glial cells adherent to the retinal surface in some of the surgically excised ARMD-related CNVMs suggest a possible role for glial-derived VEGF in the evolution of these CNVMs. Adherent retinal fragments were seen only in those advanced CNVMs that extended from the subRPE space (external to the normal RPE monolayer) to the subretinal space (internal to the RPE normal monolayer). In such eyes, it is possible that chemotactic or haptotactic VEGF gradients between the subRPE and subretinal space generated by glial cell expression of VEGF might stimulate the subretinal extension of the initial subRPE CNVM into the subretinal space. In addition, once CNVM extension into the subretinal space occurs, glial cell VEGF expression might maintain and promote the growth of the subretinal portion of the choroidal neovascular process. Both processes may be further abetted by the pronounced increase in microvascular permeability caused by VEGF. Indeed, VEGF is 50,000 times more potent than histamine at increasing microvascular permeability. The VEGF-induced serum leakage from the neovascular channels in the CNVM may not only increase the local ingress of serum growth factors into the subretinal space, it may lead to subretinal fibrin gel matrix formation, which has been previously described clinically and which has been suggested to provide haptotactic gradients for the subretinal extension of subRPE CNVMs. In addition, microheterogeneity of VEGF expression within the CNVM complex may, in part, explain the regional variations in microvascular permeability detected clinically in different portions of the CNVM complex by fluorescein and indocyanine green angiography. Further studies are necessary to determine the role of glial cell-derived VEGF in the evolution of complex CNVMs (with both subRPE and subretinal components) in ARMD.  

Variable numbers of CD11c-positive macrophages were found in the highly vascularized (most active) regions of the CNVMs. Macrophages are potential sources of inflammatory cytokines that may, even if derived from small numbers of cells, dramatically alter the stromal environment. Growth factor secretion by these and other cells may act as positive or negative modulators in the evolution of choroidal neovascularization in ARMD. Some of these mediators may, at least in part, exert their regulatory activity by their effects on VEGF-related pathways. TGF-β, for example, recently has been found to be expressed in surgically excised ARMD-related CNVMs. This growth factor has angiogenic effects in vivo, even though it directly inhibits the growth of cultured endothelial cells in vitro. This apparent paradox has been explained by the finding that TGF-β has an indirect angiogenic effect of stimulating paracrine induction of VEGF in other surrounding cells. The angiogenic effects of VEGF also may be considerably enhanced by the synergistic activity of nonsecreted basic or acidic fibroblast growth factor at areas of cellular disruption or death, where these molecules may be released. Recent
Transdifferentiated RPE and VEGF in ARMD-Related CNVMs

immunopathologic studies have confirmed the presence of alpha and beta fibroblast growth factor in surgically excised ARMD-related CNVMs. In addition, partial proteolysis by plasmin of the larger cell-surface or basement membrane heparin-bound forms of VEGF may release active, diffusible, shorter VEGF surface or basement membrane heparin-bound forms. This hypothesis is supported further by recent observations in patients with ARMD using fluorescein angiography and indocyanine green videoangiography, which suggest that the surgically excised ARMD-related CNVMs are similar to that described in ischemic areas surrounding necrotic regions of glioblastoma tumors, in which it has been suggested that tissue hypoxia induces VEGF expression and consequent angiogenesis. This hypothesis is supported further by recent observations in patients with ARMD using fluorescein angiography and indocyanine green videoangiography, which suggest that the surgically excised ARMD-related CNVMs are similar to that described in ischemic areas surrounding necrotic regions of glioblastoma tumors, in which it has been suggested that tissue hypoxia induces VEGF expression and consequent angiogenesis.

Key Words

age-related macular degeneration (ARMD), choroidal neovascularization (CNV), retinal pigment epithelium (RPE), transdifferentiated, vascular endothelial growth factor (VEGF)

Acknowledgments

The authors thank Christine Spee and Shikun He for technical assistance and Susan Clarke for editorial review.

References


87. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth fac-

---

Transdifferentiated RPE and VEGF in ARMD-Related CNVMs


