Expressions of Angiopoietins and Tie2 in Human Choroidal Neovascular Membranes

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Abstract

purpose. To elucidate the potential role of angiopoietins and the Tie2 system in choroidal neovascularization.

methods. Surgically excised choroidal neovascular membranes (CNVMs) were obtained at vitrectomy from five eyes with age-related macular degeneration, three eyes with idiopathic neovascular maculopathy, and two eyes had degenerative myopia and two eyes had angiod streaks. Light microscopic immunohistochemistry was performed to detect cytokines such as vascular endothelial growth factor (VEGF), Ang1, and Ang2 and cellular components such as retinal pigment epithelial (RPE) cells, macrophages, and endothelial cells. Immunofluorescent double staining using confocal microscopy was performed to identify the cell types that secrete specific cytokines.

results. Ang1 and Ang2 were positive in all surgically excised CNVMs, regardless of the primary disease. Double staining revealed that many of the cytokeratin, CD68 and factor VIII positive cells also had Ang1 and Ang2 immunoreactivities. In contrast to Ang1, Ang2 immunoreactivity tends to be higher in the highly vascularized regions of many CNVMs, and the localization was very similar to that of VEGF staining. Almost all vascular structures had...
prominent immunoreactivity for Tie2, which was confirmed by double staining for Tie2 and factor VIII. Tie2 immunoreactivity was also observed in the RPE monolayer and in pigmented, polygonal, and fibroblast-like cells in the stroma.

Present findings that Ang2 and VEGF are co-upregulated and that Tie2 is expressed in a variety of cell types in CNVMs further support a crucial role of the interaction between VEGF and Ang2 in pathologic angiogenesis of CNVM formation.

Two receptor tyrosine kinase subfamilies are characterized by their endothelium-specific expression: one is the family of vascular endothelial growth factor (VEGF) receptors, which consists of Flt-1/VEGFR-1, Flk-1/VEGFR-2, and Flt-4/VEGFR-3; the second is the Tie receptor family which consists of Tie1 and Tie2. VEGF and its relatives, ligands for the former receptors, are required for vasculogenesis and angiopoietins, ligands for the latter receptors appear to be involved in later stages of vessel growth and remodeling. Tie2 or angiopoietin-1 (Ang1) knockout mice have a similar phenotype and die because of immature vessels and absence of microvessel formation. Different from the VEGF receptor knockout mice, the number of endothelial cells was normal, and tubular formation was detected in Tie2 knockout mice. These findings suggest that the Tie2 system regulates maturation and stability of vessel structure. Ang1 phosphorylates Tie2 in cultured endothelial cells; however, Ang2 does not induce phosphorylation of Tie2, but rather inhibits Ang1-induced phosphorylation of Tie2. Ang2-overexpressing transgenic mice die with vascular defects similar to those of Tie2 or Ang1 knockout mice. These observations suggest that Ang2 acts as a natural antagonist of Tie2 by blocking receptor activation by Ang1. In vitro studies show that Ang1 is not as effective as VEGF in the induction of endothelial cell proliferation and tube formation but that it can induce sprouting and branchlike phenomena. Transgenic overexpression of Ang1 in the skin of mice produces larger and more highly branched vessels. A study using a corneal angiogenesis model showed that Ang1 and Ang2 facilitates VEGF-induced neovascularization and that Ang1 promotes vascular network maturation, whereas Ang2 works to initiate neovascularization. These data support the hypothesis that VEGF and angiopoietins have distinct yet complementary roles.

In contrast to these physiological roles of the angiopoietin–Tie2 system, its involvement in pathologic angiogenesis has not been investigated very thoroughly, although a possible role in tumor angiogenesis and wound healing has been suggested.

Choroidal neovascular membrane (CNVM) formation is a critical occurrence that causes severe loss of central vision in patients with age-related macular degeneration (ARMD), ocular histoplasmosis, high myopia, and other diseases. The initial step in CNVM formation is considered to be new vessels growing from the choriocapillaris through Bruch's membrane and into the subretinal space. Recent histologic and immunohistochemical studies of experimentally produced and surgically excised CNVMs have indicated that VEGF, transforming growth factor beta (TGFβ), acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) are involved in the mechanisms of CNVM formation associated with ARMD. Because VEGF has great selectivity for endothelial cells, it is considered to be a critical angiogenic factor in the development of CNVM, even though the mechanism of CNVM formation is not fully understood.

In the present study, we sought to elucidate the role of the angiopoietin–Tie2 system, and particularly its interaction with VEGF, in pathologic angiogenesis of human ocular neovascular diseases. For this purpose, we performed immunohistochemical analysis with the surgically excised CNVMs to investigate the expressions of Tie2, angiopoietins, and VEGF and determined the cellular origin that has immunoreactivity for these factors.

**Materials and Methods**

CNVM, associated with ARMD, myopia, angioid streaks, and idiopathic choroidal neovascularization were obtained after surgical excision. Clinical characteristics of the patients are summarized in Table 1. Tenets of the Declaration of Helsinki were followed. Surgical excision of subfoveal CNVMs was performed in 12 eyes of 11 patients after obtaining informed consent. Surgical indications and procedures have been reported previously. According to the guidelines of the Macular Photocoagulation Study (MPS) classification, CNVMs in each patient were classified into classic or occult. The size of each CNVM was expressed as the percentage of the corresponding optic disc area after correlation with preoperative MPS.
Tissue Preparation
Each surgically excised CNVM specimen was fixed in 3.7% formalin immediately after excision and then placed in phosphate-balanced salt solution (PBS) at 4°C for 4 hours, dehydrated with a graded alcohol series, and then embedded in paraffin. Each specimen was serially sectioned into 5-μm paraffin-embedded sections on aminopropyltriethoxysilane-coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining. Hematoxylin and eosin staining were performed to determine the histologic orientation.

Immunohistochemistry
Immunohistochemical staining was performed according to the manufacturer's protocol (Elite ABC kit; Vector Laboratories, Burlingame, CA). After removal of the embedded paraffin by xylene, sections were rehydrated with a graded series of alcohol and rinsed with PBS. To block endogenous peroxidase activities, 3% hydrogen peroxide was applied to each section for 10 minutes, and the sections were then incubated with blocking serum (Vector) for 20 minutes. The specimens were incubated overnight at 4°C with one of the primary antibodies: goat polyclonal anti-angiopoietin-1, 1:500 dilution; goat polyclonal anti-angiopoietin-2, 1:500 dilution; rabbit polyclonal anti-Tek, 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-pancytokeratin, 1:100 dilution (Sigma, St. Louis, MO); mouse monoclonal anti-CD-68, 1:100 dilution (Elm, Rome, Italy); mouse monoclonal anti-glial fibrillary acidic protein (GFAP), 1:100 dilution (Dako); and rabbit monoclonal anti-factor VIII, 1:100 dilution (Dako). Specimens were then washed for 10 minutes with PBS. A standard indirect immunoperoxidase procedure using the ABC kit (Vector) was performed with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dako) as the substrate. For negative controls, the primary antibody was omitted. Other staining procedures were the same as described earlier.

Double immunofluorescence staining was performed by overnight incubation with the primary antibodies followed by a second incubation for 4 hours with the corresponding fluorescent dye-conjugated secondary antibodies. Slides were washed with PBS for 30 minutes, mounted (Vectashield; Vector), and examined and photographed in a laser scanning microscope (LSM 10 BioMedical, Carl Zeiss, Oberkochen, Germany).

Results
Histologic Examination
We classified the surgically excised CNVMs by their fibrotic and vascular status (Table 2). Vascular status was determined by immunoreactivity for von Willebrand factor VIII. There were several variations, ranging from fibrotic membranes with rare vascular cells to membranes with diffuse and severe vascularization. We could not, however, find any distinct difference based on their associated primary diseases; ARMD, high myopia, angioid streaks, or idiopathic neovascularization (Table 2). An intact retinal pigment epithelium (RPE) monolayer was present in most cases (Table 2). Stromal cells were present in all specimens, and we classified them into three groups: polygonal cells, fibroblast-like cells, and cells with pigment granules (Table 3).

Immunohistochemistry
To identify the cellular origin of the cells making up the CNVMs, immunoperoxidase staining was performed using a series of cell type-specific antibodies. Immunoperoxidase staining revealed that many of the stromal cells were positive for cytokeratin. Both polygonal and fibroblast-like cells were cytokeratin positive. Most of the pigmented stromal cells were also cytokeratin positive. Immunoperoxidase staining revealed that many of the stromal cells also were positive for CD68; both polygonal and fibroblast-like cells were positive. Furthermore, some of the pigmented cells were CD68-positive. Adherent retina attached to the CNVM was found in one case by immunoperoxidase staining for GFAP (Table 2). There was no clear difference in cellular distribution based on the primary disease.

Vascular endothelial growth factor (VEGF) was found in all surgically excised CNVMs. VEGF immunoreactivity tended to be most prominent in highly vascularized lesions, although RPE and perivascular cells also had strong immunoreactivity for fluorescein angiograms.
Ang1 and Ang2 were positive in all surgically excised CNVMs, regardless of the primary disease (Table 3). Most pigment-containing cells in and adjacent to the RPE monolayer had strong Ang1 and Ang2 immunoreactivities, and many stromal cells were also Ang1 and Ang2 positive (Figs. 1, 2). Ang1- and Ang2-positive cells included polygonal, fibroblastic, and pigmented cells (Table 3; Figs. 1, 2). Double staining for Ang1, Ang2, cytokeratin, and CD68 revealed that many of the cytokeratin and CD68-positive cells also had Ang1 and Ang2 immunoreactivities (Fig. 3). In contrast to Ang1, Ang2 immunoreactivity tended to be higher in the highly vascularized regions of many CNVMs, and the localization was very similar to that of VEGF staining. In vascular cells, Ang1 and Ang2 could both be immunoperoxidase positive, which was confirmed by double staining for Ang1, Ang2, and factor VIII (Fig. 3). In all cases, Ang2 immunoreactivity was stronger than Ang1, and some Ang2-positive vascular cells failed to show Ang1 immunoreactivity. The difference was most prominent in highly vascularized specimens (Table 3; Figs. 1, 2, 3). All specimens were strongly positive for Tie2 (Table 3). Almost all vascular structures had prominent immunoreactivity for Tie2, which was confirmed by double staining for Tie2 and factor VIII. Tie2 immunoreactivity was also observed in the RPE monolayer and in pigment, polygonal and fibroblast-like cells in the stroma (Figs. 1, 2, 3). Interestingly, Tie2 was present in adherent retinal plaques that showed no Ang1 or Ang2 immunoreactivities (Fig. 3).

**Discussion**

The angiopoietin–Tie2 system has been studied because of its potential role in physiological angiogenesis, such as vascular development. Although it has been reported to be involved in tumor angiogenesis and wound healing, little is known about postnatal pathologic angiogenesis. Because recent studies have shown that angiopoietins promote postnatal neovascularization by potentiating other angiogenic cytokines, such as VEGF, the system is thought to play a key role in pathologic angiogenesis in which VEGF is involved. Herein, we have demonstrated the expression of the angiopoietin–Tie2 system in surgically excised human specimens of pathologic angiogenesis (i.e., CNVMs). The localization of Ang2 was similar to that of VEGF, which is more abundant in highly vascularized regions. In addition to our previous finding that VEGF upregulates Ang2 mRNA in retinal microcapillary endothelial cells, these data suggest that the angiopoietin–Tie2 system acts as a crucial mediator and that its interaction with VEGF contributes substantially to the pathologic angiogenesis of CNVM formation.

Characterization of cells in CNVMs is very important in determining the mechanism of CNVM formation, because these cells are considered to be the source of growth factors that induce the CNVM formation. The origin of CNVM stromal cells are not identified, but the origins of several cell types have been reported: Choroidal pericytes or fibroblasts grow through Bruch's membrane, blood-borne monocytes transdifferentiate into macrophages, glial cells migrate from adjacent retina, and RPE cells also migrate into CNVMs. Previous studies have shown that inflammatory cells and macrophages are common components in subretinal fibrovascular membranes, and activation of these cells stimulates collagen synthesis and fibrovascular growth by inducing VEGF, FGF, and other growth factors. Lopez et al. reported that transdifferentiated RPE cells in the highly vascularized regions were the principal sources of VEGF, and these cells were thus very important for CNVM formation. In our specimens, we found strong VEGF immunoreactivity in RPE and vascular cells and, similar to previous reports, VEGF immunoreactivity was prominent within the cytoplasm that surrounded the neovascular channels within the CNVM stroma. These findings suggest that VEGF is a potent stimulator in the formation and progression of CNVMs, and the main source of VEGF may be RPE cells.

Our immunohistochemical study showed numerous Ang1- or Ang2-positive cells in all specimens. Positive cells included RPE monolayer, pigmented stromal cells, polygonal cells, stromal fibroblast-like cells, and vascular cells. Interestingly, the distribution of Ang1 and Ang2 was similar. Ang2 staining was observed in most vessel walls; a few, however, had no Ang1 immunoreactivity. There was not much difference in the level of Ang1 staining among the specimens, but Ang2 immunoreactivity tended to be greater in active, highly vascularized CNVMs and appeared to correspond to the activity of choroidal neovascularization. The physiological expression of Ang1 and Ang2 in adult tissue is reported to be different. In the embryonic stage, both Ang1 and Ang2 are widely present, but in the adult, the expression of Ang2 is limited to tissues subject to physiological angiogenesis, such as the uterine cycle of gestation. During ovulation, Ang2 expression is
Ang2 expression was recently shown by immunohistochemical analysis to be present in quiescent adult tissues, including vascular endothelial cells. Tie2 expression is reported to occur only on endothelial cells and early hemopoietic cells, but positive cells in our specimen, were not limited to endothelial cells and included RPE cells. Tie2 upregulation in vascular endothelium of metastatic melanomas and during ovulation and wound healing suggest that Tie2 expression can be regulated by angiogenic stimuli. The degree of staining for Tie2 in this study was stronger in highly vascularized specimens, and its expression was observed in a variety of cell types, and suggests that Tie2-dependent signaling plays a role in angiogenesis in CNVMs. Further studies including the elucidation of Tie2 expression and function in nonendothelial cells, must be performed to clarify the role of the angiopoietin–Tie2 system in CNVM formation.

Ang2 has been thought to be a naturally occurring antagonist for Tie2 and in human endothelial cells was not shown to activate Tie2. However, recent studies have shown that Ang2 could phosphorylate the Tie2 receptor and promote the same functions as Ang1 when transiently expressed in nonendothelial cells, suggesting that the angiopoietin–Tie2 system changes its functions depending on cell type. Further, Asahara et al. recently showed the modulatory effect of Ang2 on VEGF-induced corneal neovascularization in a mouse model. Ang2 was shown to stimulate VEGF-induced corneal neovascularization, probably through promotion of vascular destabilization and sprouting, which are probably required to initiate neovascularization. Present findings that Ang2 and VEGF were co-upregulated and that Tie2 was expressed in a variety of cell types in CNVMs, in addition to our previous report that VEGF upregulates Ang2 expression, further support a crucial role of the interaction between VEGF and Ang2 in pathologic angiogenesis of CNVM formation.

Table 1.
Clinical Characteristics

Table 2.

View Table

Histologic Characteristics

Table 3.

View Table

Distribution of Ang1, Ang2, and Tie2/Tek1

Figure 1.

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Histology and immunohistochemistry of an idiopathic choroidal neovascular membrane (CNVM; specimen 10). (A) Surgically excised CNVM is shown stained with hematoxylin and eosin. Remnants of an intact RPE layer that was confirmed by immunohistochemistry for pancytokeratin were present at the edge of the outer surface (arrowheads). The CNVM contained new vessels (arrows), as well as nonpigmented and pigmented stromal cells. (B) Immunohistochemistry for CD68 revealed that some pigmented stromal cells were positive (arrowheads). (C) Immunohistochemistry for pancytokeratin revealed that pigmented stromal cells (arrowheads) and nonpigmented polygonal cells (open arrowheads) were positive. (D) Immunohistochemistry for Tie2/Tek1 revealed that most of the vessels were positive (arrowheads), and some of the stromal cells, including pigmented cells (open arrowheads), were strongly positive. (E) Immunohistochemistry for VEGF revealed that immunoreactivity for VEGF was prominent in the highly vascularized lesions. (F, G) Immunohistochemistry for Ang1, Ang2, and VEGF. Pigmented (arrowheads) and nonpigmented (open arrowheads) stromal cells were positive. (H) Immunohistochemistry for Ang1 revealed that RPE and stromal cells are positive (open arrowheads). Magnification, ×40 (A); ×200 (B, C); and ×160 (D, F, G, H).

Figure 2.

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Histology and immunohistochemistry of an age-related CNVM (specimen 4). (A) Surgically excised CNVM is shown stained with hematoxylin and eosin. The CNVM was rich in new vessels. (B) Immunohistochemistry for pancytokeratin revealed that pigmented (arrowheads) and nonpigmented (open arrowheads) stromal cells were positive. (C) Immunohistochemistry for CD68 revealed that pigmented stromal cells (arrowheads) and nonpigmented polygonal cells (open arrowheads) were also positive. Immunohistochemistry for Ang1 (D), Ang2 (E), and VEGF (F). Pigmented (arrowheads) and nonpigmented (open arrowheads) stromal cells were positive. Note that, instead of Ang1, immunohistochemistry showed that VEGF and Ang2 were
prominent in highly vascularized lesions. (G) Immunohistochemistry for Tie2. Many stromal cells, including fibroblast-like cells (open arrowheads) and pigmented stromal cells (arrowheads), were positive for Tie2. Magnification, ×160.

Figure 3.

Double immunofluorescent immunohistochemistry of CNVM (specimen 4) revealed an extensive overlap between cells positive for factor VIII (green) and those positive for (A) Ang1 (red) and (B) Ang2 (red). Ang1 (red) is not positive in some vessels that are positive for factor VIII (green). Double immunofluorescent immunohistochemistry revealed an extensive overlap between cells positive for (C) pancytokeratin (red) and Ang1 (green) and (D) Ang2 (green) and between cells positive for (E) CD68 (red) and Ang1 (green) and (F) Ang2 (green). Double immunofluorescent immunohistochemistry for (G) Tie2 (green) and pancytokeratin (red) and (H) GFAP (red). In many cells, the overlap resulted in yellow stain (arrows). Original magnification, ×52.


Sub retinal choroidal retinal membrane is a disease of the retina that affects elderly patient with degenerative pathologies. This case outlines a brief sketch of pathogenesis and treatment protocol of this disease in current era. It is known for self-limited and regressive behavior that can be observed thoroughly and typically has a favorable visual prognosis. Keywords: Metamorphopsia; RPE; Choroidal rupture; Central serous chorioretinopathy; Spergnitous choroiditis. A focus of inner choroidal lymphocytic infiltration was found in the left eye despite the absence of overt clinical intraocular inflammation. This is the first pathological study employing human tissue that points to pericytes as a potential critical therapeutic target with the aggravating influence of inner choroidal chronic inflammation in PIC. In the right eye, lumens of many neovascular channels were occluded by microfibrils and pericytes.
were infrequent. In the left eye, patent CNV units with pericytes were present. There were scattered macrophages but no lymphocytes in either membrane.