Localisation of vascular endothelial growth factor and its receptors to cells of vascular and avascular epiretinal membranes

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Abstract

**Aims/background**—Epiretinal membranes (ERMs) arise from a variety of causes or, in some cases, for unknown reasons. Once established, ERMs tend to progress, becoming more extensive and exerting increasing traction along the inner surface of the retina. One possible cause for their progression is the production of growth factors by cells within ERMs that may provide autocrine or paracrine stimulation. Platelet derived growth factor (PDGF) and its receptors have been localised to cells of ERMs and may play such a role. In this study, comparative data were sought for several other growth factors that have been implicated in ERM formation.

**Methods**—Immunohistochemical staining of ERMs was done for PDGF-A, PDGF-B, basic fibroblast growth factor (bFGF), three isoforms of transforming growth factor β (TGF-β), and vascular endothelial growth factor (VEGF) and its receptors, flt-1 and flk-1/KDR. Expression of flt-1 and flk-1/KDR was examined in cultured retinal pigment epithelial (RPE) cells and retinal glia from postmortem eyes by immunohistochemistry and by reverse transcription coupled to polymerase chain reaction (RT-PCR).

**Results**—Staining was most intense and most frequently observed for VEGF and PDGF-A, both in vascular and avascular ERMs. The majority of cells stained for VEGF in nine of 11 (81.8%) diabetic ERMs and in 14 of 24 (58.3%) proliferative vitreoretinopathy (PVR) ERMs. The receptors for VEGF, flt-1, and flk-1/KDR were examined in cultured RPE cells and retina glia that are major components.1–5

**Conclusions**—These data show that VEGF and its receptors are localised to both vascular and avascular ERMs and suggest that VEGF, like PDGF-A, may be an autocrine and paracrine stimulator that may contribute to progression of vascular and avascular ERMs.

**References**


nal neovascularisation regresses after retinal hypoxia is decreased by panretinal photocoagulation (PRP), but well established retinal neovascularisation is less likely to regress and often progresses after PRP. Likewise, there is a tendency for progression of PVR and idiopathic ERMs. One possible reason is that the cells within ERMs produce growth factors that recruit other cells and stimulate cell proliferation. It has previously been demonstrated that cells of both vascular and avascular ERMs produce PDGF and have PDGF receptors; therefore, PDGF may be one factor that contributes to the progression of ERMs. However, several other growth factors have also been localised in cells of ERMs (for review, see Campochiaro et al). In this study, we performed immunohistochemical staining for several growth factors in vascular and avascular ERMs to obtain comparative information.

### Materials and Methods

**EPIRETINAL MEMBRANE COLLECTION AND FIXATION**

ERMs were promptly fixed after surgical excision and processed as previously described. They were incubated in 2% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, containing 5% sucrose for 1 hour at room temperature. The sucrose concentration was increased stepwise to 20% and the specimens were frozen in a 2:1 ratio of 20% sucrose in PBS to OCT embedding compound (Miles, Elkhart, IN, USA) as previously described. Sections of 6 µm were immersed in methanol containing 0.75% hydrogen peroxide for 10 minutes at −20°C, washed with 0.05 M TRIS buffered saline, pH 7.6 (TBS), and blocked with 10% normal goat serum (NGS) in TBS for rabbit antibodies and 10% normal rabbit serum (NRS) in TBS for mouse antibodies before incubation with the primary antibody. Each ERM was stained for as many growth factors as possible, but when sufficient tissue was not available, priority was given to VEGF, TGF-β1, TGF-β3, or bFGF.

### Cell Culture

RPE cell cultures were established by a previously described technique. Primary cultures were subcultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Upstate Biotecntologies, Inc, Lake Placid, NY, USA). Cultures were demonstrated to be pure populations of RPE cells by immunohistochemical staining for cytokeratin (Dako, Santa Barbara, CA).

### Immunohistochemical Staining

Sections were incubated at 4°C overnight with one of the primary antibodies listed in Table 1. When sufficient tissue was available, each ERM was stained for all growth factors and receptor types. The slides were warmed to room temperature and washed. Immunolabeling was visualised by HistoMark Red (Kirkegaard and Perry, Gaithersburg, MD, USA), which is an alkaline phosphatase based system that yields a brilliant red reaction product or by 3-amino-9-ethylcarbazole (AEC, Sigma, St Louis, MO, USA), which reacts with peroxidase to yield a brick red reaction product. HistoMark Red was developed according to the manufacturer's instructions. For AEC reaction, sections were immunoreacted as previously described or as follows. The slides were incubated for 30 minutes with a 1:25 dilution of rabbit anti-mouse immunoglobulin in 1% NGS-TBS (Dako; Santa Barbara, CA, for mouse antibodies) or a 1:40 dilution of goat anti-rabbit globulins (Arnel, Brooklyn, NY) for rabbit antibodies. The slides were rinsed twice and incubated for 30 minutes with a 1:400 dilution of mouse peroxidase antiperoxidase complex (Arnel; for rabbit antibodies). After thorough washing with 0.05 M TRIS, pH 7.6,
the immunoreaction products were visualised by immunoperoxidase using freshly prepared 0.178 mg/ml AEC in 0.89 M sodium acetate, pH 5.1, containing 2.1 x 10^{-4} % hydrogen peroxide for 10 minutes. Sections were coded so that the observer graded the specimens without knowledge of the type of ERM being evaluated. Immunohistochemical staining of each ERM with one of the growth factor antibodies was graded as: (1) 'none', if no cell stained, (2) 'sparse', if only occasional cells stained or if cell clusters stained, but only weakly, (3) 'prominent', if most cells stained or if clusters of cells stained intensely. Double labelling for VEGF and class III β tubulin, which is a marker for identifying RPE cells in epiretinal membranes, was performed using AEC and HistoMark Black (Kirkegaard and Perry) as previously described.

Cultured RPE or retinal glia were grown in slide wells and fixed in methanol. The wells were incubated with a 1:200 dilution of an affinity purified antibody directed against an amino terminal peptide fragment of VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a 1:100 dilution of an affinity purified rabbit antibody raised against a peptide corresponding to a fragment of human flt-1 (Santa Cruz Biotechnology), or a 1:200 dilution of one of two affinity purified rabbit antibodies to flk-1 (Santa Cruz Biotechnology). One of the antibodies was an IgG generated against a 20 amino acid sequence from the carboxy terminus of mouse flk-1 and was used on the cell cultures. The other antibody was an IgG raised against a large peptide fragment that overlapped the first and showed a better cross reactivity with human flk-1/KDR. The second anti-flk-1 antibody was used on ERMs. The anti-flk-1 antibodies specifically immunoprecipitated flk-1 and immunocytochemical staining with the anti-flk-1 antibody co-localises with detection of flt-1 mRNA by in situ hybridisation in cytrophoblasts. After overnight incubation at 4°C with the primary antibody, the slide wells were rinsed and the colour reaction was developed using Histo Mark Red according to the manufacturer's instructions (Kirkegaard and Perry).

For controls, the primary antibody was omitted and replaced with non-immune rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA), non-immune rabbit serum (Arnel), or for VEGF, TGF-β, flt-1, and the flk-1/KDR antibodies generated against the smaller peptide fragment, the primary antibody was preincubated with a tenfold molar excess of the peptide against which the antibody was generated for 2.5 hours at room temperature or overnight at 4°C before its application to the slide. Immunohistochemistry for controls was otherwise performed identically. Statistical significance was determined by the χ² test.

**Results**

**GROWTH FACTORS IN ERMS**

Table 2 summarises the grading of cellular staining for each growth factor in ERMs from patients with PVR, PDR, or idiopathic macular pucker. Each growth factor studied (except TGF-β3 in idiopathic macular pucker) showed some staining in all three types of ERMs, but the frequencies and intensities of staining varied considerably. As noted previously, most ERMs (regardless of the underlying disease process) stained for PDGF-A, many in clusters of cells. Prominent staining for PDGF-A was noted in five of six PDR ERMs and in four of eight PVR ERMs, with most of the cells positive for PDGF-A. PDGF-B staining was less conspicuous. Staining for VEGF was very similar to that for PDGF-A in PVR membranes, but was often more intense in PDR membranes (Fig 1A-C). The majority of cells stained for VEGF in nine of 11 (81.8%) PDR membranes compared with 14 of 24 (58.4%) PVR membranes (Fig 1D and E), and...
two of eight (25.0%) idiopathic ERMs (Figs 1F and 2A). At least occasional cellular positivity for VEGF was seen in more than 80% of all ERMs examined. VEGF localisation in pigmented cells (Fig 3A) and its co-localisation with class III β tubulin (Fig 3C) indicates that VEGF is present in RPE cells. Staining for bFGF, TGF-β1 (Fig 2B) and TGF-β2 (Fig 2C) was much less frequent and less extensive than staining for VEGF and PDGF-A in the ERMs studied and very few ERMs stained with the antibody for TGF-β3. However, occasional ERMs did show prominent staining for one of the less frequently observed factors.

GROWTH FACTOR RECEPTORS IN ERMS

Receptors for PDGF, as well as PDGF itself, are localised to vascular and avascular ERMs, suggesting that PDGF could play an autocrine and/or paracrine role in the progression of ERMs. Because the receptors for VEGF (flt-1 and flk-1/KDR) had been felt to be specifically expressed on vascular endothelial cells, we anticipated staining for flt-1 and flk-1/KDR in vascular, but not avascular, ERMs. However, flt-1 and flk-1/KDR were localised to both vascular ERMs from patients with PDR (3/4 for flt-1; 2/4 for flk-1) and avascular ERMs from patients with PVR or idiopathic macular pucker (15/20 for flt-1; 8/12 for flk-1) (Fig 4). When one type of VEGF receptor was found in a particular ERM, it was likely that the other type was also present (p = 0.013). Flt-1 was visualised on pigmented cells in ERMs (Fig 3B), indicating the presence of VEGF receptors on RPE cells. Prominent labelling for flt-1 also occurred on nearly all cells in ERMs consisting almost entirely of glial cells, as demonstrated by GFAP staining (Fig A).

Table 2 Immunohistochemical staining for growth factors in cells of epiretinal membranes

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>N</th>
<th>None (%)</th>
<th>Sparse (%)</th>
<th>Prominent (%)</th>
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<tr>
<td>Proliferative diabetic retinopathy (PDR):</td>
<td></td>
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<td></td>
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<tr>
<td>VEGF</td>
<td>11</td>
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<td>1 (33.3)</td>
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<td></td>
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<td>9 (37.5)</td>
<td>14 (58.3)</td>
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<td>4 (57.1)</td>
<td>1 (14.3)</td>
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</table>

Figure 1 VEGF immunostaining in PDR, PVR, and idiopathic ERMs is visualised with a red reaction product. (A) An ERM from a patient with PDR shows prominent staining around a newly formed vessel (arrow), as well as in non-vascularised areas. (B) An ERM from another patient with PDR. VEGF positivity is not limited to areas of neovascularisation, but is visualised in numerous other cells, including RPE cells (brown). (C) Preincubation of the primary antibody with the peptide against which it was generated (peptide control) eliminates VEGF immunostaining as seen on another section from the same ERM shown in (B). (D) VEGF staining is also demonstrated in a PVR ERM. (E) Preincubation of the primary antibody with the control peptide eliminates immunostaining in the same ERM shown in (D). (F) Most of the cells from an idiopathic ERM are also conspicuously stained for VEGF (HistoMark Red/haematoxylin; A, B, D, E, F x145, C x70).

Figure 2 Immunostaining for multiple growth factors in a simple idiopathic ERM is visualised with a red reaction product. (A) Cellular positivity for VEGF (B) TGF-β, positivity (arrows) in cell processes from the same ERM. (C) Cellular staining for TGF-β2 is also demonstrated in the same ERM. (A, C: HistoMark Red/haematoxylin; B: AEC/haematoxylin; A x145; B, C x360).
3C–E), suggesting that the flt-1 receptors are also expressed by retinal glial cells.

**CELL CULTURES**

Immunohistochemistry for flt-1 and flk-1/KDR was performed on cultured RPE and retinal glia. RPE cells showed staining for flt-1 and flk-1/KDR along cell surfaces (Fig 5). The staining for flt-1 and flk-1/KDR was equivocal in retinal glial cells because of high background staining not seen in RPE cells, but VEGF positivity was clearly demonstrated, both in RPE cells and retinal glia (not shown). The expression of flt-1 and flk-1/KDR was also investigated by RT-PCR. Reaction products with the correct size for flt-1 (521 bp) and flk-1/KDR (537 bp) were amplified from RNA isolated from cultured RPE and from RPE isolated from postmortem human eyes (Fig 6). They were confirmed to be flt-1 and flk-1/KDR fragments by sequencing. RNA harvested from cultured retinal glia showed an appropriate reaction product for flt-1, but not flk-1/KDR.

**Discussion**

Growth factors appear to be involved in the formation and progression of epiretinal membranes. One of the most studied of these factors is PDGF. Immunohistochemical staining for PDGF and its receptors has been demonstrated in both vascular and avascular ERMs. This is an expected finding because PDGF is produced by vascular endothelial cells and RPE cells, and...
PDGF receptors are found on RPE \(^{11}\) and retinal glial cells. \(^{30}\) PDGF has been shown to be an autocrine growth stimulator for RPE cells and it may play an important role in the progression of ERMs.

However, several other growth factors have been localised to ERMs \(^{30,31}\) and so for comparative purposes, we performed immunohistochemical staining for PDGF-A, PDGF-B, VEGF, TGF-\(\beta_1\), TGF-\(\beta_2\), TGF-\(\beta_3\), and bFGF. Staining for VEGF was just as prominent as staining for PDGF, both in terms of the total number of ERMs that showed VEGF immunoreactivity and the number of cells within individual ERMs that were VEGF positive. Compared with PDGF and VEGF, staining for the TGF-\(\beta\) isoforms and bFGF was less frequent and less extensive in ERMs. While VEGF positive cells were particularly abundant and intensely stained in vascular membranes, VEGF staining was also prominent in avascular membranes. In retrospect, this is not that surprising because retinal glia and RPE each produce VEGF \(^{52-54}\) and both cell types are present in vascular and avascular ERMs. However, this finding raises two questions: (1) If VEGF is present in avascular membranes, why are there no blood vessels? (2) Is it possible that there are cells other than vascular endothelial cells that are targeted by VEGF?

With respect to the first question, there is strong evidence implicating VEGF in the development of retinal neovascularisation in ischaemic retinopathies \(^{14-21}\) and it may even be a necessary component, but it may not be the only factor involved. There is mounting...
Localisation of vascular endothelial growth factor and its receptors to cells of vascular and avascular epiretinal membranes

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evidence suggesting that while VEGF is angiogenic on chick chorioallantoic membrane and in the rabbit corneal pocket model, high levels may be needed to cause neovascularisation in the retina in some species. Immunoreactive VEGF is increased in the retina in several conditions in which neovascularisation does not occur and repeated injection or sustained release of VEGF in the vitreous cavity of primates does not cause unequivocal neovascularisation. It is possible that the presence of endothelial growth inhibitory factors, such as TGF-β, which may be secreted by glial cells, may prevent VEGF from exerting its angiogenic activity. The presence of VEGF in ERM s in the absence of neovascularisation is consistent with these other observations.

To address the second question, we investigated the expression of flt-1 and flk-1/KDR in RPE and retinal glia which, as noted above, are the predominant cell types in ERM s. As demonstrated by immunocytochemistry and RT-PCR, flt-1 and flk-1/KDR are expressed in cultured RPE. Appropriate fragments for flt-1 and flk-1/KDR are also amplified from RPE RNA from postmortem human eyes. While RNA from photoreceptors may contaminate RPE RNA, there is not likely to be any RNA from vascular endothelial cells because retinal vessels are located in the inner part of the retina and the retina was removed before isolation. In addition, Bruch’s membrane was examined and found to be intact after RPE isolation, precluding contamination by choroidal vessels. This suggests that RPE in situ, as well as RPE in epiretinal membranes and in culture, express flt-1 and flk-1/KDR. The situation is less clear for retinal glia, but based upon RT-PCR, cultured retinal glia can express flt-1 and staining for flt-1 in the majority of cells in predominantly glial ERM s suggests that the same is true for retinal glial cells in ERM s.

These findings conflict with the dogma that flt-1 and flk-1/KDR are endothelial cell specific; they add to the growing body of evidence indicating that cell types other than vascular endothelial cells including neural progenitor cells of the retina, corneal endothelial cells, renal mesangial cells, and melanoma cells possess VEGF receptors. In addition, our data strongly support the recent findings of Guerin et al who demonstrated that cultured RPE cells express flt-1 and flk-1/KDR and contain an autocrine loop for VEGF. In many ERM s, VEGF and its receptors were co-localised, suggesting that an autocrine and/or paracrine mechanism may be operative. The present study suggests that autocrine loops for both PDGF and VEGF may be involved in progression of both vascular and avascular ERM s.

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