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

Ying-Shan Chen, Sean F Hackett, Carl-Ludwig Schoenfeld, Melissa A Vinores, Stanley A Vinores, Peter A Campochiaro

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 pigmented 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 also identified on cells in ERMs and on cultured RPE cells. By RT-PCR, mRNA for flt-1 was identified in RPE cells and retinal glia, and mRNA for flk-1/KDR was identified in RPE cells.

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.

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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-β, TGF-β, and 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 Biotechnologies, Inc, Lake Placid, NY, USA). Cultures were demonstrated to be pure populations of RPE cells by immunohistochemical staining for cytokeratin.

Human retinal glial cells were cultured and characterised as previously described. The cells were maintained in medium containing 40% DMEM/40% Ham’s F-12/20% FBS and stained uniformly for glial fibrillar acidic protein, indicating that they contain a pure population of glial cells.

**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. Immunolabelling 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 wereimmunoreacted 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,

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**Table 1: Primary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mouse monoclonal (M) or rabbit polyclonal (R)</th>
<th>Dilution</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth factors:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>R</td>
<td>1:20–1:1000*</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>R</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>TGF-β</td>
<td>R</td>
<td>1:400 or 1:500</td>
<td>Ref 35</td>
</tr>
<tr>
<td>TGF-β</td>
<td>R</td>
<td>1:400 or 1:500</td>
<td>Ref 35</td>
</tr>
<tr>
<td>TGF-β</td>
<td>R</td>
<td>1:500</td>
<td>Ref 35</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>R</td>
<td>1:100</td>
<td>Ref 36</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>M</td>
<td>1:50</td>
<td>Ref 37</td>
</tr>
<tr>
<td>bFGF</td>
<td>R</td>
<td>1:3000</td>
<td>Ref 38</td>
</tr>
<tr>
<td>bFGF</td>
<td>M</td>
<td>1:50</td>
<td>Ref 39</td>
</tr>
<tr>
<td><strong>Receptors:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flk-1</td>
<td>R</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Flk-1 (smaller peptide fragment)</td>
<td>R</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Flk-1 (overlapping, larger peptide fragment)</td>
<td>R</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td><strong>Cell type identification markers:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>R</td>
<td>1:1000</td>
<td>Dako, Santa Barbara, CA; Ref 40</td>
</tr>
<tr>
<td>Class III β tubulin</td>
<td>M</td>
<td>1:300</td>
<td>Ref 41</td>
</tr>
</tbody>
</table>

*Immunostaining for VEGF was seen at all dilutions of primary antibodies ranging from 1:20 to 1:1000 (1:20, 1:50, 1:100, 1:200, 1:500, and 1:1000). A dilution of 1:20 was used on specimens that were photographed because it enhanced the crispness of the staining without adding any background or false positive staining.
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 × 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,41 was performed using AEC and Histomark Black (Kirkegaard and Perry) as previously described.42

Cultured RPE or retinal glia were grown in slide wells and fixed in methanol.34 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 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 immunoprecipitate flk-131,32 and immunocytochemical staining with the anti-flk-1 antibody co-localises with detection of flt-1 mRNA by in situ hybridisation in cytostrophoblasts.33 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,35 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 ß tubulin (Fig 3C) indicates that VEGF is present in RPE cells. Staining for bFGF, TGF-ß1 (Fig 2B) and TGF-ß2 (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-ß1. 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.11 29 Because the receptors for VEGF (flt-1 and flk-1/KDR) had been felt to be specifically expressed on vascular endothelial cells,24 48 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 consisting almost entirely of glial cells, as demonstrated by GFAP staining (Fig 3B, C). Staining for bFGF, TGF-ß1, TGF-ß2, TGF-ß3, PDGF-A, PDGF-B, and bFGF was much less frequent in ERMs studied than VEGF and PDGF-A in the ERM.

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

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>proliferative diabetic retinopathy (PDR)</th>
<th>Proliferative vitreoretinopathy (PVR)</th>
<th>Idiopathic macular pucker</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>N: 11 None (18.1) Sparse (44.4) Prominent (81.8)</td>
<td>N: 24 None (4.2) Sparse (26.7) Prominent (58.3)</td>
<td>N: 8 None (12.5) Sparse (62.5) Prominent (25.0)</td>
</tr>
<tr>
<td>TGF-ß1</td>
<td>9 None (44.4) Sparse (44.4) Prominent (11.1)</td>
<td>15 None (53.3) Sparse (50) Prominent (20)</td>
<td>7 None (14.3) Sparse (85.7) Prominent (0)</td>
</tr>
<tr>
<td>TGF-ß2</td>
<td>9 None (22.2) Sparse (44.4) Prominent (33.3)</td>
<td>14 None (42.9) Sparse (50) Prominent (7.1)</td>
<td>8 None (28.6) Sparse (57.1) Prominent (14.3)</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>3 None (16.7) Sparse (0) Prominent (83.3)</td>
<td>6 None (16.7) Sparse (0) Prominent (83.3)</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>4 None (25.0) Sparse (75.0) Prominent (0)</td>
<td>4 None (25.0) Sparse (75.0) Prominent (0)</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
</tr>
<tr>
<td>bFGF</td>
<td>8 None (37.5) Sparse (62.5) Prominent (0)</td>
<td>8 None (37.5) Sparse (62.5) Prominent (0)</td>
<td>8 None (37.5) Sparse (62.5) Prominent (0)</td>
</tr>
<tr>
<td>TGF-ß3</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
<td>5 None (100) Sparse (0) Prominent (0)</td>
<td>3 None (100) Sparse (0) Prominent (0)</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
<td>3 None (33.3) Sparse (33.3) Prominent (33.3)</td>
</tr>
<tr>
<td>bFGF</td>
<td>7 None (28.6) Sparse (57.1) Prominent (14.3)</td>
<td>7 None (28.6) Sparse (57.1) Prominent (14.3)</td>
<td>7 None (28.6) Sparse (57.1) Prominent (14.3)</td>
</tr>
</tbody>
</table>
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 ERM$s^{11,28,29}$ This is an expected finding because PDGF is produced by vascular endothelial cells$^{49}$ and RPE cells,$^{11,50}$ and...
PDGF receptors are found on RPE and retinal glial cells. 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 and so for comparative purposes, we performed immunohistochemical staining for PDGF-A, PDGF-B, VEGF, TGF-β1, TGF-β2, TGF-β3, 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-β 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 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 and it may even be a necessary component, but it may not be the only factor involved. There is mounting evidence...
Vascular endothelial growth factor is a ubiquitously expressed growth factor that plays a crucial role in the development and maintenance of vascular systems. Its expression is upregulated in various pathological conditions, including neovascularization and angiogenesis.

In the retina, the expression of VEGF is tightly controlled, and its presence is often associated with the development of choroidal neovascular membranes (CNVM) in diseases such as age-related macular degeneration (AMD). The presence of VEGF in these membranes suggests an autocrine loop for VEGF, where VEGF is produced by the endothelial cells and acts on the same cells to promote their proliferation and survival.

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27. Pournaras CJ, Tsiapoulos M, Strommer K, Gildoni N, Leuenberger PM. Scatter photocoagulation restores tissue
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