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Novel growth factors involved in the pathogenesis of proliferative vitreoretinopathy

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Abstract

Aims **To determine whether hepatocyte growth factor (HGF) and connective tissue growth factor (CTGF) are expressed in human specimens of proliferative vitreoretinopathy (PVR) and to propose a model of PVR pathogenesis based upon the known activities of these growth factors.**
Methods **Immunohistochemical methods (ABC Elite) were used to demonstrate the presence of HGF and CTGF in cryostat sections of five human PVR membranes.**
Results **In each of the five PVR membranes, stromal cells were immunohistochemically positive for both HGF and CTGF. Based upon this information and the known actions of these growth factors, a model of PVR pathogenesis was developed. In this model, injury of the retina induces an inflammatory response that upregulates HGF expression inducing the formation of multilayered groups of migratory retinal pigment epithelial cells (RPE). These RPE, present in a provisional extracellular matrix, come in contact with vitreous containing TGF-β. The TGF-β is activated, upregulating expression of CTGF. Under the influence of TGF-β and CTGF, RPE become myofibroblastic and fibrosis ensues. Retinal traction induces further detachment continuing the cycle of retinal injury.**
Conclusions **HGF and CTGF are expressed in PVR membranes and may play important roles in the pathogenesis of PVR. The expression and function of these growth factors should be critically examined in human PVR specimens, in *in vitro* cultures of RPE, and in animal models of PVR.**
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Introduction

Proliferative vitreoretinopathy (PVR) is the most common cause of failed repair of a primary rhegmatogenous retinal detachment (RRD).[1,2] It occurs when traction-generating cellular membranes develop in the vitreous and inner or outer surfaces of the retina after RRD or major ocular trauma.[1,3] A critical cell type involved in this epiretinal membrane formation is the RPE cell.[4,5] These cells proliferate and migrate from the RPE monolayer to form sheets of dedifferentiated cells within a provisional extracellular matrix (ECM) that contains fibronectin and thrombospondin.[5–7] The cellular membrane becomes progressively paucicellular and fibrotic over time.[7] Normal ocular wound healing involves a tightly coordinated series of events including recruitment and activation of inflammatory cells, release of cytokines, activation and proliferation of ocular cells, secretion of extracellular matrix, tissue remodeling and repair.[8] The mechanisms of protracted wound healing, as is found in PVR, are unknown but are presumed to include either sustained or dysregulated growth factor responses.[4]

In the normal eye, the RPE forms a monolayer of immobile, polarized, non-proliferating cells joined by apical tight and adherent junctions.[9] Within 24 hours of retinal detachment in the cat, RPE cells begin to proliferate and form multi-layered groups of dedifferentiated cells.[10] The signal for this dramatic change in the RPE monolayer may be related to loss of contact or signaling from

photoreceptors.[4,10] Alternatively, it may be a response to signals, such as tumor necrosis factor-alpha (TNF), secreted by inflammatory cells drawn to the lesion.[11] Previous work has established the important adjunctive role of TNF as a mediator of RPE activation and platelet-derived growth factor (PDGF) as an important mitogen, chemoattractant, and mediator of cellular contraction.[4,5,12–17] The cytokine TGF-β is a pivotal contributor to tissue fibrosis, and the TGF-β 2 isoform is predominant in an inactive form in normal vitreous.[18,19] Thrombospondin-1 represents a likely activator of TGF-β within tissue.[20,21] TGF-β levels are elevated in PVR vitreous and they correlate with the presence of intraocular fibrosis, however little is known about the downstream mediators of this effect.[20] In this paper, we propose that novel growth factors (hepatocyte growth factor and connective tissue growth factor) mediate two critical, and in many ways opposing, activities in PVR; the development of multilayered groups of dedifferentiated RPE from an intact monolayer early in the process, and their subsequent transformation into a fibrotic paucicellular membrane later in the process.

Methods

PVR membranes were surgically excised during vitrectomy surgery in five patients. The tenets of the Declaration of Helsinki, Finland, were followed—informed consent was obtained, and institutional human experimentation committee approval was granted for this study. The basic characteristics of the excised membranes are described in Table 1.

Each of the fresh membranes was placed in isotonic saline at 4°C, then snap-frozen in optimum cutting temperature compound (Ames/Miles, Elkhart, IN, USA) within 1 h. Each specimen was sectioned on a cryostat into 6 μm frozen sections on glass slides for immunohistochemical analysis. The sections were fixed in reagent grade acetone for 5 min at room temperature and stored at −80°C. Thawed tissue sections were air-dried, rehydrated with phosphate-buffered saline (PBS; pH 7.4), and incubated with blocking serum for 15 min. Sections were incubated for 60 min with the polyclonal primary antibody of interest then washed for 15 min with PBS. Immunoperoxidase detection was performed using the ABC Elite kit (Vector, Burlingame, CA, USA) with aminoethylcarbizole as the red chromogen. Slides were then rinsed with tap water, counterstained with hematoxylin, and mounted with glycerin-gelatin medium. Polyclonal antibodies against HGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1:200, while antibodies against CTGF (Fibrogen, South San Francisco, CA, USA) were used at a dilution of 1:400.

Table 1 Immunohistochemical staining of PVR membranes for HGF and CTGF

Case number	*HGF*	*CTGF*
1	++	++
2	+	+++
3	+	+++
4	+	++
5	+++	+++

1+, weak, focal staining (<10% of cells).
2++, moderate to strong staining, (up to 50% of cells).
3+++, intense staining (>50% of cells).

Results

All five membranes showed expression for both HGF and CTGF (Table 1). HGF expression was generally higher in cellular regions of the membrane than in fibrotic regions (Figure 1 a, b). HGF immunoreactivity was predominantly associated with the cytoplasm of the stromal cells. CTGF expression was prominent in both cellular and fibrotic regions of the membranes (Figure 1c, d). CTGF staining was present both in association with the stromal cells and the extracellular space.

Discussion

Hepatocyte growth factor (HGF)

We hypothesize that HGF is a major mediator of early PVR, inducing the separation of RPE from the monolayer and (along with TNF and PDGF) the formation of an invasive, cellular, non-fibrotic membrane containing dedifferentiated RPE. HGF, also known as scatter factor, is a pleiotropic growth factor with marked cross species activity that was originally derived from platelets.[22,23] HGF is a heparin-binding glycoprotein that is secreted as a single-chain (pre-HGF) precursor that in situations such as tissue damage is proteolytically cleaved to form a functional heterodimer joined by a single disulfide bond. HGF acts predominantly on epithelial cells where it has mitogenic, morphogenic and motogenic activities.[24–26] These effects are mediated through a transmembrane tyrosine kinase surface receptor known as c-Met or HGFR.[24]

We previously reported that RPE secrete HGF and express a functional HGFR making the HGF/HGFR system a potential autocrine loop for RPE.[27] As shown in this paper and in work by others, increased expression of HGF/HGFR is present in human PVR

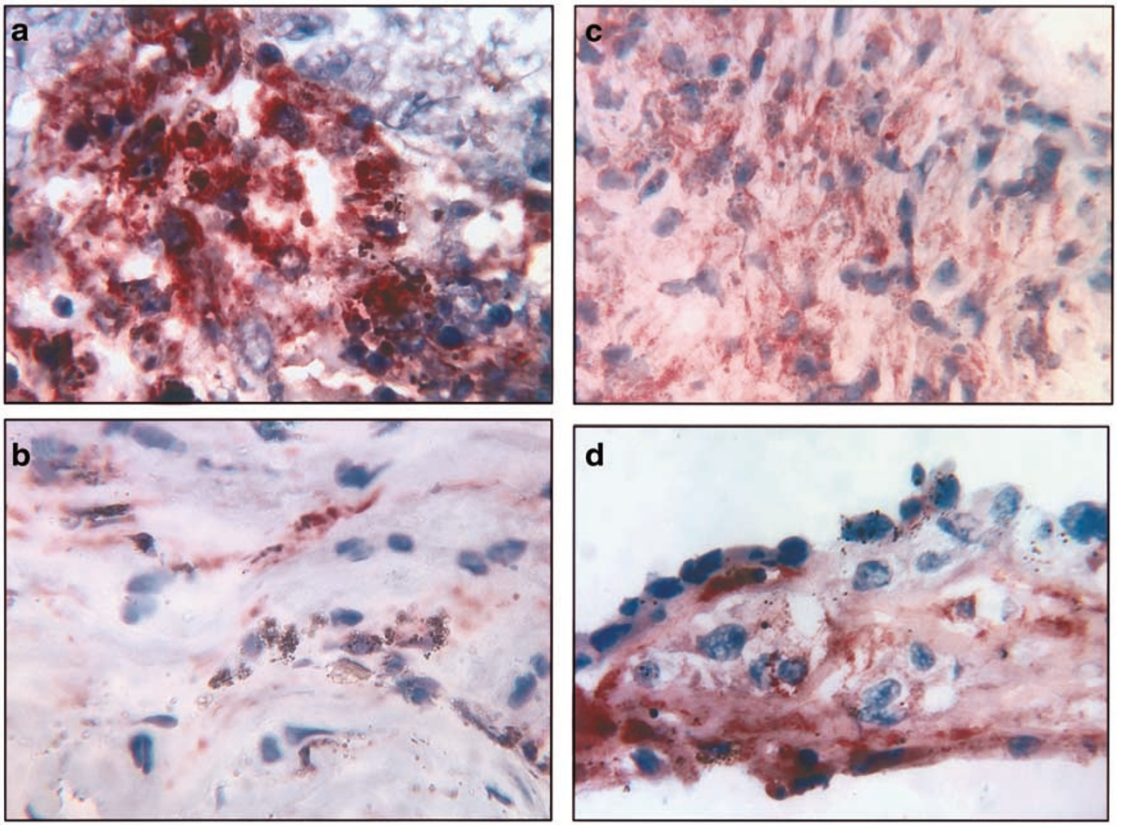

Figure 1 HGF and CTGF are expressed in surgically excised human PVR membranes. The cryostat tissue sections were stained immunohistochemically using polyclonal antibodies against HGF and CTGF. Detection was achieved with the ABC Elite kit using aminoethylcarbizole as the red chromogen. A weak hematoxylin counterstain is present. HGF is prominent within the stromal cells of cellular membranes (a) and to a lesser extent in stromal cells of fibrotic membranes (b). CTGF is found both associated with cells and in the extracellular space in cellular membranes (c) and fibrotic membranes (d). Magnification $\times 400$.

membranes and HGF is increased in the vitreous of PVR patients.[28–30] Interestingly, HGF scatter activity is higher in patients with RRD than PVR while total HGF increases with severity of disease suggesting that this growth factor may have a critical role early in the course of the disease.[29,30] Consistent with this finding, our results show that HGF expression was more prominent in cellular regions of membranes than those that were predominantly fibrotic. HGF interacts with a variety of extracellular matrix molecules and has high affinity interactions with thrombospondin-1.[31] In cultured epithelial cells of varied phenotype, HGF has been shown to modulate the localization and function of intercellular junctional proteins.[32–35] HGF has also been shown to be anti-apoptotic[36] and anti-fibrotic.[37–40] Pro-inflammatory cytokines such as TNF and interferon-gamma upregulate expression of HGF,[41] while HGF itself upregulates expression of TGF-β.[42] We have shown that HGF induces major phenotypic changes in RPE.[43] This is seen within an intact monolayer in explants and *in vivo*.[44] It results in phosphorylation and then loss of intercellular junctions, and facilitation of migration from the monolayer.[43] We predict that these morphologic changes will promote retinal detachment *in vivo* and will be associated with widespread alterations in patterns of RPE gene expression.

Connective tissue growth factor (CTGF)

We hypothesize that CTGF, in the presence of TGF-β, is the major mediator of retinal fibrosis. CTGF is a secreted cysteine-rich heparin-binding polypeptide growth factor.[45,46] It is a member of the CCN family of genes (ctgf/cyr61/nov) that includes six members encoding proteins that participate in fundamental biologic processes including wound healing and fibrosis.[46] Like other CCN members, CTGF contains four functional domains including an IGF binding protein, von Willebrand factor, thrombospondin-1, and cystine knot domains.[46] CTGF is rapidly upregulated after serum or TGF-β stimulation; however it is unstable *in vitro* with a $t_{1/2}$ of 60–90 min.[47] Little is known about the mechanism of CTGF degradation;

however, various CTGF fragments have been shown to accumulate in tissue culture or body fluids and they may retain biologic activity.[46,48–50] CTGF functions as a downstream mediator of TGF-β action on fibroblasts where it stimulates cell proliferation and cell matrix deposition (collagen 1 and fibronectin).[45–46,51] In contrast to HGF, CTGF induces apoptosis.[52,53] The receptor mediating CTGF's growth factor action is unknown.[46] Along with its action as a growth factor, CTGF has recently been implicated in fibroblasts as a *bona fide* adhesive substrate with activity mediated through $\alpha 6\beta 1$ integrin.[54] Importantly, CTGF is co-ordinately expressed with TGF-β and demonstrates increased expression in numerous fibrotic disorders including systemic sclerosis,[55,56] lung fibrosis,[57] renal fibrosis,[58,59] myocardial fibrosis,[60] and atherosclerosis.[61]

CTGF has not been previously reported in the retina, vitreous, or vitreoretinal disease tissue, although it has been shown that vascular endothelial growth factor induces expression of CTGF in retinal vascular cells.[62] We show here that there is strong immunoreactivity for CTGF in human PVR membranes. Much of the CTGF appears to be extracellular suggesting that the secreted protein may be bound to the extracellular matrix. We predict that since many of the CTGF-positive cells are also positive for cytokeratin (results not shown), that RPE will be found to produce CTGF. We also predict that CTGF will be found to be a major mediator of fibrosis in PVR. In the human PVR membranes that we examined, CTGF expression was prominent in both cellular and fibrotic areas of the membranes.

Model for pathogenesis of PVR: the PVR cycle

We propose a new model for the pathogenesis of PVR, based on the potential roles played by HGF and CTGF (Figure 2). In this model, injury to the retina induces an inflammatory response leading to the activation of RPE and upregulation of HGF expression. HGF induces the separation of RPE from the monolayer, inhibits apoptosis and promotes the formation of multilayered groups of dedifferentiated, migratory RPE. These RPE are present within a provisional extracellular matrix that includes thrombospondin (TSP). The RPE come into contact with vitreous that contains abundant inactive TGF-β. TGF-β is activated by mechanisms including TSP, thus stimulating upregulation of CTGF in the RPE. Under the influence of TGF-β and CTGF, the RPE become myofibroblastic and fibrosis ensues. The lesion becomes less cellular due to the pro-apoptotic effects of CTGF. The

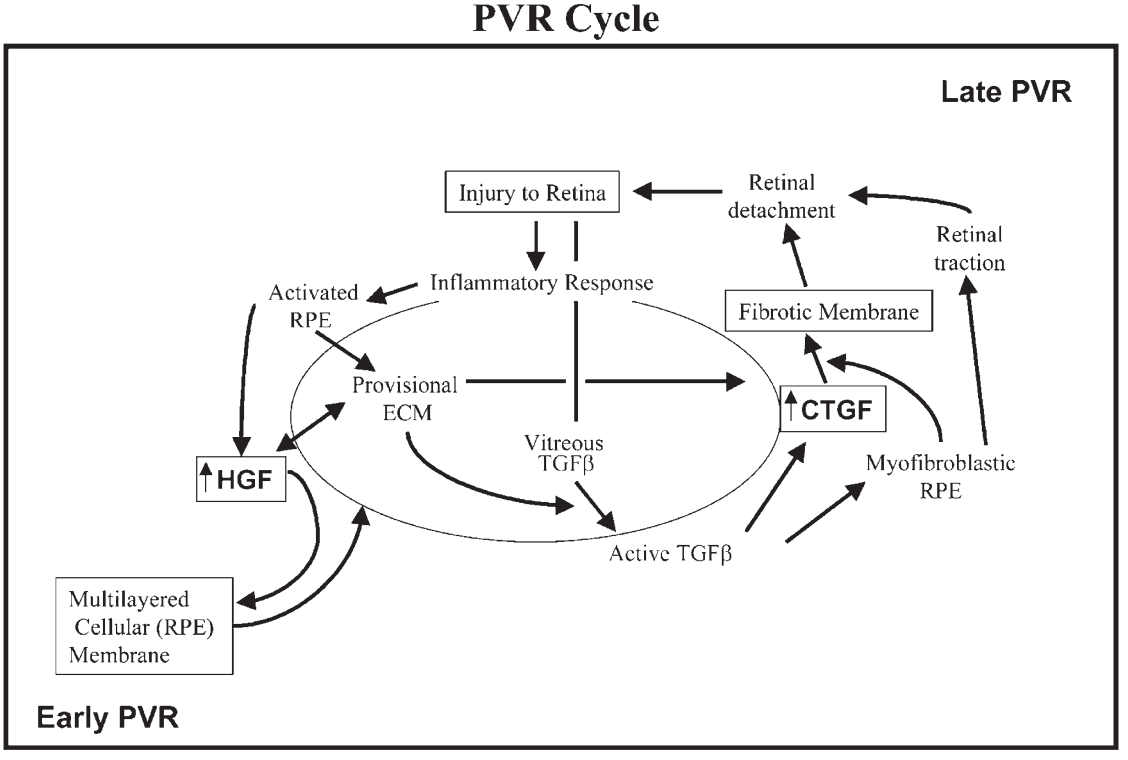

Figure 2 The PVR cycle. A model of pathogenesis of PVR is diagrammed showing the potential roles of the growth factors hepatocyte growth factor (HGF) and connective tissue growth factor (CTGF) in this process.

myofibroblastic RPE contract and in conjunction with the fibrotic ECM induce traction on the retina. Retinal traction induces further retinal detachment, continuing the cycle of retinal injury.

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