Fibronectin synthesis in subretinal membranes of proliferative vitreoretinopathy

P Hiscott, H A Waller, I Grierson, M G Butler, D L Scott, Z Gregor, I Morino

Abstract

In situ hybridisation and immunohistochemical studies were conducted on six surgically excised subretinal membranes of proliferative vitreoretinopathy to investigate whether displacement of retinal pigment epithelial and glial cells to subretinal membranes was associated with fibronectin production by the subretinal membrane cells. Fibronectin messenger RNA (mRNA) and fibronectin immunoreactivity were observed in some cells in all of the subretinal membranes studied and up to 30% of the cells in individual specimens showed intense labelling for fibronectin mRNA. The results support the concept that the cells in subretinal membranes produce fibronectin. Locally produced fibronectin may play a role in subretinal membrane cohesion, and displacement of retinal pigment epithelial and glial cells from their normal location may induce the cells to manufacture fibronectin. Fibronectin production may be more prominent in migrating subretinal cells.

Proliferative vitreoretinopathy (PVR) is a complication of retinal detachment and is characterised by the development of contractile, scar-like fibrocellular membranes on either side of the detached neuroretina (epiretinal and subretinal membranes). The contractile epiretinal and subretinal membranes of PVR have a similar cellular composition, although contractile subretinal membranes appear to have a larger retinal pigment epithelial component and smaller glial component than epiretinal membranes, and the extracellular matrix of PVR membranes at both sites contains collagens and glycoproteins, including fibronectin. Fibronectin has a number of important biological functions such as a role in cell-cell and cell-substratum adhesion, and the adhesive properties of fibronectin may contribute to the cell-cell and cell-extracellular matrix cohesion in early PVR membranes.

In PVR, epiretinal glial and retinal pigment epithelial cells are replete with fibronectin messenger RNA (mRNA) whereas cells in the subjacent detached neuroretina and retinal pigment epithelial monolayer exhibit little or no fibronectin message, suggesting that the ectopic retinal pigment epithelial and glial cells produce abundant glycoprotein while those in situ in the retina do not. The 'up regulation' of fibronectin production by the displaced cells probably contributes to the glycoprotein in epiretinal membranes but the mechanism for the change in gene expression for fibronectin is obscure.

One possible explanation for the activation of fibronectin production in the ectopic cells is that displacement of the retinal pigment epithelial and glial cells from their normal location stimulates, or removes inhibitions from, the cells to produce fibronectin, in which case fibronectin should be produced by the cells supported to sites other than the vitreoretinal junction. One such location is within the contractile subretinal membranes of PVR where retinal pigment epithelial and glial cells are displaced into the developing fibrocellular tissue. Therefore, to investigate further the hypothesis that displacement of pigment epithelial and glial retinal cells causes them to produce fibronectin, we examined the cells in PVR subretinal membranes for the presence of fibronectin mRNA employing an in situ hybridisation technique. The results were correlated with the distribution of fibronectin immunoreactivity in the specimens.

Materials and methods

CONTROL TISSUE

Formalin fixed, paraffin embedded synovial tissues, from patients with rheumatoid arthritis known to contain abundant fibronectin mRNA and show strong fibronectin immunoreactivity, were used as positive controls for the hybridisation experiments and immunohistochemical procedures.

SUBRETINAL MEMBRANES

Six subretinal membranes removed during pars plana vitrectomy for PVR were used in the study.

PREPARATION OF SINGLE-STRANDED RNA PROBES

A cDNA clone (pFH1) representing about one quarter of the estimated size of human cellular fibronectin mRNA was provided by Dr F E Baralle, Sir William Dunn School of Pathology, University of Oxford and subcloned into pGEM blue (Promega, WI, USA) to produce single-stranded RNA anti-sense and sense (control) probes, as previously described.

Transcription was conducted incorporating digoxigenin-11-UTP (Boehringer Mannheim, GmbH) with the standard transcription reaction using SP6 or T7 RNA polymerase (Boehringer). To achieve optimal probe length for tissue penetration alkaline hydrolysis was carried out to give an average probe length of 100 bases. To prevent digoxigenin loss into the organic phase phenol/chloroform extraction was omitted during probe purification. To ensure the correct size of transcripts and the success of hydrolysis the probes were checked by 2% denaturing agarose gel electrophoresis and northern blotting. Nitrocellulose filters were blocked with...
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3% normal sheep serum in phosphate buffered saline (PBS; pH 7.4) and subsequent immunohistochemical detection of probes was carried out using sheep anti-digoxigenin alkaline phosphatase conjugate (Boehringer) at 1:5000 in 3% normal sheep serum/PBS. Development was with NBT/BCIP (Sigma, Poole, UK) in TRIS buffered saline, pH 9.5 (0.3 mg/ml NBT; 0.2 mg/ml BCIP; 0.1 M TRIS; 0.1 M sodium chloride; 0.005 M magnesium chloride). Probes were stored at -20°C until used.

HYBRIDISATION HistoCHEMISTRY

Subretinal membranes were fixed for between 4 and 48 h in 10% formal-saline, dehydrated in graded concentrations of ethanol and embedded in paraffin wax.

Sections (6 µm thick) of wax-embedded tissue were cut, dewaxed, permeabilised, treated with proteinase K, post-fixed, dehydrated in methanol and air-dried. The anti-sense (SP6, fibronectin transcript) probe was then applied at a concentration of 1:100 (approximately 0.1 µg/ml) in hybridisation medium and the preparations were hybridised overnight under coverslips at 37°C in a humid chamber. During all the procedures up to and including hybridisation, RNase free conditions were employed. The coverslips and unbound probe were removed as previously described.

Sites of probe binding were visualised immunohistochemically using the antibody to digoxigenin. Non-specific antibody binding was blocked by a 20 minute incubation with 3% normal sheep serum/PBS, sheep anti-digoxigenin antibody-alkaline phosphatase conjugate was applied to the sections for 30 minutes at a concentration of 1:200 in 3% normal sheep serum/PBS. The sections were washed in PBS and the reaction sites visualised blue/black in colour using NBT/BCIP (as described above) overnight. The preparations were washed, counterstained with haematoxylin and mounted in glycerol.

Procedural controls included (1) pre-treatment of sections with 20 µg/ml RNase in TRIS/EDTA buffer (0.5 M sodium chloride, 10 mM TRIS hydrochloride pH 8.0, 1 mM EDTA) for 30 minutes at 37°C to remove all tissue RNA, and (2) incubation of sections with the sense (T7) probe at a concentration of 1:100 instead of the anti-sense probe.

FIGURE 1 Serial sections from a subretinal membrane seen by differential interference contrast microscopy. (a) Stained with the immunoperoxidase technique for fibronectin (no counterstain). Much of the membrane shows intense fibronectin immunoreactivity with a distinct fibrillar pattern (white arrows). (b) Labelled with the anti-sense probe for fibronectin mRNA and counterstained with haematoxylin. Many of the cells in the section show intense labelling for fibronectin mRNA (black arrows). (c) Labelled with the sense (control) probe. No labelling is seen. A group of pigmented cells is seen in the top left corner. All ×230.
labelling', Labelling between background and intense levels was called 'moderate labelling'.

Results

CONTROLS
Sections of rheumatoid synovium hybridised with the anti-sense probe showed moderate or intense labelling in the synovial lining cells whereas sections hybridised with the sense probe or pretreated with RNAse exhibited only background labelling. The synovium showed moderate or intense immunoreactivity for fibronectin while no immunolabelling occurred in the negative procedural controls. Subretinal membrane sections labelled with the sense probe showed only background labelling levels (Fig 1).

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<tr>
<th>Subretinal membrane no</th>
<th>Proportion of cells showing moderate labelling for fibronectin mRNA (%)</th>
<th>Proportion of cells showing intense labelling for fibronectin mRNA (%)</th>
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CELLS IN SUBRETINAL MEMBRANES
All six subretinal membranes contained cells which labelled with the anti-sense probe (Figs 1 and 2). There was some variation between the membrane specimens in the numbers of cells which contained fibronectin mRNA. Between 20% to 50% of cells showed levels of labelling above background with the anti-sense probe (Table 1). In four membranes between 10% to 30% of the total cells in the specimen showed intense labelling with the probe, the remaining positive cells labelling at moderate levels. Labelling was seen in both isolated cells and cells aggregated in layers in the subretinal tissue, including cells in layers at apparent surfaces (Fig 2). Moderate to strong fibronectin immunoreactivity was also observed in all the subretinal membranes (Fig 1). Enough tissue was present in the specimens to confirm that some of the cells which contained fibronectin mRNA were retinal pigment epithelial (four membranes; Fig 2) or glial (one membrane) in origin. It was not possible to detect a quantitative difference between cell types in the intensity of labelling for fibronectin mRNA.

Discussion
Our results confirm that fibronectin contributes to the extracellular matrix in the contractile subretinal membranes of PVR, demonstrate that many subretinal cells contain mRNA for the glycoprotein, and affirm that cells in subretinal membranes produce fibronectin. Moreover, the results are in keeping with the observation that fibronectin is synthesised by retinal pigment epithelium in vitro,23,24 since retinal pigment epithelial cells are a major component of subretinal membranes.1,4

The production of fibronectin by cells in subretinal membranes represents a marked behavioural change from the activities seen in the cells in situ in attached and even detached adult human retina, since retinal pigment epithelial and glial cells in situ lack appreciable fibronectin immunoreactivity and mRNA.1 This 'up regulation' of fibronectin production by retinal pigment epithelial and glial cells in subretinal membranes may be caused by one of several mechanisms. One possibility is that inflammatory mediators within the vitreous and subretinal fluids of eyes developing PVR subretinal membranes, or factors derived from inflammatory cells in evolving membranes, may stimulate fibronectin production. It has been shown that hepatocytes manufacture fibronectin in the presence of interleukin-6.25 However, the apparent absence of fibronectin production in the adjacent

Figure 2. Differential interference contrast micrographs of sections from a subretinal membrane. (a) Stained by the immunoperoxidase technique (no counterstain) for glial fibrillary acidic protein reveals a small glial component in the membrane (open arrows). Pigment is present in the tissue (asterisk). (b) Stained by the immunoperoxidase technique (no counterstain) for cytokeratins shows prominent retinal pigment epithelial layers (arrows). (c) Labelled with the antisense probe for fibronectin mRNA and counterstained with haematoxylin. Many of the pigment epithelial cells show moderate or intense labelling for fibronectin message (arrows). (d) Labelled with the sense (control) probe and counterstained with haematoxylin. No labelling is seen. All ×290.
neuroretina and retinal pigment epithelial monolayer is counter to this theory. Alternatively, the observation of fibronectin synthesis in the subretinal, as well as the epiretinal, component of PVR is in keeping with the theory that it is the displacement of retinal pigment epithelial and glial cells from their normal location which induces, or removes constraints from, the cells to produce fibronectin.

Some of the fibronectin in subretinal membranes may also be derived from plasma fibronectin, since fibronectin enters the vitreous and subretinal space following breakdown of the blood retina barrier in retinal detachment and PVR.26-28 The developing subretinal 'scars' may then incorporate the plasma-derived fibronectin. Indeed, moderate to strong fibronectin immunoreactivity was observed in all the subretinal membranes studied irrespective of the proportion of cells expressing fibronectin mRNA. However, much of the fibronectin content of the membranes is likely to emanate from the subretinal cells themselves since cell surface fibronectin is thought to be insoluble and therefore is likely to accumulate in the tissue. Thus both plasma and locally-produced fibronectin probably contribute to the tissue.

The accumulation of both plasma and locally-produced fibronectin has been described in reparative processes elsewhere in the body and in this respect subretinal membrane formation may be likened to an evolving scar. However, in other respects subretinal membranes differ from healing wounds – for example, the absence of a prominent vascular component,4 and it remains to be seen whether the fibronectin is as important in subretinal membrane formation as it is wound healing in general.

The production of fibronectin by subretinal membrane cells does not imply that fibronectin is important in the pathobiology of the tissue per se. However, the abundance of message and cell surface glycoprotein in some subretinal membranes and the recent demonstration of fibronectin receptors on cells displaced into PVR epiretinal membranes15 is in keeping with the concept that the glycoprotein plays a role in subretinal membrane development.

In early PVR, plasma-derived fibronectin is likely to be involved in chemotactic recruitment of cells into the developing subretinal tissue. As the subretinal membrane cells themselves begin to produce fibronectin, the insoluble cell surface glycoprotein may contribute to the cohesion of the tissue and potentiate the chemotactic stimulus in the membrane. Indeed, work in our laboratory suggests that retinal pigment epithelial cells may be migrating into already well established subretinal membranes.29,30 Migrating cells may not only be attracted into developing subretinal membranes by fibronectin but could specifically be producing the glycoprotein since migratory cells in other pathological tissues can produce fibronectin.31 Interestingly, fibronectin mRNA was particularly noted in cells at apparent surfaces of the subretinal membranes where migratory cells may be expected. If migrating cells produce fibronectin in evolving membranes, it is possible that a single agent could prevent both recruitment of cells into and cohesion within developing membranes and provide a new avenue of therapeutic intervention in the process.

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27 Campochiaro PA, Jordon JA, Glaser BM. Vitreous aspirates from patients with proliferative vitreoretinopathy stimulate


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