THROMBOSPONDIN AS A COMPONENT OF THE EXTRACELLULAR MATRIX OF EPIRETINAL MEMBRANES: COMPARISONS WITH CELLULAR FIBRONECTIN

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SUMMARY

We compared the distribution of the adhesive extracellular matrix glycoproteins thrombospondin and cellular fibronectin in epiretinal membranes. A total of nine membranes were investigated with immunohistochemical techniques. Thrombospondin and cellular fibronectin immunoreactivity were observed in seven of the specimens and immunostaining for the two glycoproteins was co-localised in four of the membranes. The findings indicate that thrombospondin contributes to the extracellular glycoprotein content of epiretinal membranes and is frequently co-distributed with cellular fibronectin in the tissue. As a consequence, thrombospondin may play a role in the assembly of the extracellular matrix of epiretinal membranes.

Epiretinal membranes are scar-like proliferations which occur on the surface of the retina as a complication of a variety of conditions including retinal detachment (when epiretinal membranes form part of the spectrum of proliferative vitreoretinopathy, PVR)[1] and proliferative diabetic retinopathy (PDR). PDR membranes usually contain a prominent vascular component whereas the membranes of PVR are generally avascular.[2–4] Both avascular and vascularised epiretinal membranes are thought to cause tractional retinal detachment by virtue of cell-mediated contraction[5] and both types of membrane become successively more fibrous with increasing clinical duration.[6,7] The progressive fibrosis has been viewed as the final stage in the natural history of membrane formation, may result in the membrane being fixed in a contracted state and is due to the deposition of collagen (notably types I and III to V) in the extracellular matrix (ECM) of the tissue.[6,8–13]

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In the last decade it has become apparent that the ECM of epiretinal membranes does not consist solely of collagens but also contains a variety of other proteins including the ECM glycoproteins. The first of the ECM glycoproteins reported in epiretinal membranes was laminin,[14] which was described as a low solubility ECM basement membrane glycoprotein.[15] Subsequently, the ECM glycoproteins fibronectin and vitronectin were reported in epiretinal membranes and, more recently, thrombospondin.[7,16,17] ECM glycoproteins raise interest because of their multiple biological roles.[18–21] The functions of ECM glycoproteins, which include cell–cell and cell–substratum adhesion, are largely mediated by a family of heterodimeric cell surface receptors known as integrins (reviewed by Hynes[22] and Ruoslahti[23]). Integrins have been reported in epiretinal membranes and the integrins expressed by epiretinal cells include receptors for fibronectin and vitronectin.[24] The vitronectin receptor ($\alpha_v\beta_3$) is thought to bind a number of ligands such as vitronectin itself, fibronectin and thrombospondin (see review by Hogg[25]).

Thrombospondin is a glycoprotein which was first identified in platelets, from which it is released following exposure of the platelets to thrombin.[26] It is now recognised as an important ECM glycoprotein consisting of three polypeptide chains with a total molecular weight of about 450 000.[19,27] Thrombospondin is thought to bind to fibronectin fibrils secreted by cells (cellular fibronectin), thus contributing to the assembly of ECM in general[19] and, perhaps, to the ECM of epiretinal membranes. In view of the possible importance of interactions between thrombospondin and cellular fibronectin in the assembly of the ECM in epiretinal membranes, we compared the distribution of thrombospondin and cellular fibronectin immunoreactivity in surgically excised epiretinal membranes.

MATERIALS AND METHODS

Controls

Blood vessel lumina in sections of human retina were used as the positive control for thrombospondin. Monolayers of cells, including scleral fibroblasts, grown in tissue culture provided positive controls for cellular fibronectin.[28]

Epiretinal Membranes

Nine epiretinal membrane specimens were obtained during closed microsurgery for PVR (5 eyes), PDR (3 eyes) or complications of central retinal vein occlusion (1 eye). An estimate of the clinical duration was recorded for each specimen.

Immunohistochemistry for Thrombospondin and Cellular Fibronectin

Fragments of retinal tissue and the epiretinal membrane specimens were placed in OCT embedding medium, frozen in liquid nitrogen and 7 μm thick sections prepared as previously described.[29] Monolayers of cells were thoroughly washed with phosphate-buffered saline (PBS, pH 7.5) to remove culture medium. The washed cultures were fixed in methanol and acetone (2 minutes each) at −20 °C. the sections and monolayers were stained using an indirect immunofluorescent technique as described previously.[29] The primary antibodies used were: (1) monoclonal anti-human thrombospondin (Sigma, Poole, Dorset) and (2) monoclonal anti-cellular fibronectin (which recognises the 240 kDa component of human cellular fibronectin; Sigma). Both antibodies were used at a dilution of 1 : 1000 in 1% normal goat serum PBS. Controls included the replacement of primary antibody with normal serum or an irrelevant antibody.[29] In addition, some sections were stained with haematoxylin and eosin. The preparations were examined by bright field, differential interference contrast and epifluorescence microscopy. The presence or absence of immunoreactivity was recorded and a comparison made between the distribution of thrombospondin and cellular fibronectin immunostaining in the specimens.

RESULTS

Thrombospondin or cellular fibronectin immunoreactivity

Table I. Immunoreactivity of nine epiretinal membranes to thrombospondin and cellular fibronectin

Membrane number	Clinical duration (months)	Aetiology	TSP	cFN	Co-localisation of TSP/cFN
1	<2	RRD	+	+	Yes
2	<2	RRD	+	+	Yes
3	<4	DR	+	+	Yes
4	<4	RRD	+	+	Yes
5	<4	RRD	+	+	No
6	<4	RRD	−	−	−
7	<4	CRVO	−	−	−
8	>4	DR	+	+	No
9	>9	DR	+	+	No

Key: **RRD, rhegmatogenous retinal detachment; DR, diabetic retinopathy; CRVO, central retinal vein occlusion; TSP, thrombospondin; cFN, cellular fibronectin; +, positive staining; −, negative staining.**

was observed in the lumina of retinal (and membrane specimen) vessels and in the pericellular matrix of the cell monolayers respectively. No immunostaining occurred in specimens processed without primary antibody.

The results of the investigation concerning epiretinal membranes are summarised in Table I. Overall, seven of the epiretinal membranes showed immunoreactivity for thrombospondin and the same seven also immunostained for cellular fibronectin. These included two membranes of more than 4 months clinical duration (Table I). The glycoproteins were observed in cellular and fibrous areas of the specimens. The distribution of the glycoproteins varied within and between membranes. Immunoreactivity for thrombospondin and cellular fibronectin was co-distributed in four specimens and all of these membranes were of less than 4 months clinical duration (Table I, Fig. 1). In three membranes, including two of more than 4 months clinical duration, thrombospondin and cellular fibronectin immunolabelling did not co-localise (Table I, Fig. 2). Two epiretinal membranes, one PVR membrane and a membrane arising after a central retinal vein occlusion, did not immunostain for either of the two ECM glycoproteins under investigation (Table I). The number of specimens

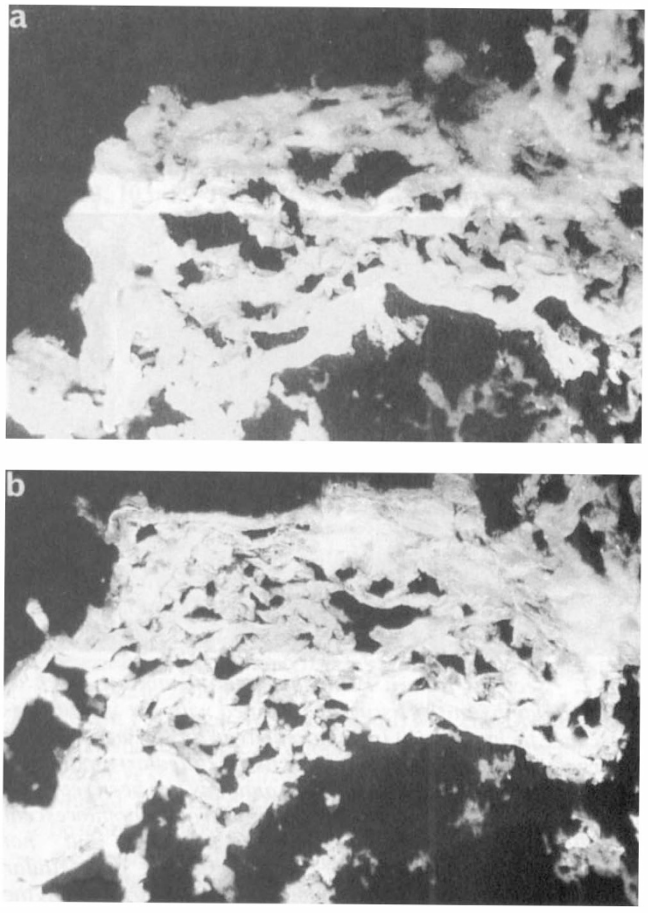

Fig. 1. *Epifluorescence micrographs of two sections from a PVR epiretinal membrane immunostained for (a) thrombospondin and (b) cellular fibronectin. Immunoreactivity for the two ECM glycoproteins co-localises in the specimen and is shown here in a predominantly fibrous portion of the membrane. (Both ×200.)*

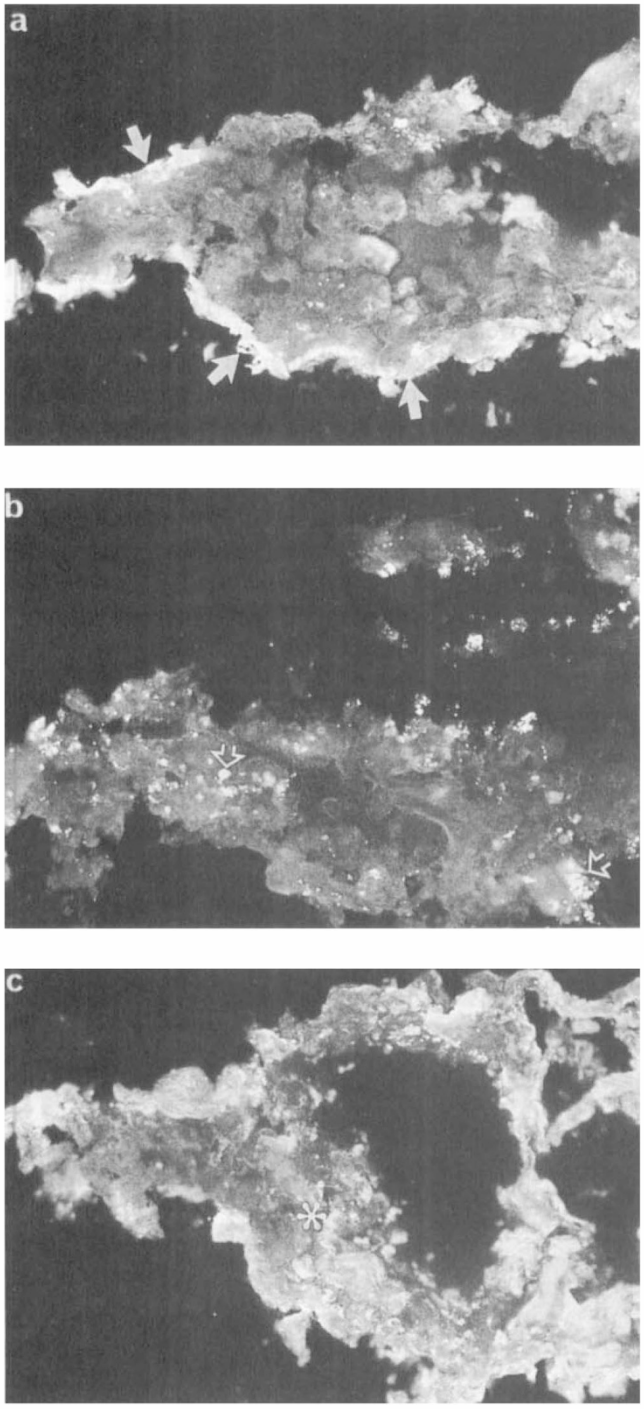

Fig. 2. *Sections from a PDR epiretinal membrane taken at different depths in the tissue and stained with the immunofluorescence technique. (a) Immunoreactivity for thrombospondin is largely restricted to layers in the tissue (filled arrows). (b) A control section in which the primary antibody has been replaced by an inappropriate antibody reveals numerous autofluorescent granules in the specimen (open arrows) but not immunolabelling. (c) Another section stained for cellular fibronectin discloses patchy immunostaining throughout the section (asterisk) and that the distribution of cellular fibronectin differs from that of thrombospondin. (All ×200).*

was too small to detect a difference between membrane aetiology or clinical duration and the presence of thrombospondin or cellular fibronectin.

DISCUSSION

Our results confirm that thrombospondin is present in the ECM of epiretinal membranes and demonstrate that thrombospondin is frequently co-localised with cellular fibronectin in the tissue. Since the fibronectin antibody used in this investigation recognises cellular but not plasma fibronectin, immunoreactivity for this antibody in the epiretinal membranes adds further support to the concept that at least some of the fibronectin present in the membranes is produced by the epiretinal cells themselves.[7,30] The epiretinal cells may also be responsible for some of the thrombospondin in the tissue since the glycoprotein is synthesised by a variety of cells including vascular endothelium, fibroblasts, smooth muscle cells, alveolar epithelium, monocytes and macrophages (reviewed by Lawler[19]). Macrophages and fibroblast-like cells are components of epiretinal membranes[31] and could therefore produce thrombospondin in the tissue.

Conversely, it is possible that epiretinal thrombospondin is derived from the plasma since low levels of thrombospondin are present there.[32,33] Plasma thrombospondin could reach the vitreous cavity and become incorporated in developing epiretinal membranes in the same way as plasma fibronectin is thought to contribute to some epiretinal membranes; for example, where a retinal detachment or detachment surgery leads to a breakdown of the blood–retina barrier.[34,35] Moreover, whole blood sometimes enters the vitreous cavity during retinal detachment – an event which is a risk factor for PVR development[36]– culminating in the periretinal release of platelet thrombospondin.

Thrombospondin accumulating in the ECM of early epiretinal membranes is likely to influence profoundly the behaviour of the epiretinal cells, since, like fibronectin and laminin, it is recognised by the integrin family of cell surface receptors which mediate cell–ECM attachment and modulate various cell activities.[22,23] Cellular migration is one activity which is influenced by ECM–integrin interactions and which is important in epiretinal membrane development.[37] Interestingly, thrombospondin has been linked to cell migration in other tissues. For example, bovine corneal endothelial cells deposit thrombospondin in their migration tracks and this is thought to play a role in the migration of the endothelial cells on basement membrane material.[38] Perhaps thrombospondin is involved in the migration of epiretinal cells on the inner limiting lamina of the retina in early epiretinal membrane formation.

Thrombospondin is also thought to play a role in the assembly of the ECM by binding to a variety of ECM macromolecules such as fibrinogen and fibronectin.[39,40] Thus the co-distribution of cellular fibronectin and thrombospondin in many of the specimens of less than 4 months clinical duration might be of functional importance. Moreover, the multifunctional nature of these ECM glycoproteins suggests that the ECM, far from merely consolidating membrane contraction at an end-stage in the disease, is actively involved in early membrane development and represents a potential therapeutic target in the management of epiretinal membrane formation.

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