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Immunohistochemical study of extracellular matrix components in epiretinal membranes of vitreoproliferative retinopathy and proliferative diabetic retinopathy

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PURPOSE. The migration, proliferation, differentiation, and adhesion of cells and other cellular functions are influenced by the surrounding extracellular matrix in normal and wound healing conditions. The formation of epiretinal membranes, a wound healing process, is a serious complication of retinal diseases, the most important being proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR). In the present study, the authors investigated the expression of various extracellular matrix components and in particular tenascin, fibronectin, laminin, collagen IV, and MMP-3 glycoprotein as well as the expression of glial fibrillary acidic protein in each type of epithelial membrane in order to elucidate the role of these molecules in the formation of these two types of membranes.

METHODS. The authors performed immunohistochemistry in 14 PVR and 14 PDR membranes, using antibodies against the above mentioned extracellular matrix components. Tenascin and fibronectin were observed as major components in the extracellular matrix, while laminin and collagen type IV were detected as minor components in both types of membranes. A higher fibronectin expression in PVR compared with PDR membranes was found ($p=0.0035$). A positive relationship of its expression with the proliferative activity ($p=0.15$) and collagen type IV expression ($p<0.0001$) was also observed.

RESULTS. Tenascin expression was positively correlated with glial fibrillary acidic protein positive cells in PDR membranes ($p=0.04$). Collagen type IV localized around vessels was observed with high levels in PDR membranes ($p=0.0031$).

CONCLUSIONS. The results indicated that the extracellular matrix components seem to be involved in PVR and PDR, contributing to tissue remodeling and perhaps by different pathogenetic pathways, which could reflect different stages of development in these two types of membranes. (Eur J Ophthalmol 2005; 15: 384-91)

KEY WORDS. Tenascin, Fibronectin, Collagen type IV, Laminin, MMP-3, Glial fibrillary acidic protein, Epiretinal membranes

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INTRODUCTION

Epiretinal membranes (ERMs) are pathologic contractile membranes on the surface of the retina that can be either asymptomatic or cause a spectrum of symptoms, from mild distortion and metamorphopsia to visual loss. They grow on the inner retinal surface, as a result of many conditions (1), such as inflammatory eye disease, ischemic retinopathy, proliferative retinopathy, or nonproliferative retinal vascular disorder, or following blunt or penetrating injuries, vitreous hemorrhage, retinal photocoagulation, or cryotherapy. According to the presence or absence of vascular endothelial cells, they are divided into vascular, associated with ischemic retinopathies, and avascular, which are observed in cases of idiopathic retinopathies or in complicated retinal detachment and penetrating ocular trauma cases. This vitreoretinopathy is a serious human intraocular disorder characterized by fibrocellular sheets of connective tissue proliferating on both retinal surfaces, which causes structural and functional damage on the retina. The fibrocellular sheets (ERMs) are composed of different cell types, essentially retinal pigment epithelial cells, glial cells, fibroblasts, macrophages, and myofibroblast-like cells surrounded by a fibrous matrix with an extensive amount of collagen (2, 3). Pathogenesis is poorly understood, especially the mechanisms of cellular migration, adhesion, and proliferation. Previous studies have suggested the participation of growth factors (4, 5) and serum proteins (6-8). The contribution of extracellular matrix components in the formation of ERMs has been studied (8-13). The matrix metalloproteinases (MMPs) are a family of enzymes that degrade and remodel the extracellular matrix and, thus, play a central role in the wound-healing process. Several reports point out that MMPs may be involved in the pathogenesis of proliferative diabetic retinopathy (PDR) and other vitreoretinal (14-19), wound-healing processes in the retina.

The aim of the present study was to examine the distribution and the possible role of some adhesion multifunctional glycoproteins of the extracellular matrix such as tenascin, fibronectin (FN), tenascin, collagen type IV (CIV), laminin (LN), and MMPs in view of the potential modulating effects of matrix remodeling in ERMs from patients who underwent vitrectomy due to proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR). In addition, these expressions were correlated with the microvessel density (MVD), the glial fibrillary acidic protein (GFAP) positive cells (astrocytes), and the proliferative activity as estimated with the proliferative associated index Ki-67, in order to clarify the potential role of these extracellular molecules in the development of these types of membrane.

MATERIALS AND METHODS

Patients

In this study, 28 ERMs that were surgically removed during vitrectomy were studied. Fourteen were from patients with PDR followed by vitreous hemorrhage and tractional retinal detachment and 14 were from patients with PVR. The surgical procedure included three-port pars plana vitrectomy, removal of posterior hyaloid face, and subsequent removal of the ERMs with vitreous picks and forceps. Immediately after excision the membranes were placed for fixation in 10% formaldehyde solution.

Immunohistochemistry

Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the labeled streptavidin avidin biotin (LSAB) method. The time from the fixation of the specimens to embedding is about 3 to 4 days. In brief, tissue sections were deparaffinized in xylene and dehydrated. They were immersed in citrate buffer (0.1 m, pH=0.6) and subjected to microwave irradiation twice for 15 min. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. All dilutions were made in TBS-1% BSA solution and followed by overnight incubation. The antibodies sources and dilutions are shown in Table I. After washing in TBS, the paraffin sections were incubated for 30 minutes with a rabbit antimouse and for another 30 minutes with mouse antirabbit peroxidase conjugate. Between each conjugation, sections were washed for 10 minutes with TRIS buffer. After final washing with TBS, a diaminobenzidine (DAB) containing 0.05% H2O2 solution was used to visualize the immunoreactivity. All secondary reagents were purchased from Dako.

Immunohistochemical evaluation

Fibrovascular tissues were classified as positive with regard to immunoreactivity for tenascin, FN, MMPs, CIV,

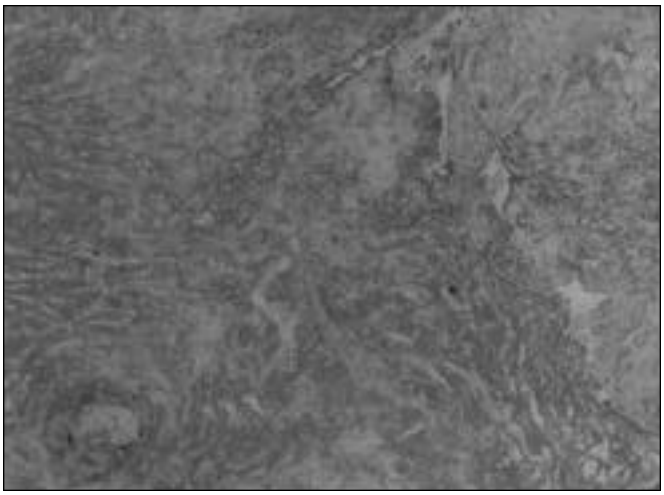

Fig. 1 - Proliferative vitreoretinopathy membrane displaying strong tenascin immunostaining in the extracellular space (original magnification, LSAB x200). LSAB= Labeled streptavidin avidin biotin.

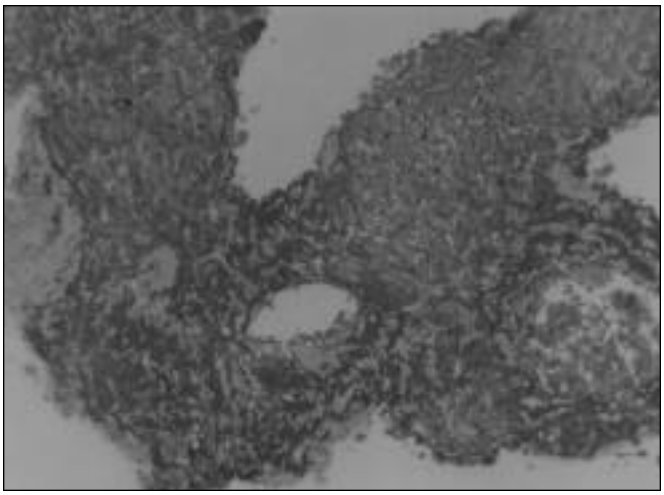

Fig. 2 - Strong and diffuse fibronectin immunostaining in the stroma of a case of proliferative vitreoretinopathy membrane (original magnification, LSAB x200). LSAB= Labeled streptavidin avidin biotin.

and LN when there was equivocal immunostaining of the matrix components in at least one representative area of the membrane. Analysis of staining was mainly restricted to stromal reactions. The extent and intensity of these proteins' expression was scored semiquantitatively corresponding to negative (-), weak to moderate (+), and strong (++) immunoreactivity according to the previous study (13). The thickness of the sections was the same and the specificity of antigen binding was controlled using positive and negative controls. The negative controls were included to rule out nonspecific binding of antibodies; tissue sections were also subjected to the whole procedure, but omitting incubation with the primary antibody.

The pattern of staining was also estimated. GFAP (a glial specific protein) used as a glial cell marker, especially for astrocytes. GFAP-positive cells formed a monolayer on one side of the membrane or in to a lesser degree GFAP positive cells were observed singly in the fibrous stroma. The nuclear antigen Ki-67, a marker of proliferation, was evaluated semiquantitatively and scored as +, ++, and +++, corresponding to small, moderate, and high amounts of positive cells.

Immunohistochemical staining for CD34, which stained stromal vascular endothelial cells, was conducted to evaluate the microvascular density (MVD). Individual microvessel counts were then made on x400 power magnification. Results were expressed as the mean MVD per field for the three hot spots areas. The specimens that were too small, and provided insufficient immunohistochemical results, were not included in the study. The immunoreactivity was interpreted by means of light microscopic examination and evaluated independently by two

TABLE I - ANTIBODIES USED

Antibodies	Supplier	Dilution	Incubation time, hr
Tenascin (TN2, M0636)*	Dako	1:50	1
Fibronectin (NCL, clone, 568)*	Novocastra	1:100	1
Collagen type IV (clone, CIV22)*	Dako	1:50	1
Laminin (clone, 4C7)*	Menarin	1:1000	1
MMP-3†	Oncogene	1:200	1
FGFAP	Dako	1:50	1
CD34	Novocastra	1:50	1
Ki-67 (M0722)†	Dako	1:10	1

†With microwave oven antigen retrieval; *Incubation with pronase; MMP-3 = Matrix metalloproteinase 3; GFAP = Glial fibrillary acidic protein

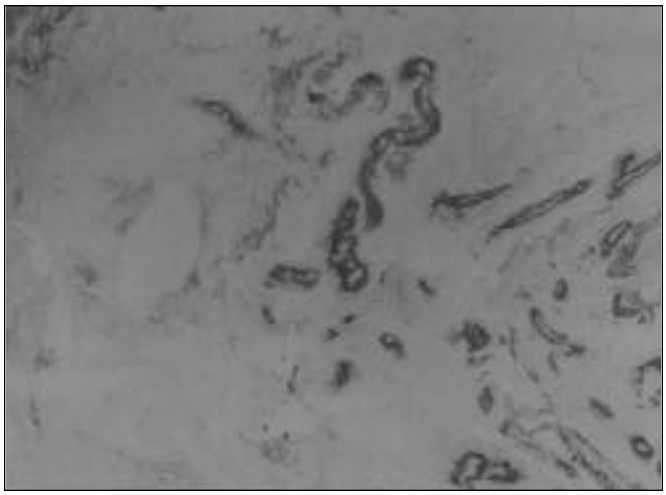

Fig. 3 - Collagen type IV-defined basement membranes seen around vessels and in the stroma (upper left) in small amount, in a case of proliferative diabetic retinopathy membrane (original magnification, LSAB x200). LSAB= Labeled streptavidin avidin biotin.

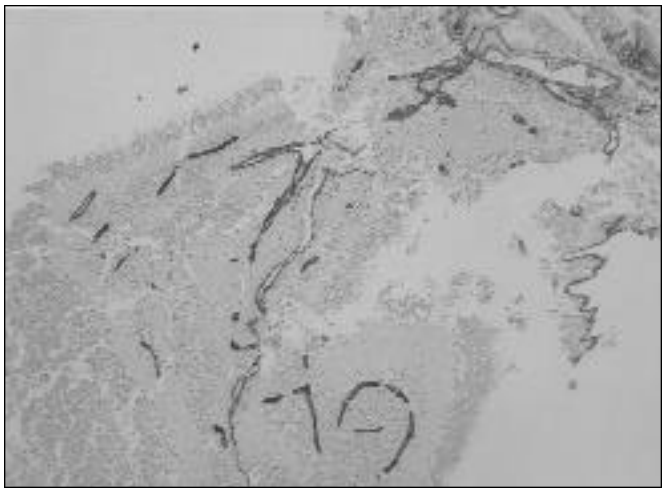

Fig. 4 - Laminin expression mainly around vessels in a proliferative diabetic retinopathy membrane (original magnification, LSAB X 200). LSAB= Labeled streptavidin avidin biotin.

pathologists (E.I., E.T.) Differences in interpretation were reconciled by re-review of slides separately or jointly at a double-headed microscope.

Statistical analysis

Superior Performance Software System (SPSS) software 10.0 for windows (SPSS Inc., 1989-1999) was used by the authors to compare morphologic features and protein expression data. Significant differences between the expression of the target proteins with regard to clinicopathologic features were computed by the t-test for paired or non-paired values or analysis of variance test if the data were normally distributed. If the data did not show a normal distribution, differences were analyzed by the Wilcoxon signed rank test for paired values or the Mann-Whitney U test and the Kruskal-Wallis H test for independent values. Correlation between these proteins was computed using Pearson's correlation coefficient for normally distributed data or Kendall's tau rank correlation coefficient where the data did not show a normal distribution. p Values < 0.05 were considered statistically significant.

RESULTS

Tenascin expression

Tenascin showed a diffuse stromal distribution. In PVR membranes this expression was relatively increased in

TABLE II - ECM COMPONENTS EXPRESSION IN PDR AND PVR MEMBRANE

	Type of membrane		
	PDR	**PVR**	**p value**
Tenascin			
0	—	1/14	—
+1	3/14	7/14	
+2	5/14	6/14	NS
+3	6/14		
Fibronectin			
0			—
+1	6/12	4/11	
+2	4/12	7/11	NS
+3	2/12		—
Laminin			
0	1/9	1/14	
+1	6/9	8/14	0.0035
+2	1/9	3/14	
+3	1/9	2/14	
Collagen IV			
0	6/9	2/8	—
+1		3/8	0.0031
+2	1/9	3/8	—
+3			
2/9			

ECM = Extracellular matrix; PDR = Proliferative diabetic retinopathy; PVR = Proliferative vitreoretinopathy

comparison to PDR membranes, an observation which proved to be of no statistical significance (Fig. 1). In detail, weak TN expression was detected in 1/14 (7.1%) cases, moderate in 7/14 (50%) cases, and extensive in 6/14 (42.9%) cases of the PVR membranes while the respective cases for PDR membranes were 3/14 (21.4%), 5/14 (35.7%), and 6/14 (42.9%) (Tab. II). A positive relationship of TN expression with the proliferative associated index Ki-67 ($p=0.015$) and CIV expression ($p<0.0001$) in PVR membranes was observed. This expression was also positively correlated with GFAP positive cells in PDR membranes ($p=0.04$).

Fibronectin expression

Microscopy revealed two patterns of FN expression. One concentrated around vessels and basic membranes and another showed stromal diffuse distribution (Fig. 2). In PVR membranes there was moderate FN expression in 4/11 (36.4%) cases and strong FN expression in 7/11 (63.6%) cases, while in PDR membranes there were 6/12 (50%) cases of weak FN expression, 4/12 (33.3%) cases of moderate, and 2/12 (16.7%) cases of strong FN expression. The above demonstrated a statistically significantly ($p=0.0035$) stronger expression of FN in PVR rather than PDR expression (Tab. II).

Collagen IV expression

The expression of CIV was detected in two patterns, around vessels and in the stroma (Fig. 3). The first pattern gave results of statistical significance ($p=0.0031$) with the expression of CIV being dominant in PDR membranes. In detail, in PVR membranes, 2/8 (25%) cases showed weak, 3/8 (37.5%) cases moderate, and 3/8 (37.5) cases strong expression, while in PDR membranes 2/9 (22.2%) cases showed weak, 6/9 (66.7%) moderate, and 1/9 (11.1%) cases showed strong expression of CIV (Tab. II).

Laminin expression

Several cases showing weak LN expression, but with no statistical significance, were observed in both PVR and PDR membranes. LN was also expressed in two patterns: around vessels and in the stroma. In both patterns the basic membrane was stained (Fig. 4). No expression in 1/14 (7.1%) cases, weak in 8/14 (57.1%) cases, moderate in 3/14 (21.4%) cases, and strong in 2/14 (14.3%) cases of PVR membranes and 1/9 (11.1%) cases of no expression, 6/9 (66.7%) cases of weak expression, 1/9 (11.1%) cases of moderate expression, and 1/9 (11.1%) cases of strong expression in PDR membranes sums the results of LN expression (Tab. II).

MMP-3 expression

Although MMP-3 expression was not characterized by intensity in staining, results between PDR and PVR membranes revealed no statistical significance. In detail, 6/21 (29%) cases showed no expression of MMP-3 and 4/21 (19%) showed weak expression of MMP-3 in PVR membranes, while the respective cases for PDR membranes were 8/21 (38%) cases of no expression and 3/21 (14%) cases of weak expression.

DISCUSSION

Significant progress has been made in elucidating physiopathologic mechanisms of ERM, although many fundamental questions remain unanswered. The molecular mechanisms of ERM formation are not fully understood; however, it has become clear that stromal cells and their products play a significant role in their pathogenesis and further evolution. The process of membrane formation requires complex changes in the normal cell to cell and cell to matrix interactions, which in turn are reflected in variable up- and downregulation of significant molecules.

Tenascin-C (TN) is an extracellular relative large matrix glycoprotein consisting of a pair of trimeric segments modulating adhesion of cells to FN and can be classified as an anti-adhesive or adhesion-modulating extracellular matrix protein (20-22). Its molecular structure is unique in that it contains domains homologous to epidermal growth factor FN and fibrinogen (23). However, this clone of tenascin (TN2) does not cross-react with these proteins. To our knowledge there are limited reports concerning TN expression in ERM (10). Tenascin has been found to be expressed in cornea (24) and in the limbal area of fetal and adult eyes (25). It has been reported that TN antagonizes the attachment of some cells to FN, by weakening the attachments of ERM cells to underlying retinal tissue and thus facilitating the growth of the membrane (10). In the current study we found no statistically significant differences in TN expression in PVR and PDR. In vitro, TN participates in the control of cell proliferation and migra-

tion, and it has been found that TN expression was correlated with the proliferative index Ki-67 in intraductal breast carcinoma (26). Moreover, it is known that the ECM acts as a reservoir for a number of growth factors and their binding proteins, which are selectively accumulated and released (27). It has also been suggested that growth factors in the ECM are particularly active when complexed with other ECM molecules, this interaction often being essential to their activity (28). The results of our study showed a positive relationship of TN expression with the proliferative activity as estimated with the proliferative index Ki-67 in PVR membranes. In addition, this expression was also correlated with the expression of CIV in this type of membrane. Taking these results together, we can speculate that although TN expression takes place in both PVR and PDR, there are different phases of cell adhesion and interactions with extracellular matrix proteins in initiating and controlling these two types of membranes.

FN belongs to a class of high molecular weight proteins (440 KD), is the cellular type of FN, which stimulates migration of fibroblasts toward the wound-chemotactic recruitment (29). Thereafter FN is secreted by the fibroblasts themselves and produces a fibrillar extracellular matrix that has important relationships across all membranes (30).

FN was found to be a significant component of ERMs and the local production of FN occurs at the early stage of development of membranes (9, 11, 12, 31, 32). The source of FN in ERMs is unclear, although many of the constituent cell types (macrophages, glial cells, epithelial cells, and fibroblasts) are known to be capable of FN synthesis (33).

Previous studies showed that FN is expressed in both PVR (11) and PDR (12) membranes and not in healthy tissue. Our study provides additional information that this expression is higher in PVR compared to PDR membranes. We attribute this finding to higher concentration of stromal cells that produce FN in PVR membranes rather than PDR, because the latter are mainly vascular. It has been reported that FN appears to control endothelial cell growth by direct and active chemical signaling pathways (34). Moreover, in vivo experiments showed that FN, but not LN (which is thought to serve as a marker of endothelial differentiation), is expressed in the zone of vasculogenesis immediately prior to vessel formation. In addition, it has been indicated that astrocytes influence the FN component of the extracellular matrix during retinal vasculogenesis and that expression of FN precedes that of LN in this process (35, 36). It was also found that LN was observed as a minor component in extracellular matrix and LN, like FN, seems to have a key role in the structural arrangement of newly formed capillaries in PDR, and that receptor expression could be involved in events of endothelial cell adhesion and proliferation (12). In the present study, we found no relationship of FN or LN expression with the vascularity or the GFAP positive (astrocytes) cells. We speculate that these processes take place at the early stage of vasculogenesis and the development of membranes.

In the present study we used the monoclonal mouse antihuman 4C7 antibody, which recognizes the A-chain of human LN and CIV22 monoclonal mouse antibody, which is directed against CIV. LM and CIV constitute the major intrinsic components of basement membranes (BMs) and are involved in cellular adhesion to BMs and the extracellular matrix. LM promotes epithelial cell adhesion to collagen (37) and has the ability to convert embryonic mesenchymal cells into polarized-shaped form (38), especially the type of laminin A chain.

It has been found that both PDR and PVR membranes express CIV and LN in the stromal and in a perivascular distribution (9, 11, 12, 14, 31). The results of the current study showed in addition that no statistically significant differences of their expression exist between the two types of membranes. CIV expression was detected in two expression patterns: stromal and perivascular. In the first pattern no statistically significant difference of its expression between the two types of membranes was observed. However, the second pattern of CIV expression was mainly observed in PDR membranes. This finding is in accordance with previous observations, indicating that the BM of newly developed vessels in ERM is similar to the normal BM of normal vessels (9, 14, 32).

The MMPs are a family of enzymes that degrade and remodel the extracellular matrix and, thus, play a central role in the wound-healing process. Proliferative retinopathy is considered a would-healing process in the retina and the role of MMPs in its pathobiology is unknown. Several studies have reported that unlike normal presence of MMPs (MMP-1, MMP-2, MMP-3, or MMP-9) in both PVR and PDR may contribute in the process of matrix remodeling according to our results (16-19) MMP-3 (stromelysin-1) is capable of degrading various connective tissue matrix macromolecules including cartilage proteoglycan, FN, CIV, and LN (39, 40). However, we found no significant correlations of MMP-3 with the other extra-

cellular matrix proteins or with the tissue vascularity, indicating probably different pathways involving the regulation of these molecules.

In conclusion, our data indicate that the contribution of extracellular matrix components in the development of PDR and PVR may be by different pathogenetic pathways in tissue remodeling and progression in both types of membranes. These differences perhaps reflect different stages of tissue expansion.

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