The Role of Fibronectin, Laminin, Vitronectin and Their Receptors on Cellular Adhesion in Proliferative Vitreoretinopathy

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Purpose. To examine the possible role of some adhesion multifunctional glycoproteins of the extracellular matrix, such as fibronectin (FN), laminin (LN), vitronectin (VN) and their receptors (β1-subunit complex and αvβ3 integrins) in events of cell migration and adhesion in proliferative vitreoretinopathy (PVR).

Methods. Optical and electron-immunocytochemical techniques were carried out on epiretinal membranes. Electrophoretic immunoblotting methods and densitometric analysis of normal and PVR vitreous were also undertaken. Chi-square (χ²) and unbalanced analysis of variance were employed for statistical analysis.

Results. FN was detected as a major component in the extracellular matrix in both fibrillar and pericellular arrangement. A change in pericellular distribution to more fibrillous organization was related to the time of intraocular proliferative tissue development (P < 0.001). LN and VN were observed as minor components in extracellular matrix. A colocalized pattern between VN and FN in collagenic bundles of the matrix was often observed. Beta-1 subunit and αvβ3 receptors were usually localized in a position that could mediate the interaction of FN, VN, and/or LN to the cell plasma membrane. Increased levels of FN concentration were observed in both subretinal fluid and pathologic vitreous; intravitreal FN concentration tends to increase with clinical stages of the evolution of PVR, whereas intravitreal VN levels tend to decrease.

Conclusions. Results suggest that FN could mediate the initial events involved in epiretinal membrane formation, and VN could modulate the adhesion mechanisms in established membranes. Invest Ophthalmol Vis Sci. 1994;35:2791–2803.

The biologic activities of the extracellular matrix (ECM) reside both in its special components and in its structural integrity. Most ECM contain a fibrous collagenic network or other fiber-forming proteins, such as glycoproteins. Noncollagenous extracellular glycoproteins have important roles in many cell-surface interactions, such as adhesion, migration, phenotype differentiation and polarization, wound healing, tumor-cell invasion, and metastasis. For example, fibronectin (FN; MW 440 kD) is a multifunctional interactive glycoprotein found as a soluble protein and an insoluble form organized into extensive ECM, produced and secreted by a wide variety of cell types. Laminin (LN; MW 850 kD) is a major component of basement membranes and possesses multiple functional sites that mediate its interactions with cells and other ECM elements. Finally, vitronectin (VN; MW 65 to 78 kD) is present in fibrillar pattern in the ECM of a variety of tissues and also in circulation. The regulation of ECM assembly and cellular response often requires adhesion and specific interactions of a cell with its substrate. These interactions involve specific cell-surface proteins that bind adhesive ligands of the ECM recognized by both Arg-Gly-Asp (RGD) peptide cell-binding sequences and non-RGD cell-binding sequences in adhesive ECM macromolecules such as those described above. In particular, integrins—heterodimeric molecules of cell-adhesion receptors—have been implicated in a variety of cell-to-cell and cell-to-matrix interactions and
are also active in transmitting signals from the extra to the intracellular compartment.\textsuperscript{16-21} All integrins are α subunits, noncovalently associated with a β subunit, and can be expressed in a wide variety of cell types.\textsuperscript{19}

Many of the integrins that share the β₁ subunit are known to recognize FN,\textsuperscript{27,23} LN,\textsuperscript{24,25} and collagen.\textsuperscript{26} Cell attachment to VN occurs by any of several β₂ subunit associations, including the classical VN-receptor: the α₅β₁ integrin.\textsuperscript{27} These receptors also bind to FN, fibrinogen, von Willebrand factor, and collagen.\textsuperscript{19,28}

Proliferative vitreoretinopathy (PVR) is a serious human intraocular disorder characterized by fibrocellular sheets of connective tissue proliferating on both retinal surfaces, which causes structural and functional damage to the retina. PVR is most likely to develop after rhegmatogenous retinal detachment, perforating trauma of the posterior segment of the eye, and after surgery for retinal reattachment.\textsuperscript{29} These fibrocellular sheets (epiretinal membranes, ERM) are composed of different cell types, essentially retinal pigment epithelial cells, glial cells, fibroblasts, macrophages, and myofibroblast-like cells\textsuperscript{30-32} surrounded by a fibrous matrix with an extensive amount of collagen.\textsuperscript{30,35}

Pathogenesis is poorly understood, especially the mechanisms of cellular migration, adhesion, and proliferation. Previous studies have suggested the participation of growth factors\textsuperscript{34,35} and serum proteins.\textsuperscript{36-38}

In the present work, we have studied the distribution of extracellular matrix glycoproteins and their receptors in PVR. The results suggest that FN could have an important role in the earliest pre-membranogenic stages of this pathologic condition and later in the mechanisms of cell-ECM adhesion.

**MATERIAL AND METHODS**

**Human Tissue and Samples**

Human ERM (n = 54) were dissected and removed by appropriate intraocular vitreous forceps (Grieshaber, Switzerland; Cat. No. C-612.08, C-612.13, and C-612.98) from patients with retinal detachment complicated by PVR who were undergoing intraocular surgery. Specimens were immediately fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for at least 12 hours at 4°C. They were then rinsed in PBS 0.1 M pH 7.4 and immersed in 2.1 M sucrose in PBS solution for 2 to 4 hours. Part of them (n = 28) were mounted on a metal stub, rapidly frozen in liquid nitrogen and maintained at −196°C before sectioning by cryoultramicrotomy. The remainder (n = 26) were embedded in OCT (Miles, Elkhart, IN), frozen in isopentane and stored at −35°C for cryostat sections. Pathologic vitreous (n = 15) was obtained during surgery under visual control by aspirating liquefied vitreous from the center of the vitreous cavity with a tuberculin syringe before the vitrectomy infusion was opened. Subretinal fluid aspirates (n = 10) were obtained by external drainage. Samples (200 µl to 500 µl) were centrifuged (13,500g for 8 minutes at room temperature), divided into aliquots, and then stored at −35°C. Control samples (n = 9) were obtained from normal human eyes donated for corneal transplant (Eye Bank, Barraquer Ophthalmological Centre, Barcelona, Spain), as described above. ERM, subretinal fluid, and vitreous aspirates were classed into A to D according to Hilton et al classification.\textsuperscript{39} ERM were also classed arbitrarily according to the time since ERM onset: up to 2 months; 2 to 6 months; and more than 6 months. ERM onset was determined by vitreoretinal interface examination, including fundus biomicroscopy and indirect ophthalmoscopy.\textsuperscript{29}

**Antibodies**

For immunocytochemical detection of ECM glycoproteins a rabbit antiserum against human fibronectin (Dakopatts, Glostrup, Denmark) and a rabbit antiserum against murine LN (Sigma, St. Louis, MO) were used, both at a dilution of 1:200. A mouse mAb directed against human VN (mAb VN7) was a generous gift from Dr. Klaus Preissner and was used at a dilution of 1:150. A polyclonal rabbit anti-murine β₁ integrin subunit (anti-ASβ₁) was provided by Dr. Carles Enrich and his coworkers at a dilution of 1:50. The α₂β₃ mAb LM609, prepared as described,\textsuperscript{36} was a kind gift from Dr. David Cheresh and was used at a dilution of 1:30. Rhodamine (TRITC) (Dakopatts) or FITC-conjugated anti-rabbit or anti-mouse immunoglobulin antisera (Dr. David Cheresh and was used at a dilution of 1:30. Rhodamine (TRITC) (Dakopatts) or FITC-conjugated anti-rabbit or anti-mouse immunoglobulin antisera (Boehringer, Penzberg, Germany) were used for optical immunodetection at a dilution of 1:25. For electron microscopic immunocytochemistry, protein A/colloidal gold 16 nm (pA-Au 16 nm), produced in our laboratory, and rabbit anti-mouse immunoglobulin-gold (IgG-Au 10 nm) (Amersham, UK) were used at a dilution of 1:50. In several electronic immunocytochemical experiments, we used rabbit anti-mouse secondary immunoglobulin (Dakopatts) at a dilution of 1:75 to amplify the mAb detection. Both primary and secondary reagents were diluted in 1% ovalbumin in PBS-glycine 0.1 M (pH 7.4).

**Indirect Immunofluorescence Procedure**

For immunofluorescence techniques, ERM were obtained by both consecutive serial cryostat (Frigocut 2800 E, Reichert-Jung, Wein, Austria) sections (6 µm to 10 µm) and semithin sections (0.3 µm to 0.4 µm) at −70°C (Ultracut FC4D, Reichert-Jung), prepared on 0.5% gelatin-coated slides and placed in humidified chamber at 4°C before optical immunocytochemical
procedures. Indirect immunofluorescence for cryostat sections was performed following Vilaró et al. Semithin frozen sections were air-dried at room temperature, washed (3 × 5 minutes) in PBS-glycine 0.1 M, and blocked (10 minutes) in 1% ovalbumin in PBS-glycine 0.1 M solution in a humidified chamber at room temperature. Primary antibodies were incubated for 2 hours, slides were rinsed with PBS-glycine 0.1 M, and the secondary conjugated antibodies (IgG-FITC or IgG-TRITC) were applied for 1 hour at room temperature in darkness. After the last incubation, slides were washed and mounted with 70% glycerol in 5% n-propyl gallate-buffered mounting medium.

Negative control sections were prepared by omission of the primary antibodies. Immunostaining was visualized with an epifluorescence microscope (Polyvar II, Reichert-Jung).

For each specimen, 9 to 20 whole sections were studied and interpreted at low magnification (×10 to ×25) microscopy. Indirect immunofluorescence results were always visualized in association with interferential microscopy of the same field.

Electron Immunocytochemical Procedure

For electron immunocytochemical staining, consecutive serial ultrathin sections (90 nm to 0.1 μm) at −105°C were obtained by cryoultramicrotomy (Ultracut FC4D, Reichert-Jung). placed on gold grids (200 mesh) formvar-coated for TEM and maintained in PBS 0.1 M pH 7.4 at 4°C before the electron immunocytochemical study. The grids were pretreated in ammonium chloride in PBS 0.1 M solution (10 minutes) to eliminate nonspecific radicals, rinsed in PBS-glycine 0.1 M solution (4 × 2 minutes), and then blocked in 0.5% ovalbumin in PBS-glycine 0.1 M solution (10 minutes) at room temperature. Primary antibodies were incubated for 30 minutes at the dilutions mentioned above and washed in PBS-glycine 0.1 M. Secondary anti-mouse IgG-Au 10 nm or pA-Au 1(5 nm was then applied for 20 minutes. After successive washes, first in PBS 0.1 M (4 × 2 minutes) and then in double distilled water (6 × 2 minutes), grids were contrasted in 0.03% uranyl acetate solution (10 minutes), and a thin surface membrane of methyl-cellulose was applied. Double-staining procedures for VN/FN were carried out in a two-step method. First mAb VN7 was applied as described, then grids were fixed in 2% paraformaldehyde in PBS 10 mM pH 7.4 (20 minutes) and successively rinsed in PBS 10 mM. The second step was then carried out for antisera against FN. Negative control sections were prepared by omission of the primary antibodies, to observe anti-mouse IgG-Au specificity and pA-Au affinity for human tissues. Results were observed in conventional TEM (Hitachi 600 AB, Hitachi, Japan).

Hemoglobin and Protein Sample Measurement

Control for blood plasma contamination in both subretinal fluid aspirates and normal vitreous samples was carried out by hemoglobinometry. On the basis of normal vitreous concentrations, we excluded all pathologic samples that showed hemoglobin levels higher than 0.2 ng/ml (Coulter Counter Model S, Beds, UK). Protein concentration of all samples was determined by protein-dye binding.

Electrophoresis and Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described. Briefly, normal and pathologic vitreous and subretinal fluid samples were mixed in a 1:1 (vol/vol) electrophoresis sample buffer (1% SDS/10% 2-mercaptoethanol/10% [wt/vol] glycerol/0.001% bromophenol blue/0.125 M Tris-HCl, pH 6.8), kept at 100°C for 5 minutes, and electrophoresed (Mini-Protein II 200/2.0 Electrophoresis Apparatus, Bio-Rad, Richmond, CA) for 2.5 to 4 hours at 50 to 60 V, on a 5.7% or 10% polyacrylamide gel in SDS. Protein bands were visualized by Coomassie blue or silver staining. Western blotting of proteins on nitrocellulose and detection using antibodies were performed as described. SDS-PAGE proteins were transferred (Trans-Blot 200/2.0 Transfer Apparatus, Bio-Rad) at 20 V, overnight at 4°C onto a nitrocellulose sheet (Hy-Bond-c, Amersham). Primary antibodies were incubated overnight at 4°C in 1% nonfat dry milk in 50 mM Tris-HCl (pH 7.4) at 1:1000 dilution. Secondary anti-rabbit or anti-mouse peroxidase-conjugated immunoglobulin (Dakopatts) diluted 1:2000 in 0.05% Tween 20 (Sigma) in 50 mM Tris-HCl (pH 7.4) was applied for 3 to 4 hours at room temperature. The nitrocellulose strips were stained with diaminobenzidine (0.1% DAB in 50 mM Tris-HCl pH 7.4).

Slot Blotting and Densitometrical Quantification

To quantify FN and VN, we used a slot blotting method (Bio-Dot SF Microfiltration Apparatus, Bio-Rad) on nitrocellulose sheets for each dilution bank of normal and pathologic vitreous samples. Nitrocellulose sheets were incubated with primary and secondary antibodies and stained using the DAB technique described above. A direct densitometrical reading was performed by digitalized images of nitrocellulose sheets (IBAS II, Interactive Analysis System, Kontron, Munich, Germany). Standard concentrations of purified VN and FN (0.2 μg/μl; Boehringer) were analyzed simultaneously with each sample.

Statistical Analysis

Chi-square analysis ($\chi^2$) was used to estimate statistical differences between the variabilities of frequency...
RESULTS

FN Matrix Assembly in ERM, Glycoproteins, and Integrin Expression

The time course and pattern of distribution in vivo of FN fibril formation in ERM matrix was established in cryostat sections (n = 26 ERM) by indirect immunofluorescence. Large amounts of FN were evident in most specimens, in pericellular or fibrillar arrangement in matrix, with short and thin or longer and thicker fibrils (Fig. 1). A pericellular pattern of distribution was more frequently noted in the group of ERM with only 2 months of evolution (Figs. 1A and 1B) than in the group of ERM aged between 2 to 6 months (P < 0.005, by chi-square analysis test). Pericellular pattern was rarely observed in the third group of ERM greater than 6 months of evolution, and differences were established with the first group (P < 0.05, by chi-square analysis). No significant difference was found between the intermediate group and the last. Electron-immunocytochemistry with immunogold labeling techniques confirmed the FN in its pericellular pattern and in collagen fiber arrangement. Individual cells presented large amounts of FN deposited along the plasma membranes and on cytoplasmic processes when present (Figs. 1C and 1D); cells with a wide variety of morphology showed large amounts of this glycoprotein.

Dense fibrillar fluorescent labeling was the most frequent finding for FN in matrix material, sometimes localized (Figs. 1I and 1J), sometimes generalized distributed across surface of the sample (Figs. 1E–H). The first group of ERM (<2 months of evolution) showed a clear localized distribution when compared with the group of intermediate time of evolution, which showed a generalized pattern in the extracellular medium (P < 0.001, by chi-square analysis). FN appeared to decrease in matrix material with the time of ERM evolution and showed a tendency to assume a localized distribution in specimens with more than 6 months of evolution when compared with the intermediate group (P < 0.01, by chi-square analysis).

FIGURE 1. Fibronectin expression in ERM. (A) Semithin sections prepared by indirect immunofluorescence stain showed moderate amounts of pericellular FN (arrows) (X990, bar = 20 μm). (B) A wide variety of morphologic cell types presented immunoreactive FN (arrows) (X800, bar = 20 μm). (C) Immunoelectron microscopy showed labeling (16 nm gold) (arrows) closely related with plasma membrane (X19,200, bar = 1 μm). (D) Higher magnification of bracketed area in C; immunoreactive FN binds a peripheral electron-dense material (arrows) related to cell membrane observed in some cell types. Collagenic matrix composed by fibers with clear periodicity present variable amounts of immunogold label (X92,500, bar = 1 μm). (E) Cryostat-sectioned specimens analyzed by interference contrast microscopy revealed abundant and dense fibrillar matrix in regular arrangement (X390). (F) By immunofluorescence, abundant amounts for this protein in generalized and regular thicker fibrillar pattern across the specimen surface were seen (X390). (G) Generalized and fine fibrillar FN irregular distributed in matrix material were also common pattern of labeling (X315). (H) The same section examined by interference contrast microscopy reveals areas of regular and random collagenous fiber arrangement (X390). (I) Localized areas of regular pattern of immunofluorescence stain (X390) contrast with (J) areas of irregular fibrillar distribution in the same specimen (X315, bar = 30 μm).
FIGURE 2. Laminin expression in ERM specimens. (A) Cells distributed in matrix (arrows) visualized by interference contrast microscopy (B) presented variable immunoreactivity staining for LN (arrows) (×800, bar = 20 μm). (C) Slight labeling in a fine fibrillar pattern of distribution (D) in small localized areas in the same sectioned specimen examined by interference contrast microscopy (×360, bar = 40 μm). (E) By immunoelectron microscopy, pA-Au (16 nm) particles were noted both related to the plasma membrane (arrow) and the extracellular material surrounding cells (×19,200, bar = 1 μm). (F) Collagenic fibers with periodicity presented immunoreactivity for this glycoprotein (×39,500, bar = 0.5 μm). (G) Scanty immunoreactivity for LN was common feature in matrix (19,200, bar = 1 μm).

In all ERM examined (n = 54), LN was present in 31 specimens (57%) but always as a minor component in the tissue. Its pericellular distribution was also observed (Fig. 2B), but most frequently a fine fibrillar pattern was noted in localized areas of the specimen (Fig. 2C). Although the third group of ERM (>6 months of evolution) tended to lack this glycoprotein, no significant difference was found among groups. By electron-immunocytochemistry, less extensive amounts of LN were occasionally noted around the plasma membrane but were not directly related with it (Fig. 2E). The distribution of LN in collagenic matrix showed a similar pattern to that observed for FN, but was restricted in confluent areas of immunogold labeling, which in most cases occupied small areas of the specimen (Fig. 2F and 2G).

In several consecutive serial cryostat sections, a clear pericellular preference for determinate cell types was manifest for both proteins (FN and LN), but

FIGURE 3. Identification of pericellular immunoreactive FN and LN in ERM. Consecutive serial cryosections were incubated with specific antisera for FN and LN in indirect immunofluorescence experiments. (A) Specimens incubated with anti-FN antisera showed high cell population that present strong pericellular staining. (B) The same section examined by interference contrast microscopy revealed a group of pigment-laden cells localized at the edges of sample (between open arrows). This area was negative for FN immunostaining; therefore, some pigmented cells at the inner aspect of the specimen showed positive label (complete arrows). (C) Consecutive cryosection obtained for the same specimen and incubated with anti-LN antisera showed positive label for cells at the periphery of sample corresponding the marginal area nonreactive for FN (between arrows) (×315, bar = 40 μm).
FIGURE 4. Expression of β1 subunit complex and αβ3 integrin in ERM. Semithin cryosectioned specimens were analyzed by indirect immunofluorescence after incubation with anti-ASβ1 antisera and mAb LM609 directed against αβ3 receptor. (A) Wide variety of cells presented fluorescent staining for β1 complex receptor (arrows) (×730, bar = 20 μm). (B) The VN receptor (αβ3) was occasionally found in isolated cells or organized in clusters or nest (arrows) (×360, bar = 40 μm). (C) VN immunolocalization in the same sample shows a pericellular pattern of distribution (arrow) as its receptor (×450, bar = 30 μm). (D) High magnification showed immunofluorescence for β1 subunit complex closely related to the plasma membrane (arrow) (×1,130, bar = 10 μm). (E) Beta-1 labeling for plasma membrane (pm) was corroborated by immunoelectron microscopy (×39,500, bar = 0.5 μm).

mainly for LN (Fig. 3). Areas with pigment-laden cells appeared to show a preference in labeling LN (Figs. 3B and 3C), although there were regions in sections that had significant labeling in the absence of pigmented cells.

VN was demonstrated in 6 out of 17 (35%) specimens tested for this glycoprotein. Its pericellular distribution (Fig. 4C) was more frequently seen than its generalized fine fibrillar arrangement in the matrix. As expected, by electron-immunocytochemical procedures the labeling for VN was distributed around the cell membrane but was not restricted to it (not shown). A low level of immunogold reactivity was also present in dense or loose bundles of collagen fibers in localized areas (not shown). Immunofluorescence labeling for αβ3 and β1 subunit complex integrins was occasionally identified (Fig. 4). The latter was observed more frequently, but it was observed less frequently in ERM with more than 6 months' evolution. By electron microscopy methods, anti-β1 subunit complex labeling was achieved infrequently but always correlated with cell cytoplasmic membrane (Fig. 4E); immunolabeled cells did not show any particular morphologic characteristic. Alpha-V Beta-3 integrin was infrequently identified and was associated with slight immunogold labeling of plasma membrane (not shown).

In collagenous matrix, densely or loosely arranged, variable quantities of FN were also noted (Figs. 5A and 5B). Double-labeling experiments showed a clear colocalization of VN and FN over collagenous bundles (Figs. 5C and 5D) and occasionally in a pericellular pattern, with predominant immunoreac-
Electrophoresis and Immunoblotting for Normal and Pathologic Vitreous, and Subretinal Fluid Samples

Pathologic vitreous and subretinal fluid samples of PVR patients were compared with pathologic vitreous samples from other intraocular proliferative disorders. Controls were normal human serum and normal vitreous samples obtained from human eyes after death. Protein concentration of pathologic PVR vitreous for each stage of disease were substantially increased when compared with normal vitreous samples (0.506 ± 0.04 μg/μl) \( (P < 0.001, \) by unbalanced ANOVA test). Normal vitreous separated by 7.5% SDS-PAGE under reducing conditions and Coomassie blue or silver staining revealed a main band around 66 kD, corresponding to the albumin fraction when normal human serum was processed in the same experiment. In the pathologic vitreous and subretinal fluid samples, main bands of low molecular weight (≤66 kD) were noted, which coincided with the electrophoretic band pattern of the low-weight proteins observed in the normal human serum samples analyzed. Proteins with a molecular mass higher than 66 kD were less evident, and similarities were found with the serum band profile (Fig. 6). By immunoblotting assays, FN was detected in 210 to 230 kD bands in 7.5% SDS-PAGE gels, coincident with the electrophoretic mobility of purified FN used as a mass marker. In normal vitreous samples, little FN immunoreactivity was detected (Fig. 7a). In 10% SDS-PAGE gels, VN immunoreactivity was present in all the samples analyzed as a double band (Fig. 7b) corresponding to the VN 75 kD and 65 kD bands. Several pathologic samples immunoblotted with antibodies against FN or VN showed low molecular mass bands that may represent degraded fragments for intact FN or VN. LN immunoreactivity was not detected in any subretinal fluid or vitreous sample.

**FN and VN Concentration Measurement in Normal and Pathologic Samples**

Densitometry by IBAS indicated significant differences in the FN concentration among the stages of PVR samples and other intraocular pathologic proliferative conditions. In the early stage of disease (stage A), low FN levels (0.188 ± 0.04 ng/μg protein) were observed similar to normal samples (0.129 ± 0.03 ng/μg protein). At the next stage of disorder (stage B), a substantial raise in the intraocular FN levels (0.490 ± 0.08 ng/μg protein) was detected, and this difference was significant when compared with both the normal and the previous pathologic stage samples \( (P < 0.05, \) by unbalanced ANOVA test). Similar increased concentrations of FN were noted in the later PVR stages. Decreased levels of intraocular VN concentration were observed in pathologic samples when compared with normal samples (0.195 ± 0.05 ng/μg protein). VN concentration appeared to decrease with development of intraocular proliferative disorder. The VN levels at stage A (0.144 ± 0.08 ng/μg protein) and B (0.163 ± 0.08 ng/μg protein) were significantly higher than the later stage D (0.061 ± 0.03 ng/μg protein) \( (P = 0.05, \) by unbalanced ANOVA test). Similar VN levels were noted in stage C (0.093 ± 0.04 ng/μg protein), and when the later stages (C and D) were compared with the normal VN levels, statistical differences were apparent \( (P < 0.05, \) by unbalanced ANOVA test). Table 1 provides quantitative measurements of total proteins, FN, and VN relative amounts in normal and pathologic conditions. The total variation of FN and VN concentrations at each stage of PVR are presented in Figure 8.
DISCUSSION

Significant progress has been made in elucidating physiopathogenic mechanisms in PVR, although many fundamental questions remain. One such question concerns the events of cell-matrix interaction in periretinal proliferative fibrocellular tissue formation.

Several studies have evaluated the involvement of various cell types and the possible role of soluble and insoluble proteins in human ERM. Here we have studied the cellular distribution of the main extracellular glycoproteins that could mediate cell-matrix interaction in PVR. We have observed that FN is the major protein involved in both extracellular and intravitreous environment, and we suggest it may have a leading role in membranogenesis. On the other hand, the decreased levels of intraocular VN observed in the development of PVR could be involved in the mechanisms of cell adhesion in the established ERM.

The regulation of extracellular matrix assembly and cellular response to these matrices are important for the control of several cellular events. For instance, cell morphology, cell attachment and migration, tissue...

FIGURE 6. Electrophoretic profile of normal (nl) and PVR vitreous proteins separated on 7.5% SDS-PAGE under reducing conditions and Coomassie blue staining. Large amounts of protein of low molecular mass (~66 kD) characterize pathologic samples in both subretinal fluid (SRF) in early stages (A and B) and vitreous aspirates in later stages (C and D) of disease. Proteins of molecular mass higher than 66 kD observed in normal human serum (S) were less evident in pathologic samples. Molecular weight (MW) standards were as follows: fibronectin (220 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), and trypsinogen (24 kD).

FIGURE 7. Immunoblot analysis of normal (nl) and PVR vitreous proteins separated by 7.5% and 10% SDS-PAGE under reducing conditions. (a) Western blot of subretinal fluid (SRF) in the early stages A (lanes 3 and 4) and B (lanes 5 and 6) of PVR and later stages C (lane 8) and D (lane 9) were positive for FN. Purified FN (lanes 1 and 2) was used as positive control. (b) On 10% SDS-PAGE, PVR samples in different stages (lanes 2, 3, and 4) and from patients with intraocular foreign body (IOFB) and proliferative diabetic retinopathy (PDR) were incubated with mAb VN7 directed against VN. Double-band profile for VN (~65 to 75 kD) was observed and low molecular mass fragments of degraded glycoprotein were also detected.
Glycoproteins and Receptors in PVR

TABLE 1. Concentrations of Total Proteins, FN, and VN in Normal (n = 9) and Pathologic Vitreous (n = 25) Samples

<table>
<thead>
<tr>
<th></th>
<th>Total Proteins (ng/μl)*</th>
<th>Fibronectin (ng/μg Protein)</th>
<th>Vitronectin (ng/μg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.506 ± 0.04</td>
<td>0.129 ± 0.03</td>
<td>0.195 ± 0.05</td>
</tr>
<tr>
<td>PVR A†</td>
<td>13.31 ± 3.37§</td>
<td>0.188 ± 0.04</td>
<td>0.144 ± 0.08</td>
</tr>
<tr>
<td>PVR B†</td>
<td>15.70 ± 5.10§</td>
<td>0.400 ± 0.08†</td>
<td>0.183 ± 0.08§</td>
</tr>
<tr>
<td>PVR C</td>
<td>15.13 ± 4.50§</td>
<td>0.523 ± 0.07†</td>
<td>0.093 ± 0.04‡</td>
</tr>
<tr>
<td>PVR D</td>
<td>14.70 ± 2.90§</td>
<td>0.454 ± 0.02‡</td>
<td>0.061 ± 0.03‡</td>
</tr>
<tr>
<td>IOFB</td>
<td>20.80 ± 6.00§</td>
<td>1.546 ± 0.50§</td>
<td>0.154 ± 0.09</td>
</tr>
</tbody>
</table>

PVR, proliferative vitreoretinopathy and stages (A, B, C, D); IOFB, intraocular foreign body.
* Mean ± SE.
† Subretinal fluid.
‡ Statistical significance from normal samples (P < 0.05).
§ Statistical significance from normal samples (P < 0.001).

stability, cell polarity, and differentiation often require adhesion and specific interactions of a cell with its substrate. ERM formation can be understood as a pathologic proliferative "model" in which FN matrix assembly shows interesting features. When intraocular proliferative tissue with less than 2 months of clinical evolution was labeled for FN, pericellular distribution was significantly more evident than in older specimens. In addition, a localized fibrillar FN pattern was more apparent in the first specimen group analyzed than in others. On the other hand, fibrillar FN generally distributed in matrix was a common feature that characterized tissue with longer evolution and was less evident in specimens with more than 6 months of clinical evolution; absence of FN immunoreactivity was noted only in the latter group. Upon analyzing the role of FN in wound repair some crucial chronologic events were observed: During the early stages of wound healing, plasmatic-soluble FN stimulates directed cell migration toward the wound, making it act as a chemotactic element, and it also has a role in cell-to-cell, and cell-to-substrate adhesion; later, insoluble FN is secreted by cells to produce a fibrillar extracellular matrix that has important relationships with cellular anchorage and interaction with this matrix; finally, FN may enhance phagocytosis in wounds but gradually disappears with maturation of collagen. The assembly of FN into fibrils was found as a cell-surface-mediated process that involves the binding of this glycoprotein by a receptor-like system, whereas the αβ, integrins, and some other β integrins seem to be required as part of the adhesion mechanism. Thus, a cell-surface-correlated FN is observed first, followed by a partial assimilation into the matrix. For these reasons, we may consider, according to the behavior of FN observed in the intraocular fibrocellular tissue examined, that PVR may represent modified intraocular wound repair because the assembly behavior of this glycoprotein agrees with the classical model described above. Based on stereologic procedures, we observed that the volume–density cell–matrix estimation in ERM tends to decrease with the time of disease evolution, which might be explained by a reduction of cellular population and/or an increase in collagen deposition in fibrocellular tissue (unpublished data, 1993). FN was abundant in most of the ERM studied, having been reduced in amount and occasionally absent in specimens with more than 6 months of evolution; at this stage, similar to wound repair, FN tends to disappear gradually whereas stabilization of the collagenic framework is noted. Cells involved in ERM formation may be considered the probable source of FN. Many cell types identified as a constituent of these membranes (retinal glia, retinal pigment epithelium, and fibroblast-like cells were largely recognized as components in the intraocular fibrocellular tissue in hu-
man PVR,\(^\text{38}\) are known to be capable of FN synthesis in vitro,\(^\text{51,52}\) and variable labeling of FN mRNA has recently been reported in cells in human detached retina and in ERM.\(^\text{38}\)

LN and VN appear to be involved in fibrocellular tissue formation, but they only have a secondary role. Their identification was occasionally possible in pericellular and fibrillar patterns. Pericellular LN labeling was noted in this study by optical and electron microscopy immunocytochemistry procedures. Pigment-laden cells were frequently observed associated to this glycoprotein, but these were not the only characteristic cell type positive for immunoreactive LN. Pigmented cells, as a component of ERM, may be represented by retinal pigment epithelial cells and macrophage-like cells.\(^\text{52}\) In addition, ultrastructural studies found that retinal pigment epithelium and retinal glial cells are able to constitute basement membranes, and they act with collagen type IV as structural proteins. Among several functions, LN promotes epithelial cell adhesion to collagen\(^\text{53,54}\), and has the ability to convert embryonic mesenchymal cells into polarized-shaped form.\(^\text{55}\) These data are sufficient evidence to justify further double-labeling assays to elucidate two major points: first, whether pigment-laden cells that label immunoreactive LN represent retinal pigment epithelial cells, and second, whether cells positive for pericellular LN have a glial origin. The significance of VN in cell events involved in epiretinal formation could be mainly sought in its ability to promote cell adhesion\(^\text{14,56}\) and its biochemical structure, which includes a domain capable of binding collagen.\(^\text{1,12,57}\) The cell attachment activity of VN is based on the RGD sequence,\(^\text{14,16}\) which is recognized by a wide variety of cell types.\(^\text{15}\) Properties of VN in hemostasis are extensively described,\(^\text{11,12}\) but, in our view, it is difficult to correlate hemostatic phenomena with ERM formation in PVR.

A fine fibrillar pattern of VN arrangement in the matrix material was observed, and colocalization of VN and FN was frequently noted in collagenic bundles by electron-immunocytochemistry procedures. Several immunofluorescence studies suggest the deposition of VN in a fibrillar pattern in loose connective tissue of many normal structures, including lung, kidney, skin, and smooth and skeletal muscle,\(^\text{6,9}\) where it is sometimes colocalized with FN. These data provide evidence that appreciable amounts of VN may be deposited far from the liver where it is biosynthesized. For this reason, the mechanisms involved in VN and FN matrix deposition during intraocular fibrocellular tissue formation in PVR could also involve the exposure of fibrillar collagenic framework and other proteins synthesized by local cellular components to plasmatic VN and FN present in the altered vitreous. Our data (see below) showed an increasing intravitreal level of FN and a slight alteration in VN concentration in subretinal fluid and vitreous aspirates, suggesting this process at least for FN.

Expression of \(\beta_1\)-subunit complex and \(\alpha_\text{v}\beta_3\) integrins is corroborated by the pericellular pattern identified by these glycoproteins. However, we do not have sufficient data to conclude that \(\beta_1\) complex was preferentially expressed in the group of ERM whose pericellular labeling for FN was most evident, or whether this integrin immunoreactivity decreased in specimens with longer clinical evolution. In addition, in immunoelectron microscopy labeling procedures, integrin immunoreactivity seems to be scanty, which could be due to normally diminished tissue antigenicity as a consequence of the method. Another explanation for the integrin activity in PVR involves its presence in the earliest stages of disease when constituted ERM are not found, but the events correlated to membrane-generation, such as cellular migration, adhesion, and proliferation, certainly do take place. Moreover, surgically excised ERM represent a final product of a complex proliferative phenomenon and look like a stabilized "scar" in the wound repair process. The distribution and presumed functional interaction of receptors, FN, and LN are generally coincident in several structures,\(^\text{4,19}\) but some peculiarities are remarkable. For example, responses to collagen and FN or LN are commonly mediated by \(\beta_1\)-class integrins, whereas nonplatelet receptors for VN and FN are composed of \(\alpha_\text{v}\)-subunit in association with \(\beta_1, \beta_2\), or \(\beta_3\).\(^\text{58-60}\) In addition, FN can bind with the classical VN-receptor (\(\alpha_\text{v}\beta_3\)) specifically and with high affinity, supporting cell adhesion to matrix proteins.\(^\text{29}\) Finally, VN-receptor (\(\alpha_\text{v}\beta_3\)) may act as an LN-receptor and can mediate cell adhesion.\(^\text{8,61}\) All this evidence is indicative that integrin receptor complexes and specific sequence domains of glycoproteins may have a major role in intraocular fibrocellular tissue formation.

We present evidence that ERM formation can represent a cell-mediated process. It is not clear how the mechanisms involved in cell migration, adhesion, and growth observed in PVR are initiated, but we hypothesize an important contribution of breakdown of the blood-retinal barrier, in this case reflected by a failure of the retinal pigment epithelium to function as a barrier.\(^\text{62}\) High concentrations of proteins in subretinal fluid (early stages) and in pathologic vitreous (later stages) characterize the PVR intraocular environment. These levels tend to be constant at the different clinical stages of disease and were 25 to 30 times higher than that of normal vitreous and about one-fourth of protein levels of normal serum. In fact, as previously described, the serum contains chemoattractant substances that act by increasing migration for retinal-derived glial cells and retinal pigment epithelial cells, in vitro; cell migration appears to be mediated in a
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PVR in a dose-dependent manner, and, among these serum elements, FN was highly active. In addition, important alterations in integrity and permeability of blood-retinal barriers have been found in eyes with diabetic retinopathy, as well as in a transient form in eyes with retinal detachment. Hemorrhage may also result in release of plasma or serum components into the vitreous cavity, and these are thought to be associated with increased incidence of PVR after penetrating ocular injuries, with or without intraocular foreign bodies.

Our findings showed increased levels of intravitreal FN and protein in traumatic PVR with intraocular foreign bodies, associated with accepted hemoglobin concentrations (<0.2 mg/ml) in samples. Breakdown of blood-retinal barriers may explain the changes in proteins and FN levels observed in pathologic intraocular milieu. Intraocular FN levels vary significantly in the earliest stages of PVR (stages A and B) when we could only analyze subretinal fluid because intraocular surgery with vitreous manipulation was rarely imperative to reattach the retina. At these stages, we may observe clinical intraocular changes but constituted ERM are not found, although mechanisms involved in membranogenesis are certainly present. Similar relative levels of FN in normal vitreous are observed in subretinal fluid obtained at stage A of disease, although important changes in protein concentrations were found. Associating experimental and clinical data, we suggest that FN may have a significant role in initial events that characterize several clinical intraocular aspects of ERM formation in PVR. An increase in FN levels could not be exclusively explained by plasmatic origin because the protein concentration remains unchanged. An attractive hypothesis is that early cell production of FN at stage A is responsible for the increased FN levels observed at stage B.

Unlike FN, intraocular VN levels decrease progressively with the clinical evolution of disease, and slight differences in concentrations were noted when comparing the earliest stages with normal vitreous samples. These findings are difficult to explain because plasmatic concentrations of FN (0.3 μg/μl) and VN (0.2 to 0.4 μg/μl) are similar. If permeability of ocular barriers reveals high concentrations of FN, which has a much higher molecular weight (450 KD) than VN (65 to 75 KD), it might be difficult to accept a selective mechanism that allows an exclusive passage of intraocular FN from circulation. For these reasons, we assume that VN could be adsorbed in some events of cellular adhesion and spreading, correlated to cell-substratum interaction. This fact might be accepted and has been demonstrated in vitro. It is likely that, in vivo, FN and VN exert differential effects in mechanisms involving tissue growth and repair, and the differential distribution of these glycoproteins in vivo supports this hypothesis.

Finally, it has recently been reported that chronic wound fluid samples, examined by immunoblotting and cell adhesion assays, showed a marked and sometimes complete degradation of VN, as well as a less evident degradation of FN that alters the cell adhesion necessary for normal wound closure. In our study, we noted the presence of some products of FN and VN degradation in immunoblotting experiments in several samples. However, our data are insufficient to determine whether degradation is time-dependent or is related to clinical stages of disease. Despite this evidence, similarities between wound fluids and PVR intraocular environment may be established, suggesting that periretinal fibrocellular tissue formation observed in this disorder may represent a final product of a specific mechanism of intraocular wound repair.

Key Words
epiretinal membranes, retinal detachment, glycoproteins, integrins, immunocytochemistry

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