UNIVERSITAT DE BARCELONA

Vitreorretinopatía Proliferativa y Retinopatía Diabética Proliferativa: Caracterización Celular y Glucoproteínas de Adhesión de la Matriz Extracelular

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Tesis Doctoral - Barcelona 1992

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FACULTAT DE MEDICINA

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En/Na RICARDO-PEDRO CASAROLI MARANO

nascut/uda el dia 11 de desembre de 1957,

a SAO PAULO (BRASIL),

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El Tribunal nomenat pel Magfc. i Excm. Sr. Rector d'aquesta Universitat ha qualificat l'esmentat treball amb la nota de:

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PER A LA POSSESSIÓ EFECTIVA DEL GRAU DE DOCTOR CAL ABONAR ELS DRETS DEL TÍTOL CORRESPONENT

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El presente trabajo ha sido realizado bajo la dirección del Dr. Senén **VILARO COMA**, Catedrático de Biología Celular de la Universidad de Barcelona y del Dr. Joaquín **BARRAQUER MONER**, Catedrático de Cirugía Ocular de la Universidad Autónoma de Barcelona, en los laboratorios de la Unidad de Biología Celular del Departamento de Bioquímica y Fisiología de la Universidad de Barcelona.

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A mis padres

"Dejamos consignado que lo inútil, aun aceptando el punto de vista humano (con las necesarias restricciones de tiempo y lugar), no existe en la Naturaleza. Y, en último extremo, aun cuando no fuera posible poner al servicio de nuestra comodidad y provechos ciertas conquistas científicas, siempre quedaría una utilidad positiva: la noble satisfacción de nuestra curiosidad satisfecha y la fruición incomparable causada en el ánimo por el sentimiento de nuestro poder ante la dificultad vencida."

Santiago **RAMON Y CAJAL**

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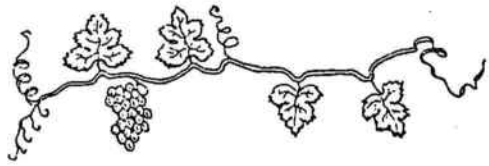

INDICE

PRESENTACION

PRESENTACION

El presente trabajo está estructurado en dos bloques bajo un tema común: ***La Enfermedad Proliferativa Intraocular.***

En el primer bloque se presenta, una revisión bibliográfica de la **Vitreorretinopatía Proliferativa** y la **Retinopatía Diabética Proliferativa**, ya que creemos esencial proponer un enfoque celular para ambas patologías a fin de poder aproximarnos a la comprensión de los mecanismos fisiopatológicos de estas alteraciones.

En el segundo bloque se presenta nuestra aportación experimental al estudio de las membranas fibrocelulares y fibrogliovasculares de ambas patologías, así como la matriz extracelular que las constituyen. El **Capítulo I** estudia la morfología y la ultraestructura de los componentes celulares, capilares y de la matriz extracelular de las membranas pre-retinianas, con el objetivo de caracterizarlas desde un punto de vista estructural. Asimismo, en este capítulo, hemos realizado un estudio estereológico de las fracciones volumétricas de los componentes de estos tejidos con el propósito de observar la relación matriz extracelular/célula según la patología y su evolución. En el **Capítulo II**, se analiza la expresión de las proteínas de los filamentos intermedios de los diferentes tipos celulares involucrados en la formación de las membranas epirretinianas, pretendiendo caracterizar los tipos celulares presentes y su comportamiento en el proceso proliferativo.

Estudiada la estructura y sus componentes celulares, cada patología es considerada en su respectivo capítulo, ya que sus características estructurales y fisiopatogénicas son distintas. En el **Capítulo III** se describe la expresión de la *fibronectina*, la *laminina*, y de la *vitronectina* en los capilares de neoformación y láminas basales que constituyen las membranas fibrogliovasculares de la Retinopatía Diabética Proliferativa. Se establece la relación glucoproteínas-receptores (β_1 y $\alpha_v\beta_3$) y se evalúan las concentraciones de proteínas totales, *fibronectina* y *vitronectina* en muestras vítreas normales y patológicas, a fin de caracterizar el medio intraocular en la patología. En el **Capítulo IV** se analizan los cambios de expresión de estas glucoproteínas y sus receptores en las membranas epirretinianas de la Vitreorretinopatía Proliferativa y se estudian las alteraciones de concentración intravítrea de *fibronectina* y *vitronectina* en los diferentes estadios clínicos de evolución de esta patología.

Por último, hemos agrupado nuestras consideraciones generales y conclusiones globales en un mismo apartado que se encuentra al final de este trabajo. La referencias bibliográficas fueron reducidas a las más significativas y esenciales con relación a cada tema y se hallan relacionadas al finalizar cada capítulo.

INTRODUCCION

Vitreorretinopatía Proliferativa: Fisiopatología, elementos celulares y extracelulares

Anales del Instituto Barraquer 21:177-190,1990.

Resumen: La Vitreorretinopatía Proliferativa (VRP), está caracterizada por una proliferación celular y la formación de membranas epirretinianas e intravítreas, que predisponen a los desprendimientos de retina traccionales. Esta es, sin duda, la complicación que se observa con más frecuencia tras la cirugía reparadora de los desprendimientos de retina y, con diferencia, constituye, la causa más común de los fracasos post-operatorios. Este proceso, cuya patogénesis es poco conocida puede entenderse como un mecanismo micro-reparacional localizado. Ciertos tipos de células, tales como las del epitelio pigmentario de la retina, las células de la glia retiniana, los fibroblastos y macrófagos, parecen tener un papel importante en los fenómenos de migración, adhesión y proliferación observados durante el desarrollo de la enfermedad, y que conllevan a la aparición de membranas fibrocelulares sobre la retina. Los numerosos elementos humorales y del propio tejido que han sido identificados según criterios morfológicos, ultraestructurales y, más recientemente, por medio de técnicas inmunocitoquímicas, coinciden en considerar la enfermedad como un complejo fenómeno multifactorial. Actualmente se atribuye a las proteínas y los componentes de la matriz extracelular un destacado papel en la patogénesis. Considerarla como un modelo proliferativo humano *in vivo* es esencial para el entendimiento de los eventos que caracterizan su fisiopatogénesis.

Palabras claves: Vitreorretinopatía proliferativa, desprendimiento de retina, epitelio pigmentario de la retina, células gliales, matriz extracelular, glucoproteínas, membranas epirretinianas.

La Vitreorretinopatía Proliferativa (VRP), puede ser considerada como un mecanismo reparativo cicatricial intraocular, en el cual la formación de un tejido conjuntivo fibrocelular, con su consiguiente contracción, da como resultado la aparición de fuerzas de tracción sobre las estructuras adyacentes. La proliferación celular con la formación de tejido conjuntivo, está asociada a un amplio espectro de complejas entidades patológicas, entre las cuales destacamos la retinopatía diabética proliferativa (RDP), el trauma ocular, las inflamaciones, las retinopatías isquémicas y el desprendimiento de retina (DR) con componente regmatógeno.

En los traumas oculares, con o sin perforación del segmento posterior, así como en las inflamaciones vítreas o tras las cirugías convencionales de reparación del DR, se pueden observar alteraciones estructurales en las interfases vítreorretinianas (hialoides posterior y superficies perirretinianas interna y externa), que con cierta frecuencia desarrollan tracciones sobre la superficie de la retina.

Con relación a la enfermedad diabética proliferativa intraocular y las retinopatías isquémicas, el contacto entre la superficie posterior del vítreo y la superficie interna de la retina, constituye una parte esencial para el desarrollo del componente vascular de neoformación, característico de estas entidades. La ausencia del contacto vítreo-retina funciona como factor protector contra la formación de membranas vítreas fibrovasculares.

En el proceso proliferativo intraocular, podemos distinguir básicamente dos situaciones principales: la proliferación en el vítreo y aquella que tiene lugar en las superficies de la retina (interna y externa). Tras un trauma ocular perforante, la invasión celular y posterior crecimiento intravítreo se origina a partir de la herida en la esclerótica; se establece una desorganización estructural del vítreo, con la formación de bridas y bandas de tejido conjuntivo cicatricial, que traccionan, desprenden y muchas veces incarceran la retina, en la herida de entrada. Asimismo, en la mayoría de estos casos, observamos una proliferación celular sobre la retina, que se traduce en la formación de membranas en sus superficies: *las membranas epirretinianas* (MER), en la interfase vítreo-retina, y más raramente las *membranas retrorretinianas* (MRR), que se sitúan entre la retina neurosensorial y el epitelio

pigmentario de la retina (EPR). Esta diferenciación nos parece oportuna, ya que poseen aspecto y comportamiento clínico distintos.

La proliferación celular que aparece como complicación de la cirugía reparadora de los DR o tras ciertos DR de larga evolución, diagnosticados o no, puede originar MER. Machemer y Laqua (1975), caracterizaron por primera vez este fenómeno como una compleja entidad clínico-patológica; ésta *Proliferación Perirretinal Masiva* implicaría una proliferación, una metaplasia fibrosa y una posterior contracción de ciertos tipos celulares involucrados en el proceso. Su traducción clínica ha sido extensamente estudiada, estableciendose una clasificación inicial (Machemer,1978). Actualmente el proceso se denomina ***Vitreorretinopatía Proliferativa*** (VRP) (The Retina Society Terminology Committee,1983) y constituye la complicación más frecuentemente observada tras la cirugía reparadora del DR.

El problema básico en el establecimiento de una terapéutica efectiva, racional y objetiva para la VRP, reside primordialmente en la falta de un mayor conocimiento de los factores intrínsecos y extrínsecos, que estimulan y modulan la migración, la adhesión, la proliferación y la contracción celulares observadas en la patología. El origen de sus constituyentes celulares, así como la interacción y la actuación del entorno extracelular y su relación con los factores humorales, poseen un papel fundamental para la mejor comprensión de sus mecanismos fisiopatológicos.

En esta revisión, centraremos nuestras consideraciones en ciertos hechos de trascendencia, dentro de la fisiopatología de la VRP, relativos a los mecanismos y al comportamiento de la célula y la interacción de esta con su matriz. Asimismo analizaremos los diferentes elementos implicados, para tratar de comprender esta manifestación, como un modelo humano *in vivo* para el estudio proliferativo intraocular reparacional.

LOS FACTORES PREDISPONENTES Y COADYUVANTES

Existen algunas ideas fundamentales, que resumen la aparición de una proliferación celular en la interfase vítreo-retina: ***no hay VRP en ausencia de DR, asimismo raramente existirá DR ante la ausencia de una dehiscencia retiniana y no se observará solución de continuidad en la retina, mientras persista el contacto de ésta con el vítreo.***

Esta será la condición anatómica indispensable hacia la que se orientará nuestra fisiopatología: la destrucción de la integridad estructural vítreorretiniana, caracterizada por la separación entre la corteza del cuerpo vítreo y la estructura que descansa sobre la pared de la cavidad vítrea, la retina, a la cual está adherida. Este desprendimiento puede presentarse con menos frecuencia en situación *anterior*, a nivel de la base del vítreo, asociado a graves traumatismos oculares. Normalmente aparece bajo la forma de un *desprendimiento del vítreo posterior (DVP)*, que puede constituir un fenómeno fisiológico primario relacionado con la edad y añadido a algunos factores que aumentan su incidencia (alta miopía, afaquia), o secundariamente a ciertos estados patológicos (uveítis, coriorretinitis, hemorragias). La despolimerización de las moléculas de ácido hialurónico con la consiguiente agregación de fibrilas de colágeno, producen lagunas acuosas intravítreas que coalescen, hasta romperse en el espacio retrohialoideo, ocupando súbita o progresivamente la interfase vítreorretiniana, estableciéndose de este modo el DVP. El cuerpo vítreo tiende a colapsarse hasta desprenderse totalmente. En su trayecto encuentra adherencias normales y, en algunas ocasiones, adherencias patológicas en la superficie retiniana, resultando un mecanismo de tracción vítreorretiniano, que provocará en ciertas eventualidades, una dehiscencia en la retina: *los agujeros o desgarros retinianos*. Las condiciones fundamentales para la presencia de un DR aparecen entonces, consistiendo en una relación causa-efecto entre la tracción ejercida sobre las adherencias patológicas (lesiones vítreorretinianas o lesiones vítreas) y las dehiscencias originadas en la retina.

La membrana limitante interna de la retina (MLI), representa la frontera entre el vítreo y la retina, comportándose como una verdadera barrera contra una posible invasión celular hacia el vítreo (McLeod and Leaver,1977). Las fuerzas que mantienen la neurorretina adherida al EPR,

incluyen un mecanismo de bombeo del mismo EPR, que drena fluido e intercambia metabolitos entre la retina y los tejidos subyacentes, así como las fuerzas osmóticas originadas de la propia coriorretina (Foulds,1976). Tras la aparición del DVP y asociado a una solución de continuidad sobre la retina, a través de la MLI, el fluido intravítreo fluye hacia el espacio subretinal (Machemer,1984). Las fuerzas osmóticas y el mecanismo de bombeo del EPR se muestran insuficientes para mantener la retina adaptada. Los elementos celulares podrían así invadir fácilmente la cavidad vítrea, ya que el fenómeno celular de inhibición por contacto dejaría de existir, y además, factores plasmáticos de exudación, aprovechándose de las alteraciones osmóticas y del fallo del EPR, se hacen presentes actuando activamente en el inicio de la migración celular. La llegada de los elementos celulares y humorales, será tanto mayor cuanto mayor sea el DR y mayor sea el tamaño del desgarro sobre la retina, aumentando los riesgos de VRP (Bonet,1987).

El mantenimiento del contacto vítreo posterior-retina, constituye la base fundamental de un gran número de alteraciones de la interfase vítreorretiniana. Desafortunadamente las bases de dicho mantenimiento, son todavía poco conocidos, en parte por la ausencia de modelos experimentales fidedignos y en parte por la falta de conocimientos de los mecanismos de adhesión entre la corteza vítrea y la MLI (Foss,1972). El aumento del espesor de la MLI relacionado con la senilidad, ha sido observado en ojos humanos (Gartner,1970). Este fenómeno podría estar implicado en la pérdida de funcionalidad del mecanismo de adhesión entre las células y de estas con su substrato en la interfase vítreo-retina, ya que el DVP está directamente relacionado con la edad, sospechándose así del papel desempeñado por el medio extracelular en la modulación de esta adhesividad (Kleinman *et al.*,1981).

Clínicamente podemos observar los efectos inflamatorios de las hemorragias en el vítreo, cuando éstas están asociadas a traumatismos o desgarros retinianos. La respuesta inflamatoria contribuye no solamente a la presencia de elementos celulares sanguíneos, sino también a la presencia de una exudación vascular, que se traduce en un vítreo de aspecto proteico. Las agresiones físicas a la coroides (crioterapia, diatermia) pueden llevar a una importante dispersión de células del EPR. Si se asocian a un desprendimiento de la coroides como consecuencia de un hematoma, afectan indirectamente a la función de bombeo del EPR. Las hipotonías oculares presentes con frecuencia en estas situaciones, en caso de ser persistentes, alteran la función normal del cuerpo ciliar, induciendo una exudación vascular. Se establecería entonces un proceso dinámico cíclico, en el cual la exudación hacia el vítreo resultaría en un aumento de la hipotonía, y ésta, a su vez, en una mayor exudación. Asimismo, en distintas situaciones clínicas, también podemos observar el paso de elementos extraños hacia la cavidad vítrea. Componentes séricos exudados de origen sistémico, parecen constituir un medio ideal para el estímulo de ciertos fenómenos en las células allí presentes. Sin duda, la interacción de los factores endógenos y exógenos desencadenarían un complejo mecanismo de migración, adhesión, proliferación y contracción celulares.

LA PROLIFERACION

Inicialmente el colapso del cuerpo vítreo ha sido implicado como fuente de contracción, del cual podrían resultar unas fuerzas de tracción que terminarían por producir el DR (Havener,1973). Estudios experimentales y clínicos resaltan la importancia del proceso proliferativo celular seguido de la contracción de sus elementos, estableciéndose con ello las bases del mecanismo de tracción (Machemer *et al.*,1978; Van Horn *et al.*,1977). Así la migración, adhesión y proliferación celular y la posterior contracción, son los estadíos que caracterizan el proceso de la VRP en su ***fase activa***. Seguramente, el punto de mayor relevancia reside en las alteraciones de las barreras (EPR, MLI) que permiten y/o estimulan la migración celular. Esta migración podría estar determinada por factores quimiotácticos y quimiocinéticos, inductores de la proliferación, que, a su vez, estaría modulada por una serie de sustancias del vítreo "enfermo" originadas a partir de la circulación a través de la exudación intravítrea.

Los elementos celulares pueden estar dispuestos directamente sobre la superficie de

la retina, generalmente en contacto con la MLI, pero también sobre la retina externa. Así se establecen dos aspectos de importancia capital, que contribuyen a la fisiopatología del fenómeno de membranogénesis observado en la VRP: *la interacción célula-célula y la interacción célula-substrato.* Ciertos tipos celulares poseen receptores en sus membranas que facilitan el contacto con otras células y con el medio que las contienen. Este hecho puede estar relacionado con la activación del fenómeno celular migratorio proliferativo y contráctil; este último, es considerado el efecto más desastroso de este complejo proceso, asemejándose al mecanismo de proliferación y contracción fibroblástica, que observamos en el curso natural de una reparación cicatricial (Gloor and Daicker,1975; Cleary and Ryan,1981).

Sabemos que los filamentos intermedios citoplasmáticos, son constituyentes normales de las células; su importancia todavía no está del todo clara, pero se ha sugerido un papel de sostén estructural (Spoones *et al.*,1971; Crawford *et al.*,1972). Estos filamentos fueron detectados como constituyentes de ciertos tipos celulares "metaplásicos", de características miofibroblásticas, involucrados en la VRP (Machemer and Laqua,1975; Laqua and Machemer,1975; Van Horn *et al.*,1977; Machemer *et al.*,1978). Las fuerzas contráctiles, que caracterizan las células miofibroblásticas, actuarían tangencialmente sobre la superficie retiniana acortando la masa celular y superando la propia fuerza estructural de adhesión entre la retina y el EPR. Cleary y Ryan (1979), describieron la importancia del papel de los vectores de fuerza en los DR traccionales, producidos en modelos experimentales de VRP por trauma ocular perforante.

En un estadío final, la "cicatriz" vítreorretiniana entraría en una ***fase de estabilización***, donde se observará la acción de proteínas como el colágeno, fibronectina y laminina (Grierson and Rahi,1981; Scheiffarth *et al.*,1988). El colágeno probablemente no participaría en el fenómeno contráctil, sino solamente en la estabilización del proceso (Peacock and Van Winkle,1970). La contracción del tejido neo-formado correspondería a los elementos celulares (Machemer,1978).

Ryan (1985) considera el concepto de "masa crítica", en el cual solamente un número suficiente de células, en una misma etapa de su ciclo vital, podrían generar una fuerza de contracción tal que supere la fuerza de adhesión retiniana.

Tras la migración celular, sea ésta hacia el vítreo o sobre las superficies interna y externa de la retina, el proceso de adhesión célula-célula y célula-substrato, origina la formación de una verdadera membrana fibrocelular (MER, MRR), totalmente contráctil y clínicamente visible tras la deposición de matriz extracelular. Las fuerzas de contracción parecen estar presentes ya en los estadíos tempranos de su desarrollo, punto en el que los cambios iniciales en la superficie de la retina no son evidentes (Machemer,1978).

Las MER, son la traducción anatomopatológica más característica de todo el proceso de la VRP. Podrían, teóricamente, ser divididas en dos categorías según sus características clínica e histológica. Las MER *simples* que pueden causar un arrugamiento de la superficie retiniana tras su contracción, ejercen poca fuerza tangencial contráctil. Frecuentemente están constituidas por una monocapa de un determinado tipo celular, y una matriz pobre en colágeno. Por otro lado, las MER *complejas*, se caracterizan por la presencia de un tejido semejante a una cicatriz fibrosa, produciendo verdaderos pliegues en la retina y DR traccional. Poseen múltiples capas pluricelulares dispuestas al azar y con abundante material extracelular (Grierson *et al.*,1987). Debemos esperar que las MER simples se transformen en MER complejas.

LOS ELEMENTOS CELULARES

La producción experimental de un DR en modelos animales fué, inicialmente, la única oportunidad para el estudio detallado del desarrollo del proceso de la VRP (Machemer and Norton,1968). El cuadro clínico que se ha observado en ojos de simios, se asemejaba aparentemente con la enfermedad proliferativa observada en ojos humanos, pero no la reproducía fielmente cuando se comparaban determinados aspectos. Los estudios por microscopía óptica y electrónica de las

primeras muestras de ojos humanos afectos de VRP y obtenidas por medio de cirugía intraocular (vitrectomía), sugirieron la discreta semejanza entre el modelo experimental y el proceso proliferativo humano (Van Horn *et al.*,1977). Conclusiones basadas en diversas observaciones mostraron que las células que proliferaban y sufrían metaplasia, derivaban, probablemente, del EPR (Machemer and Laqua,1975; Mandelcorn *et al.*,1975; Mueller-Jensen *et al.*,1975; Machemer *et al.*,1978; Machemer,1978) y de las células de la glia retiniana (Laqua and Machemer,1975a; Van Horn *et al.*,1977). A partir de estas descripciones iniciales, se han realizado infinidad de investigaciones con el objeto de analizar una simple etapa o un determinado aspecto del complejo proceso, todavía no bien comprendido, de la VRP.

Estudios con microscopía convencional, y más recientemente mediante cultivos celulares y técnicas inmunocitoquímicas, lograron mostrar la implicación en su génesis de las células del EPR, y las células gliales (astrocitos y células de Müller), así como la presencia de macrófagos (Kampik *et al.*,1981) y de fibroblastos (Hiscott *et al.*,1984; 1985), pero todavía quedan dudas en cuanto al origen de ciertas células de morfología semejante a los fibroblastos (*fibroblast-like cells*) identificadas en el proceso. Trabajos recientes, involucran la fibronectina (Miller *et al.*,1986) y la laminina (Scheiffarth *et al.*,1988), así como ciertos factores polipeptídicos de crecimiento (Campochiaro and Glaser,1985), en la patogénesis de VRP.

Células del Epitelio Pigmentario de la Retina (EPR)

Tras la aparición de un DR, se ha observado una liberación de células a partir del EPR "desnudo". Estas células, que originalmente poseen una disposición histológica en monocapa, y que son estabilizadas por el contacto célula-célula, pasan a migrar hasta la superficie externa de la retina desprendida o a través de las soluciones de continuidad de la MLI hacia el vítreo (Machemer and Laqua,1975; Machemer,1978). La presencia de estas células flotando en el vítreo, confiere a éste un aspecto punteado visible como "polvo de tabaco" (*"tabacco dust"*), descrito por Hamilton y Taylor (1972). Para ciertos autores, las células del EPR son sin duda las células clave en la formación de las MER complejas (Machemer and Laqua,1975; Johnson,1976; Machemer,1978; Newsome *et al.*,1981; Radtke *et al.*,1981), siendo también observadas frecuentemente en las MRR (Trese *et al.*,1985; Jerdan *et al.*,1987). Estas células dispersas en el vítreo, se distribuyen por acción de la gravedad, por corrientes de convección y a través de migración activa (Campochiaro and Glaser,1986), secretando factores mitógenos y quimiotácticos que inducen, a su vez, la proliferación de más células del EPR (Bryan and Campochiaro,1986).

La posibilidad de que parte de las células del EPR de tipo macrofágico puedan sufrir un proceso de metaplasia y asumir una morfología similar a fibroblastos y fibrocitos, fué propuesta por Machemer y Laqua (1975), así como en base a estudios *in vitro* realizados más recientemente (Vidaurri-Leal *et al.*,1984; Vidaurri-Leal and Glaser,1984); originalmente de forma cuboidea, las células de EPR toman una morfología elongada (semejante a fibroblastos) en presencia del vítreo.

Las células del EPR, no solamente inducirían la migración y proliferación de su propio tipo celular, sino también la de las células gliales (Campochiaro *et al.*,1984; Burke and Foster,1985; Hjelmeland *et al.*,1986), de monocitos (Robertson *et al.*,1986), así como de fibroblastos (Bryan and Campochiaro,1986).

Por otra parte, las células del EPR ejercen importantes fuerzas de tracción (Glaser *et al.*,1987). Así, poseen un sistema contráctil altamente desarrollado en su citoplasma con presencia de filamentos de actina, miosina, tropomiosina, vinculina y espectrina, conocidas por su capacidad contráctil (Opas and Kalnins,1986; Shinakawa *et al.*,1986). En su fenotipo cuboidal normal, el sistema de filamentos de actina, está organizado en forma de un anillo cortical citoplásmico (Opas and Kalnins,1986), que se presentará disperso en el citoplasma, en el caso de que estas células asuman una morfología semejante a macrófagos (Grierson *et al.*,1987). Por otro lado, bajo la forma de fibroblastos, las células del EPR agrupan sus microfilamentos como fibras de *stress* (Opas and Kalnins,1986) que

observamos normalmente en los miofibroblastos o en los fibroblastos contráctiles del tejido cicatricial (Sugita *et al.*,1980; Grierson and Rahi,1981).

Además, las inyecciones intravítreas de células del EPR en ojos de conejos (Radtke *et al.*,1981) y en ojos de simios (Grierson *et al.*,1986), producen DR traccional y alteraciones intraoculares generales semejantes a las encontradas en la VRP.

Así mismo tienen la capacidad de producir *in vivo* e *in vitro* colágeno del tipo I, II, III, IV, pero no del tipo V (Campochiaro *et al.*,1986). Rodrigues y cols. (1981), han detectado importantes cantidades de colágeno del tipo I y IV en MER traccionales. Otras glucoproteínas tales como la fibronectina y laminina, pueden también ser producidas *in vitro* por este tipo celular (Campochiaro *et al.*,1986).

Mediante la microscopía electrónica, células de características epitelioides se encontraron en muchas MER complejas (Clarkson *et al.*,1977; Kampik *et al.*,1981; Yamashita *et al.*,1986). No obstante, existe una dificultad marcada en la identificación de aquellas células que, estando presentes, tengan características intermedias entre epiteliales y fibroblastos. Las técnicas inmunocitoquímicas lograron evidenciar inequívocamente la implicación epitelial. Ciertas MER pueden estar constituidas por células del EPR en más del 90% de su población total, presentando un marcado positivo en presencia de anticuerpos contra citoqueratina, una proteína del citoesqueleto de las células epiteliales (Hiscott *et al.*,1984a). Por otro lado, la detección de citoqueratina, en muestras de membranas obtenidas quirúrgicamente, no ha podido ser confirmada por algunos autores (Weller *et al.*,1988b). En base a estos resultados, el comportamiento y el papel exacto de las células del EPR en el proceso proliferativo intraocular, todavía esta por dilucidar.

Células Gliales (Astrocitos y Células de Müller)

Ha sido descrita anteriormente (Laqua and Machemer,1975; Machemer,1978; Harada *et al.*,1981) la posibilidad de que las células de la glia retiniana puedan crecer, expandiéndose a partir de la MLI, sobre la superficie de la retina y dar lugar a la formación de MER localizadas, así como proyectarse hacia la superficie externa y cubrir grandes áreas de retina. El componente glial en la retina humana está formado por las células de Müller, verdadera "montura" de soporte retiniano, y los astrocitos confinados en las capas internas de la retina.

Resultados recientes obtenidos en modelos experimentales mediante microscopía electrónica de barrido, evidencian una capacidad peculiar de las células de Müller para proyectarse y evaginarse a través de microperforaciones en la MLI, ganando de esta manera la superficie interna de la retina (Ohsawa and Miki,1982; Hitchins *et al.*,1985). Sorprendentemente, la protrusión de estas células a través de la MLI, parece no ser un proceso puramente pasivo, según el cual la célula utilizaría una microsolución de continuidad preexistente, sino que tendrían la capacidad de ejercer fuerzas contra una MLI intacta por medio de sus pedículos.

Ha sido demostrado que la inflamación y el trauma ocular inducen la proliferación celular de la retina neural (Miller *et al.*,1986; Lean,1987) y que el citoesqueleto de las células gliales proliferativas es rico en filamentos intermedios, entre ellos la vimentina (Schnitzer,1985). Desafortunadamente la vimentina es un constituyente que ha sido identificado en muchos tipos celulares de origen mesenquimatoso y por ello tiene un valor limitado en la identificación de ciertas células de localización ectópica, como es el caso de un estudio sobre MER. Otro componente de los filamentos intermedios de la glia es la proteína glial fibrilar acídica (GFAP), fuertemente positiva en la inmunodetección y casi exclusiva para los astrocitos (Hiscott *et al.*,1984). En condiciones normales, las células de Müller son inmunonegativas para la GFAP (Bigmani and Dahl,1979), pero, tras una agresión retiniana, pasan a presentar una reacción altamente positiva en la totalidad de su citoplasma. (Hiscott *et al.*,1984; Bigmani,1979; Erickson *et al.*,1987). Este fenómeno concede una exclusividad diagnóstica con respecto a las células gliales de las MER y MRR, cuando utilizamos anticuerpos anti-GFAP para identificarlas de otros tipos celulares.

Harvey y cols. (1987), observaron que el Factor de crecimiento derivado de plaquetas (PDGF) es un factor quimiotáctico para las células gliales. El PDGF podría presentarse en el vítreo a partir de macrófagos invasores y derivados plaquetarios de la exudación plasmática. Las células gliales poseen también la capacidad de producir factores que estimulan la proliferación de células del EPR y fibroblastos (Burke and Foster,1985), lo que demuestra la multifactorialidad del proceso observado en la VRP.

Se ha propuesto que las células podrían adoptar, por medio de metaplasia, un fenotipo semejante a un fibroblasto, producir colágeno y ser responsables de las alteraciones en la superficie de la retina observadas en la VRP (Laqua and Machemer,1975; Van Horn *et al.*,1977; Kenyon *et al.*,1975). Realmente estas células pueden producir colágeno del tipo I *in vivo* e *in vitro* (Burke and Kower,1980), pero cuando células gliales en cultivo son inyectadas experimentalmente en la cavidad vítrea (Peters *et al.*,1986), observamos un DR traccional que no se asemeja en intensidad y complejidad al cuadro traccional observado cuando células del EPR (Fastenberg *et al.*,1982; Radtke *et al.*,1981) o fibroblastos (Hitchins *et al.*,1985; Sugita *et al.*,1980), son inyectadas en un experimento similar.

En base en estudios efectuados por microscopía electrónica (Kenyon *et al.*,1975; Kampik *et al.*,1981; Clarkson *et al.*,1977) y con técnicas inmunocitoquímicas utilizando anticuerpos contra la GFAP (Hiscott *et al.*,1984), se ha observado que las células gliales se encuentran presentes en una gran parte de las muestras de MER complejas, pero siempre como componente menor dentro de la población celular total (Hiscott *et al.*,1984; Yamashita *et al.*,1986). Clásicamente, están dispuestas en una fina capa celular involucrando poca matriz extracelular a su alrededor. Por otro lado, constituyen el mayor componente celular, si no el único, en las MER simples (Foss,1977; Roth and Foss,1971; Foss,1974). Este hecho podría explicar el motivo por el cual ciertas MER ofrecen menos dificultades en el acto de la disección, presentándose flexibles, con buena maleabilidad quirúrgica y algo menos adheridas sobre la superficie retiniana, contrariamente a otras membranas que en ocasiones producen dehiscencias sobre la retina cuando efectuamos su extracción quirúrgica. Esto nos haría considerar, también, el poder de contracción de estas MER simples que, formadas casi exclusivamente por células de la glia, alteran poco, o menos devastadoramente la superficie retiniana en la cual se presentan. En base a estas evidencias podríamos llegar a la conclusión de que, desde el punto de vista funcional, el proceso contráctil y el acumulo secundario de la matriz extracelular observados en las MER complejas, estarían poco relacionados con la participación de las células gliales. Aunque Hui y cols. (1988), demostraron la posibilidad de contracción de estas células, parece ser que su función principal, es la de servir como un punto de anclaje para la proliferación y la contracción de otros tipos celulares (Cleary and Ryan,1981; Hiscott *et al.*,1984).

Fibroblastos

Se presentan bajo forma fusiforme con un prominente retículo endoplásmico rugoso y un voluminoso complejo de Golgi en su citoplasma granuloso, comportamientos relacionados con intensa actividad secretora y de síntesis. Los miofibroblastos difieren de los fibroblastos normales, en que presentan grandes acumulos de filamentos de actina en su citoplasma. Gabbiani y cols. (1971), fueron quienes inicialmente observaron la presencia universal de los miofibroblastos en el tejido cicatricial de granulación. Por medio de estudios ultraestructurales, ambos tipos celulares han sido detectados como componentes de las MER complejas (Bellhorn *et al.*,1975; Kampik *et al.*,1981; Harada *et al.*,1981; Yamashita *et al.*,1986; Kampik *et al.*,1980).

Su papel en el proceso cicatricial fisiológico es evidente (Ross,1968) y parece ser importante para la reparación de la membrana de Bruch tras agresiones experimentales con fotocoagulación por laser (Pollack *et al.*,1986).

La interacción de un grupo de fibroblastos con su matriz colagénica, produciría su adhesión a la superficie retiniana y la formación de una "unidad de contracción". El conjunto de estas

"unidades" actuando de una forma no organizada, a diferencia del mecanismo observado en el curso natural del proceso cicatricial, podría explicar el fenómeno contráctil observado en la retina (Glaser *et al.*,1987; Hiscott *et al.*,1983). El origen de estos fibroblastos en las MER, es uno de los muchos puntos de controversia en la VRP. Tal como hemos considerado anteriormente, la posibilidad de que células de EPR adopten la forma de fibroblastos por medio de metaplasia o trasformándose en macrófagos ha sido descrita por Machemer (1978). Otro posible origen de la presencia de los fibroblastos, podría ser su transformación a partir de macrófagos. Gran parte de estos resultados, fueron obtenidos por medio de observaciones a partir de estudios experimentales con cultivos celulares y tisulares, por lo que puede no representar la realidad *in vivo* (Carr,1973). Inyecciones intravítreas de cierto tipo de endotoxina, fueron efectuadas experimentalmente en ojos de conejos, observándose la aparición de membranas inflamatorias similares a las MER complejas humanas (Hiscott *et al.*,1988). Las células inflamatorias son constantes hallazgos histológicos en la proliferación epirretiniana experimental (Hitchins *et al.*,1985; Sugita *et al.*,1980) y en muestras patológicas obtenidas de ojos humanos (Clarkson *et al.*,1977; Harada *et al.*,1981; Kampik *et al.*,1981).

Constatamos frecuentemente en nuestras observaciones clínicas, que en ciertas entidades intraoculares con características vítreoproliferativas, tales como la retinopatía diabética y la retinopatía de la prematuridad, los elementos vasculares se encuentran implicados en la formación de membranas fibrovasculares vítreorretinianas. Podríamos pensar que la adventicia vascular, pericitos y células endoteliales vasculares presentan una capacidad de transformación, por lo menos teóricamente, en fibroblastos o miofibroblastos contráctiles. A favor de todo ello, estaría la constatación de la presencia de abundantes filamentos citoplásmicos contráctiles en las células del sistema pericítico-endotelial (Gordon and Essner,1986) y el considerable potencial proliferativo y contráctil, similares a la musculatura lisa, de los pericitos en cultivo, así como el hecho de que también pueden asumir un fenotipo fibroblástico (Joyce *et al.*,1984).

Por último, los hialocitos constituyen la población celular original y nativa del vítreo. Son descritos algunas veces como histiocitos y otras como fibrocitos, involucrados en la producción de glucosaminoglucanos, pero no en la producción de colágeno en el vítreo maduro (Grabner *et al.*,1980). Harada y cols. (1981), los han identificado en MER humanas por medio de estudios microscópicos de su ultraestructura. Contrariamente, parecen inducir una respuesta proliferativa mínima tras las agresiones retinianas por medio de fotocoagulación (Gloor,1974). Hiscott y cols. (1984; 1985), demuestran un importante componente fibroblástico en las MER, normalmente avasculares, a pesar de que sabemos que los fibrocitos del tejido conectivo se encuentran normalmente en estrecha relación con vasos sanguíneos. En base a estas de estas evidencias ciertamente podríamos esperar que los fibroblastos identificados en las MER complejas, originadas en un proceso proliferativo a partir de una herida penetrante del globo ocular, derivarían, presumiblemente, de las capas oculares externas, e invadirían el vítreo por la misma puerta de entrada de la lesión original.

Macrófagos

El hecho de que la VRP pueda ser considerada como una forma modificada de una formación cicatricial vítreorretiniana (Gloor and Daiker,1975), caracterizada por una reacción inflamatoria inicial que conllevaría subsecuentemente a una proliferación celular (Miller *et al.*,1986), es de esperar con todo esto, que los monocitos fagocitarios puedan desempeñar un papel de importancia en el proceso. Leibovich y Ross (1975), han estudiado detalladamente el papel de los macrófagos en el curso natural de la reparación cicatricial. Tras eliminar experimentalmente los macrófagos de un tejido de granulación, por medio de antisuero anti-macrófagos o de la hidrocortisona, se observaron dos hechos importantes en el proceso cicatricial. En primer lugar se constató la dificultad del desbridamiento de la herida, prueba del protagonismo principal de estas células en la formación del tejido conjuntivo cicatricial. En segundo lugar, se observó un aumento en el tiempo de aparición del tejido fibroso, a medida que la intensidad del proceso disminuía. Por otro lado, la

inyección intravítrea experimental de una cantidad de macrófagos en cultivo, provoca, tras una reacción inflamatoria, el desprendimiento del vítreo (Hui *et al.*,1987).

Sin embargo estas células se han descrito en pocas ocasiones como constituyentes de MER complejas, en base a estudios morfológicos (Kampik *et al.*,1981) o bien identificadas por métodos inmunocitoquímicos con anticuerpos monoclonales y amplificados por medio del complejo peroxidasa-anti-peroxidasa (Weller *et al.*,1988). A pesar de la especificidad de la técnica utilizada por Weller y cols. (1988), sólo se estudiaron tres MER originadas de VRP post-traumáticas; todo lleva a pensar en la estrecha relación de este tipo celular con la formación de MER tras el trauma. Un punto fundamental a ser tenido en cuenta con relación al experimento anterior, sería la asociación o no, de cada uno de estos tres casos con una perforación ocular o la presencia de una hemorragia intravítrea como consecuencia del traumatismo ocular.

De modo diferente a los fibroblastos, los macrófagos tendrían su posible origen más claramente establecido. Podrían provenir de la sangre, por extravasación plasmática tras la ruptura de la barrera hemato-retiniana (Gloor,1974; Campochiaro *et al.*,1986a), o consistir en modificaciones metaplásicas de las células del EPR, según ciertos autores (Machemer and Laqua,1975). Debido a sus características morfo-funcionales próximas a la de los macrófagos, los hialocitos podrían representar la fuente vítrea para este tipo celular. Hasta el presente, la detección de hialocitos en MER no ha podido ser formalmente demostrada (Harada *et al.*,1981).

El relevante papel fagocitario observado en la reacción inflamatoria y en el proceso cicatricial, es de lejos la función primordial de los macrófagos activados. Estos poseen múltiples mecanismos de activación sobre los cuales ejercen su acción los mediadores humorales (Adams and Hamilton,1984). Trabajos recientes (Mayer *et al.*,1981; Akiyama and Yamada,1987), atribuyen a la fibronectina, entre otras funciones, importantes características de adhesión y estimulación de la proliferación celular. Los fibroblastos y los macrófagos, poseen en sus respectivas membranas plasmáticas, receptores para la fibronectina (Alitalo *et al.*,1980), así como la capacidad de sintetizarla *in vitro* (Freundlich and Avdalovic,1983; Akiyama and Yamada,1987). Paralelamente a estas habilidades, la fibronectina refuerza los procesos de fagocitosis opsonino-dependiente (Pommier *et al.*,1983) y opsonino-independiente (Czop *et al.*,1981). La opsonización es el proceso por el cual anticuerpos unidos o no con intermediarios del sistema del complemento (C3b), desencadenan el estímulo para la fagocitosis de un microorganismo o de un antígeno extraño. Teóricamente, podríamos pensar que las interacciones macrófago-fibronectina y fibroblasto-fibronectina puedan representar etapas vitales en los fenómenos celulares observados en la VRP.

LOS ELEMENTOS EXTRACELULARES

Las MER son sin duda, junto al vítreo patológico, las únicas fuentes de estudio directo en el intento de comprensión del proceso proliferativo intraocular humano. Contrariamente a lo que sucede en los estudios morfológicos celulares y de su estructura, la literatura carece de investigaciones con respecto al entorno extracelular y sus elementos. La matriz extracelular es fundamental para la estructura, diferenciación y proliferación de los componentes celulares de las membranas vítreorretinianas y epirretinianas. Su interacción con el componente celular hace que desempeñe un papel importante en los fenómenos de migración, proliferación y contracción que conllevan a la aparición del DR traccional.

Colágeno

La presencia de colágeno de los tipo I, II, III, IV y V ha sido observada en estudios mediante inmunofluorescencia (Hiscott *et al.*,1985; Scheiffarth *et al.*,1988). Parecen ser constituyentes frecuentes de las MER de distintas etiologías (Jerdan *et al.*,1986).

Los tipos I y III del colágeno, han sido demostrados como componentes frecuentes de la matriz extracelular en las MER de la VRP, tanto de etiología idiopática como post-traumática.

Típicamente han sido observados en el tejido conectivo y cicatricial (Furthmayr and Mark,1982), pudiendo ser sintetizados por cualquiera de los tipos celulares implicados en la fisiopatogénesis de la VRP (Campochiaro *et al.*,1986; Furthmayr and Mark,1982).

El colágeno del tipo II, es una conocida proteína de los cartílagos (Mark *et al.*,1982), siendo también el principal constituyente del vítreo y de la retina neurosensorial (Newsome *et al.*,1976). Parece estar ausente en las MER de la VRP idiopática. Está, sin embargo, presente en muestras post-traumáticas, post-inflamatorias y sobre todo en la retinopatía diabética proliferativa, y parece ser el colágeno predominante en las membranas de la VRP (Jerdan *et al.*,1987). Esto nos sugiere una cierta similitud en los mecanismos fisiopatológicos involucrados en la formación de MER de distintas etiologías.

El colágeno del tipo IV, posee estrecha relación con la laminina, otra glucoproteína de la matriz extracelular. La última es por excelencia la proteína que está estructuralmente vinculada a las láminas basales, promoviendo la adhesión célula-colágeno tipo IV (Wick *et al.*,1979). Investigaciones actuales han demostrado la capacidad de las células del EPR, de producir este tipo de colágeno *in vivo* e *in vitro* (Campochiaro *et al.*,1986; Turksen *et al.*,1985).

El colágeno del tipo V, se caracteriza principalmente por estar ampliamente presente en las lesiones fibróticas. Modesti y cols. (1984), relatan su peculiar capacidad de producir filamentos dispuestos en el intersticio pericelular, principalmente observados en células de la musculatura lisa. Llama la atención que estas microestructuras puedan tener alguna propiedad contráctil y consecuentemente estar implicadas en el mecanismo de contracción muscular. No obstante la identificación de este tipo de colágeno, por técnicas inmunocitoquímicas con fluorescencia en las MER, ha sido dudosa (Scheiffarth *et al.*,1988), así como la posibilidad de su producción en cantidades mensurables por células del EPR en cultivos o *in vivo* (Campochiaro *et al.*,1986).

Análisis morfológicos realizados recientemente, determinan una relación directa entre la cantidad de colágeno y el tiempo de la enfermedad proliferativa intraocular, con una clara predominancia de colágeno en membranas de más largo tiempo de evolución (Hiscott *et al.*,1985).

Fibronectina

La fibronectina es una glucoproteína macromolecular, responsable de gran número de funciones en el plasma y en la matriz extracelular, promoviendo la adhesión y migración celular. Otras, de entre muchas capacidades bien conocidas, son la de inducir la síntesis de proteínas del citoesqueleto celular y su estrecha participación en el proceso fagocitario (Akiyama and Yamada,1987; D'Ardenne and McGee,1984; Grinnell *et al.*,1981; Ruoslahti *et al.*,1981). Sin embargo, debido a la multitud de sus funciones biológicas, su papel en el proceso proliferativo de la VRP resulta todavía poco comprendido.

Sabemos actualmente que las glucoproteínas de la matriz extracelular y el colágeno se intercorrelacionan con los elementos celulares, permitiendo una interacción de sucesos intra y extracelulares, lo que confiere a la fibronectina un gran interés en el proceso reparacional (Ruoslahti and Pierschbacher,1987).

Esta proteína ha sido implicada en la fisiopatogénesis de la VRP por primera vez en 1985, cuando Campochiaro y cols. la detectaron en elevadas concentraciones examinando aspirados vítreos obtenidos durante la vitrectomía de pacientes portadores de VRP. Estos valores extremadamente altos no pudieron ser corroborados en una serie de pacientes recientemente estudiados (Weller *et al.*,1988a). A diferencia de los primeros resultados, Weller y cols. (1988a) incluyen en su estudio un grupo control formado por muestras vítreas obtenidas de ojos *post-mortem* en los cuales no se pudo identificar la proteína, incluso utilizando métodos para la amplificación de los resultados. Aunque con estos resultados contradictorios, los hechos nos llevan a considerar la presencia de la fibronectina como un posible factor fuertemente indicador, de un proceso vítreoproliferativo activo. Otro dato de interés en esta investigación, reside en la constatación de elevadas concentraciones intravítreas de la proteína

en los casos de VRP idiopática, traumática y en ojos revitrectomizados cuando se compararon con los casos de DR por desgarro gigante de presentación aguda. Los últimos, sometidos a vitrectomía inmediatamente tras la manifestación del cuadro clínico, no mostraron indicios de fibronectina en el vítreo. Esto indica el posible origen plasmático de esta glucoproteína, ya que en los casos traumáticos se ha asociado una hemorragia en el vítreo y, en cambio, el tiempo transcurrido tras la ruptura de la barrera hemato-ocular observada en el grupo de pacientes con DR por desgarro gigante, no ha sido suficientemente largo para permitir el paso de cantidades significativas de fibronectina hacia el vítreo.

Por otra parte, su capacidad de estimulador quimiotáctico para la migración de fibroblastos y células del EPR, es bien conocida (Mensing *et al.*,1983; Campochiaro *et al.*,1986) así como su afinidad de adherirse a receptores de la superficie celular, colágeno, heparina, actina, fibrina y otras proteínas extracelulares y polisacáridos (Ruoslahti and Pierschbacher,1987). Estudios inmunocitoquímicos revelaron su frecuente presencia en MER de distintas etiologías, sugiriendo su contribución significativa para la estabilización de la trama fibrilar producida en la interfase vítreorretiniana (Hiscott *et al.*,1985). Actuando en la interacción célula-substrato, la fibronectina es un potente mediador para la adhesión célula-célula (Pierschbacher and Ruoslahti,1984), hecho éste, básico y necesario para el inicio del proceso proliferativo con una subsecuente membranogénesis.

Su posible origen celular puede ser considerado en relación con su producción *in vivo* e *in vitro* por células del EPR (Campochiaro *et al.*,1986), por los fibroblastos (Akiyama and Yamada,1987) y macrófagos humanos (Alitalo *et al.*,1980). Así podemos hipotetizar que la presencia de la fibronectina puede ser observada, en todas las etapas de la VRP: tempranamente, promoviendo la migración y la adhesión celular para después, presumiblemente, teniendo su producción garantizada por estas mismas células, estabilizar el proceso proliferativo con el acumulo de colágeno, confiriendo así un cierto comportamiento cíclico en los eventos relacionados con los elementos celulares y extracelulares. Seguramente no es un factor patognomónico de la VRP, pero su relevancia en las alteraciones intraoculares proliferativas debe ser investigado.

Laminina

La laminina y el colágeno del tipo IV, son proteínas estructurales de las láminas basales. La primera promueve la adhesión celular al colágeno del tipo IV (Terranova *et al.*,1980). Su participación en el mecanismo proliferativo de la VRP apenas ha sido sugerido, pero al examinar los resultados presentados en trabajos recientes, esta proteína parece tener una actuación más moderada, en comparación a los otros elementos claves.

Essner y Lin (1988), la identificaron por medio de inmunolocalización en la lámina basal de los capilares retinianos, arteriolas y vénulas de ratas. Sabemos que la lámina basal circunda las células endoteliales y pericitos de los capilares retinianos, como también el endotelio y células del músculo liso de los grandes vasos. La fibronectina presente en la interfase pericíto-endotelio, sugiere un posible papel en la integridad de los vasos ya que promueve la adhesión entre células (Essner and Lin,1988). La lámina basal capilar y los pericitos adquieren particular interés en la actualidad por los cambios morfológicos y bioquímicos que sufren en ciertas enfermedades vasculares, entre ellas la diabetes. Así, ambas proteínas podrían estar involucradas en la angiogénesis y en el proceso proliferativo intraocular observados en la retinopatía diabética, entidad en la cual la formación de membranas vítreorretinianas con características fibrogliovasculares es bien conocida. Scheiffarth y cols. (1988), lograron identificar ambas proteínas y el colágeno del tipo IV, en la casi totalidad de una serie de MER (n=13) extraídas de los ojos de pacientes portadores de retinopatía diabética proliferativa, sometidos a tratamiento quirúrgico.

El EPR esta interpuesto entre la retina neurosensorial y la coroides altamente vascularizada. Esta situación estratégica, no solamente interesa a la adhesión retiniana sino también constituye un obstáculo al paso de vasos hacia la retina. En su situación basal, el EPR contacta con la membrana de Bruch, la cual ejerce la función de una barrera sólida contra la vascularización en el

espacio subretiniano (Hogan,1967). Utilizando marcadores específicos, Campochiaro y cols. (1986) demuestran que es posible identificar la laminina, entre otras varias moléculas, en la membrana de Bruch y alrededor del EPR *in vivo*, cuando son estimulados en cultivos celulares. Así las células del EPR pueden producir los componentes de la matriz extracelular encontrados en la membrana de Bruch, sugiriendo una probable relación de cooperación entre ambas estructuras, para que se conserve el mantenimiento de esta barrera física. La ruptura de esta interacción, podría llevar a alteraciones funcionales de la membrana de Bruch, como por ejemplo la aparición de un material extracelular fibrilar o no fibrilar en sus capas, conocido por el nombre de "drusas" (Green and Key,1977).

La adhesión de la corteza vítrea a estructuras vecinas, se hace principalmente por intermedio de la MLI y la membrana basal de las células de Müller. Un aumento del espesor de la MLI relacionado con la senilidad, ha sido un hallazgo común en ojos humanos (Gartner,1970); este hecho podría estar relacionado con el DVP. Este, como ya mencionamos anteriormente es condición esencial para innumerables alteraciones vítreorretinianas, tales como el DR regmatógeno traccional o la retinopatía diabética proliferativa (Yanoff and Fine,1975). Tras el estudio de ojos humanos *post-mortem*, por medio de técnicas de inmunofluorescencia, Kohno y cols. (1987) señalaron que en la región retiniana del polo posterior de los ojos jóvenes, la MLI exhibía un patrón lineal, aunque ocasionalmente bilaminar, para la laminina y la fibronectina. Además, el patrón bilaminar estuvo presente en todos los ojos seniles examinados. Este hecho podría estar relacionado con el aumento del espesor de la MLI en el polo posterior, representado por el patrón bilaminar de la MLI. Por lo tanto el DVP, fenómeno correlacionado estrictamente con la edad, podría representar el resultado de determinadas alteraciones bioquímicas en la MLI.

Polipéptidos

Actualmente, las investigaciones sobre los polipéptidos (factores de crecimiento derivados del suero) en el vítreo, producidos por células nativas, dan lugar a numerosas hipótesis sobre la regulación de la intensidad proliferativa y la síntesis de sustancias extracelulares en los variados estadíos de la VRP.

La fagocitosis de partículas extrañas es una función importante observada y realizada por varias estructuras oculares. Para tal función, las células deben ser activadas donde sea necesario, detectándose en caso de tal activación, una posible intensificación de su capacidad fagocitaria (Rohen and Van der Zypen,1968). Un ejemplo práctico es lo observado en los casos de hemorragias, espontáneas o no, en la cámara anterior donde observamos las células de la malla trabecular, fagocitando activamente los eritrocitos (Grierson and Lee,1973; 1978). El ***Factor de Crecimiento Derivado de Plaquetas*** (PDGF) es conocido por facilitar la endocitosis de varios tipos celulares (Davies and Ross,1978) y alterar la distribución intracelular de actina, hecho que parece estar estrechamente relacionado con la fagocitosis (Allison,1972). Pero su versatilidad no se resume solamente en su supuesta implicación en el proceso de la fagocitosis. Campochiaro y Glaser (1985), observaron su potencia como quimiotáctico en relación con las células del EPR humano *in vitro*, estimulando además la migración y la proliferación de las mismas (Campochiaro and Glaser,1986; Campochiaro *et al.*,1986a), así como también las de las células gliales retinianas (Harvey *et al.*,1987).

Su participación en el proceso proliferativo intraocular gana importancia tras constatarse la sustancial elevación de sus niveles intravítreos, evidenciados por radioinmunoensayo, en muestras obtenidas de pacientes portadores de VRP (Liggett *et al.*,1986). Así, un modelo experimental clínicamente semejante al cuadro de una VRP ha podido ser apreciado tras inyecciones intraoculares de PDGF y fibronectina (Yeo *et al.*,1986).

El ***Factor de Crecimiento Transformante Beta*** (TGF-β), es un polipéptido altamente activo, que fue aislado inicialmente a partir de su producción por determinadas células tumorales caracterizado por su capacidad para inducir la transformación fenotípica (Roberts *et al.*,1980). Posteriormente, se ha detectado su producción por una gran variedad de células normales y neoplásicas

(Roberts *et al.*,1983).

El efecto del TGF-β sobre cultivos celulares varía dependiendo de las condiciones del cultivo y del tipo celular, pudiendo estimular la proliferación de ciertas células mesenquimatosas. Esta estimulación parece mediarse a través de la activación de la expresión de otros factores de crecimiento, tales como el PDGF. Este hecho sugiere que la actividad mitógena de ciertos grupos celulares podría ser el resultado de la interacción entre varios factores de crecimiento, entre ellos los anteriormente citados (Leof *et al.*,1986). Por otro lado, varios investigadores han observado su efecto inhibitorio sobre el crecimiento de las células epiteliales (Tucker *et al.*,1984) y sobre la proliferación de las células endoteliales vasculares (Schroder *et al.*,1986). Más recientemente, Mustoe y cols. (1987) han evidenciado su posible papel en la potenciación del proceso cicatricial a partir de estimular una mayor presencia de macrófagos y fibroblastos en el lugar de la lesión. Ignotz y Massagué (1986) demuestran su capacidad para incrementar la síntesis de proteínas, como la fibronectina y colágenos en varios grupos celulares. Parecen existir evidencias del aumento de los niveles intravítreos de este polipéptido en ojos con enfermedad proliferativa (Glaser *et al.*,1988).

El estímulo angiogénico para la neoformación de vasos sanguíneos *in vivo* ocurre en ciertas situaciones patológicas. Un potente mitógeno para diferentes tipos de células, particularmente para las células del endotelio vascular, es el ***Factor de Crecimiento Fibrobrástico básico*** (FGFb) (Gospodarowicz *et al*,1986), el cual se nos presenta como un importante inductor de vasos de neoformación en determinados modelos animales. Baird y cols. (1985) refieren que las concentraciones vítreas de FGF están elevadas en pacientes con retinopatía diabética proliferativa, sugiriendo un supuesto papel en la neoformación vascular pre-retiniana. Sabemos que el proceso proliferativo en la VRP se caracteriza por su avascularidad, siendo infrecuentes los hallazgos de células del endotelio vascular en las MER cuando se han utilizado métodos de inmunolocalización con anticuerpos anti-Factor VIII. No obstante, su presencia en el proceso proliferativo no está descartada.

CONCLUSIONES

La Vitreorretinopatía proliferativa está caracterizada por una proliferación celular consiguiente a una agresión de las estructuras intraoculares, tales como la retina y el vítreo. La traducción histopatológica de este proceso se encuentra representada por la formación de membranas epirretinianas y vítreas, que predisponen a un desprendimiento traccional de retina. Su curso natural, con la formación de este tejido conjuntivo, podría representar un ***micro proceso cicatricial*** en el intento de restituir la integridad estructural dañada.

Los numerosos estudios clínicos propuestos acerca de su patogénesis inducen a pensar que sólo un tratamiento quirúrgico bien conducido constituye la única, aunque no definitiva, solución para esta compleja entidad clínica. En los últimos años, los estudios experimentales han contribuido a aclarar numerosos e importantes aspectos que caracterizan la peculiaridad de cada una de las etapas de este proceso multifactorial.

Las investigaciones mediante microscopía convencional y electrónica, realizadas en los últimos diez años, se han beneficiado ampliamente de la especificidad de las técnicas de inmunolocalización, reveladoras de los tipos celulares implicados en la formación de las membranas retinianas responsables de las fuerzas de tracción sobre la retina. No obstante ello, el origen de una gran proporción de los tipos celulares no identificados y clasificados como *fibroblast-like cells*, permanece oscuro. Un primer grupo de membranas epirretinianas (*Complejas*) se caracterizada por presentar varios tipos celulares, con el predominio de células del epitelio pigmentario retiniano. El segundo grupo (*Simples*), por otra parte, tan sólo posee un tipo celular.

Del mismo modo que los elementos celulares, los componentes extracelulares poseen un papel importante en la migración, la adhesión, la proliferación y la contracción de la masa celular, con la subsecuente estabilización del proceso. De entre ellos, la fibronectina y la laminina, glucoproteínas de la matriz extracelular, presentan actividades multifuncionales en las principales

etapas de la enfermedad proliferativa. Sus respectivos mecanismos de acción en la enfermedad proliferativa intraocular son aun poco conocidos.

Aparentemente, los elementos humorales (glucoproteínas y polipéptidos) funcionan en la vitreorretinopatía proliferativa como moduladores específicos en todo el proceso. Estos factores no están limitados a una simple actividad fisiológica, sino que participan y/o desencadenan las diferentes etapas del proceso.

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Retinopatía Diabética: Patogenia y fisiopatología celular.

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Resumen: Las complicaciones oculares producidas por la diabetes son consideradas actualmente como una de las principales causas de ceguera. Así mismo los mecanismos fisiopatológicos que envuelven la retinoapatía diabética son poco conocidos. Las alteraciones estructurales en el estado hiperglucémico, los factores del micro-ambiente intraocular y la acción de ciertas proteínas del plasma, así como el comportamiento de las células endoteliales y los pericitos frente a estímulos angiogénicos, contribuyen al desarrollo de la etapa proliferativa en la enfermedad intraocular. Los progresos en el ámbito clínico y farmacológico se han beneficiado de la utilización de los modelos animales, así como de las técnicas modernas de biología celular y molecular, contribuyendo a mejor comprensión del entorno celular en las condiciones del metabolismo diabético. Se han purificado y secuenciado nuevos péptidos y proteínas a partir de tejidos normales y tumorales, constituyendo nuevos progresos en el conocimiento de los fenómenos biológicos que subyacen a la neoformación vascular, migración, proliferación y diferenciación de las células endoteliales. En este trabajo revisamos algunas de estas aportaciones recientes, intentando centrar el comportamiento de la célula y su entorno en las principales situaciones fisiopatológicas que caracterizan las diferentes fases de la retinopatía diabética.
Palabras clave: Retinopatía diabética proliferativa, neovascularización, células endoteliales, pericitos, factores de crecimiento, angiogénesis, matriz extracelular.

Diversas investigaciones clínicas y experimentales sugieren en la actualidad que la aparición y la evolución de la retinopatía diabética está caracterizada por un proceso multifactorial complejo, por lo que formular un concepto unificado sobre su patogénesis constituye una tarea difícil. Aunque las manifestaciones de la retinopatía diabética la presentan como una enfermedad microvascular, su fisiopatología refleja incuestionablemente disturbios metabólicos, ultraestructurales, endocrinos y hemorreológicos. Las alteraciones celulares son consecuencia del desequilibrio del metabolismo de los hidratos de carbono, caracterizado por una hiperglucemia e hipoinsulinemia, asociadas además a los disturbios en las síntesis proteica y lipídica. Estos cambios imponen modificaciones del sistema vásculo-endotelial con un importante deterioro de su integridad y estructura. La respuesta característica de la retina frente a la diabetes mellitus es una sucesión compleja de acontecimientos que, al ser analizada como un fenómeno individual, podría ser entendida como un esfuerzo en la reparación tisular tras una agresión, con evolución y presentación clínica particulares.

A fin de considerar el comportamiento celular con respecto a aspectos clinicopatológicos y experimentales de la enfermedad, podemos considerar como única la respuesta de la red microvascular retiniana frente a la agresión diabética. No obstante, debemos pensar que las lesiones típicas de la retinopatía diabética son el reflejo de una respuesta vascular generalizada en estos organismos, que se ha modificado en el complejo ambiente intraocular.

ALTERACIONES CELULARES Y ESTRUCTURALES

Ciertas alteraciones observadas en el sistema vascular nos llevan a considerar la manera en que el entorno diabético afecta el comportamiento y la replicación celular.

El ***engrosamiento de la lámina basal*** capilar ha sido ampliamente observado en la diabetes humana y así como en la experimental (1-4). Además, se ha observado una vacuolización del colágeno normal de las láminas basales con la deposición de colágeno fibrilar, sin saberse exactamente los procesos bioquímicos y fisiológicos que conllevan a esta particular alteración (5). El colágeno es el principal elemento macromolecular de las láminas basales pero otras moléculas, presentes en menor

cantidad, son también funcionalmente importantes. De entre ellas destacamos la laminina (6) y los proteoglicanos, entre los cuales mencionamos los heparan sulfatos como responsables estructurales de las láminas basales (7). La fibronectina, una glucoproteína fundamental en el mecanismo de adhesión célula-substrato, representa otro componente estructural para las láminas basales, pero su papel es todavía controvertido (8). En la diabetes experimental en ratas (9), se ha observado que una de las principales lesiones bioquímicas en las láminas basales, es la importante reducción en la producción de heparan sulfato, con síntesis secundaria aumentada de colágeno. Sabemos que estas láminas tienen primordialmente una función de sostén, contribuyendo a la rigidez estructural de ciertos sistemas, como los vasos sanguíneos. Otra posible propiedad es la de filtro molecular de paso, relacionado con el peso molecular de las partículas, como se puede observar en la membrana de Bruch del epitelio pigmentario de la retina (10). En la actualidad también se reconoce el papel de las láminas basales en la proliferación y diferenciación celular (11). Varios componentes de la matriz extracelular, que participan en la interacción con el substrato, parecen estimular e inhibir la proliferación y crecimiento de las células endoteliales vasculares en cultivo (12). Igualmente, diferentes tipos celulares asumen morfología diferenciada cuando, *in vitro*, son cultivados en un substrato enriquecido con colágeno (13,14).

Tras el inicio de la enfermedad diabética humana y experimental se ha observado, como alteración primaria en los glomérulos renales, un rápido acúmulo de material matricial producto de un aumento en su síntesis (15). El aumento de la síntesis de los componentes de la matriz glomerular ha sido ampliamente descrito en estudios experimentales (16,17). No obstante, se desconoce si la alteración sería originaria de la propia matriz o dependería de un cambio celular en alguna de las etapas del proceso de síntesis, que estaría regulada por el ambiente diabético. Otra explicación para el acúmulo de material matricial es el aumento de su síntesis en respuesta a agresiones celulares de diversa naturaleza (18).

La ***pérdida de los pericitos intramurales*** constituye otra alteración observada en los estadíos iniciales de la patología (19,20). Los pericitos son células de origen mesodérmico que, ensamblados en la lámina basal, envuelven los capilares y se relacionan íntimamente con las células endoteliales, sin contacto directo con los procesos intraluminales (21). En la vascularización retiniana normal observamos una elevada relación pericito-célula endotelial de 1:1, alterándose precozmente en el curso de la enfermedad por la desaparición de los primeros (19). La renovación de las células endoteliales en los capilares retinianos bajo condiciones normales parece ser extremadamente pequeña (22), aumentando en situaciones de isquémia (23) o agresiones físico-químicas (24). Los pericitos muestran muy bajo (22) o ningún potencial de renovación celular (25). Por medio de técnicas histológicas, donde se ha utilizado una digestión enzimática mediante tripsina, se han podido observar espacios ocupados parcialmente por núcleos de pericitos intramurales degenerados a lo largo de la lámina basal de capilares retinianos en pacientes diabéticos (26). Estos pericitos "fantasmas" son consecuencia de un mecanismo todavía desconocido de pérdida selectiva, observado en las etapas tempranas de la enfermedad. Recientemente, se ha observado *in vitro* que los pericitos inhiben la proliferación (27) y la migración (28) de las células endoteliales por medio del factor de crecimiento transformante-β en su forma activada, sintetizado solamente cuando se establece el contacto pericito-célula endotelial (27,28).

Los ***capilares acelulares*** han sido ampliamente descritos en la retina (19,29), en la musculatura estriada (30), y en los glomérulos renales (31) de pacientes diabéticos de larga duración. Parece constituir la lesión microvascular más avanzada en la retinopatía; estudios clínico-patológicos evidencian la no funcionalidad de la microcirculación en estos capilares (32,33), constituyendo el paso inicial para la aparición de la isquémia y subsecuente proliferación neovascular. Se desconocen los motivos y la secuencia de circunstancias que llevan a la pérdida total de los elementos celulares en la red capilar retiniana. Un mecanismo de disminución de la longevidad de las células, consecuencia del deterioro celular con su desaparición precoz, asociado al bajo potencial de renovación celular,

podrían explicar esta alteración.

La observación clínica más precoz de la retinopatía son los ***microaneurismas*** (19,32,34,35), formaciones saculares debidas a dilataciones localizadas en las paredes de los capilares, y que, desde el punto de vista histopatológico, pueden ser acelulares o hipercelulares (19). Actualmente existen evidencias de que los pericitos actúan como elementos contráctiles en las paredes capilares, a semejanza de la musculatura lisa presente en los grandes vasos (36,37). El tono ejercido por los elementos fibrilares contráctiles de los pericitos es suficiente para contrarrestar la presión transmural producida por la microcirculación sanguínea; desapareciendo el pericito desaparecerá el tono, originando las dilataciones focales y localizadas de la pared vascular (microaneurisma). No obstante, se observa la formación de microaneurismas retinianos en ciertas enfermedades que frecuentemente no presentan alteraciones pericíticas (38). Muchos autores se refieren a la hipercelularidad de los microaneurismas retinianos como una "proliferación endotelial" (19,39), interpretándola como un intento fallido de neovascularización (40) o, simplemente, como un proceso reparativo desencadenado por la pérdida selectiva de los pericitos y subsecuente adelgazamiento de la pared capilar (19).

LA BARRERA HEMATO-RETINIANA (BHR)

El sistema vascular está separado de la retina neurosensorial por dos barreras anatómicas diferentes. La primera barrera se localiza a nivel de las células del epitelio pigmentario de la retina - que posee uniones intercelulares del tipo *zonulae occludentes* - que separa la coroides, altamente vascularizada, de las capas de la retina externa (*BHR externa*). Por otro lado, otra barrera se sitúa a nivel del endotelio de los capilares retinianos (*BHR interna*), que lo separa de las capas retinianas internas. La función de estas barreras, es la de impermeabilizar el ambiente intraocular de la presencia de moléculas de elevado peso molecular (41). El hecho que la ruptura de la BHR se presenta en muchos pacientes diabéticos, en ausencia de cualquier lesión vascular retiniana demostrable, sugiere que esta alteración podría ocurrir a ambos niveles y de una forma precoz (42), incluso antes de la aparición clínica de la retinopatía.

Estudios realizados mediante microscopía electrónica sugieren que una de las posibles causas de esta ruptura podría ser la apertura de las *tight junctions* existentes entre las uniones de los procesos endoteliales microvasculares adyacentes (43). Otra disfunción endotelial que podría contribuir a la desaparición de esta barrera, serían las "fenestraciones" de las células endoteliales. Normalmente estas porosidades están ausentes en el endotelio capilar retiniano normal, pero se observan en los vasos de neoformación retinianos, en los cuales la BHR se había alterado (44,45). Una posible explicación anatómica para el aumento de la permeabilidad de la BHR se basa en estudios experimentales en animales, en los cuales se ha observado un aumento temprano y progresivo de la permeabilidad a la fluoresceína sódica (P.M. 330 D). En las células del epitelio pigmentario de la retina de ratas diabéticas se han observado alteraciones ultraestructurales progresivas a medida que aumentaba el tiempo de la enfermedad. Estas alteraciones fueron caracterizadas como invaginaciones de la membrana plasmática en la superficie basal adyacente a la coriocapilar. Se ha postulado que estas invaginaciones pudieran contribuir al transporte activo o pasivo de moléculas (46).

LA MATRIZ EXTRACELULAR Y LOS MECANISMOS BIOQUIMICOS

El aumento de la síntesis de matriz extracelular por las células vasculares en la diabetes, puede ser entendido como parte de un programa de actividad biosintética alterada, en la cual la célula intenta adaptarse a un nuevo ambiente particular y nocivo que está modulado por múltiples factores. La hipoxia retiniana parece ser la agresión común en la retinopatía diabética y en otras enfermedades vasoproliferatives menos frecuentes, que evolucionan con alteraciones semejantes caracterizadas clínicamente por la triada "hipoxia-neovascularización-proliferación fibrogliovascular" (47).

Los mecanismos productores de la hipoxia retiniana han sido estudiados fundamentalmente en el contexto celular y a partir del metabolismo de los componentes sanguíneos, dando a conocer las alteraciones en el ámbito de su reología: las *alteraciones hemodinámicas* y de la

hemostasis (48). Se ha observado una reducción en las tasas de la *2,3*-difosfoglicerato en los eritrocitos con la disminución de fosfatos inorgánicos plasmáticos en los pacientes diabéticos de difícil control clínico. Esta reducción de *2,3*-difosfoglicerato se relaciona con la disminución en la liberación del oxígeno por la hemoglobina (49). La afinidad de la hemoglobina hacia al oxígeno se altera incrementandose su fracción A_{1C}. Esta es responsable de una mayor afinidad hemoglobina-oxígeno, pero actúa como un factor de bloqueo para su liberación, en la cual interviene la *2,3*-difosfoglicerato (50). La insuficiencia insulínica o la insensibilidad celular a ella, permite un desvío del metabolismo normal de la glucosa hacia la ***vía del poliol***, que conlleva la producción y acúmulo del sorbitol y fructosa. El aumento de la presión osmótica que se observa, como consecuencia del sorbitol intracelular, conlleva a un edema de las células endoteliales con la disminución de los cambios intercelulares de difusión, contribuyendo así a un estado tisular de hipoxia (51). Las alteraciones en los elementos sanguíneos son bien conocidas y se caracterizan por la hipercoagulabilidad con aumentada agregación eritrocítica (52), una hipersensibilidad funcional de las plaquetas (53), y una disminución en la respuesta fibrinolítica (54). Las alteraciones de la hemodinámica microvascular, observadas precozmente, que llevan al aumento de la presión capilar e invasión tisular por proteínas plasmáticas se ha propuesto como el mecanismo fisiopatológico más importante de la aparición de la microangiopatía (55). Aunque la complejidad de las alteraciones hemodinámicas y sus consecuencias fueron establecidas a nivel glomerular (56), existen ciertas controversias con respecto al comportamiento de la microcirculación en el ambiente retiniano (57). Estas manifestaciones hemorreológicas observadas en un microsistema circulatorio aislado e "impermeable" como es el sistema microcapilar de la retina con su barrera hemato-retiniana, junto al engrosamiento de la membrana basal capilar con estrechamiento de su lumen, pueden explicar de una forma sencilla la disminución y la lentificación del flujo micro-circulatorio. Esto es un posible y significativo mecanismo que contribuiría al origen del entorno hipóxico y sus consecuencias.

De entre los mecanismos bioquímicos supuestamente implicados en la patogénesis de la retinopatía diabética, el estado mantenido y prolongado de ***hiperglucemia***, ha sido considerado como el principal mecanismo etiológico de muchas de las eventuales alteraciones funcionales y anatómicas de la diabetes. Con el desarrollo de las técnicas de cultivo celular, esta idea es aún en la actualidad controvertida (58). Estudios recientes *in vitro*, han puesto de manifiesto que las exposiciones de las células de la microcirculación retiniana a concentraciones de glucosa por encima de los niveles fisiológicos, aumentan la síntesis proteica, pero disminuyen sustancialmente el poder de proliferación celular (59). La formación del tejido fibroglial vascularizado observado en la retinopatía, parece tener una estrecha relación con la proliferación de las células endoteliales, que estarían mediadas por factores humorales y angiogénicos, probablemente presentes en el vítreo patológico (60,61). Si desde el punto de vista celular muchos procesos deben ser todavía investigados, estudios anteriores, basados en observaciones esencialmente clínicas (62), establecieron que pacientes que presentaban un control satisfactorio del nivel glucémico en los primeros 5 años de la enfermedad tienden ha desarrollar menos retinopatía severa que aquellos en los que su control ha sido menos efectivo.

La ***glucosilación no-enzimática*** de proteínas, que parece estar relacionada con los estados de hiperglucemia, se presenta incrementada en la diabetes. Esta reacción conlleva altos niveles de hemoglobina glucosilada (63), se desconoce todavía si el proceso tiene un papel relevante en la enfermedad. Se ha postulado que la glucosilación no-enzimática de los componentes de las láminas basales y consiguiente reordenación química para formar los "productos finales de glucosilación", pueden contribuir al acúmulo secundario de material matricial. El bajo recambio de estos productos y su interacción con proteínas plasmáticas invasoras, inducen la secreción de factores de crecimiento que estimulan la proliferación celular y su matriz (64). En base a estudios realizados en glomérulos renales se sabe que el colágeno de las láminas basales de los pacientes diabéticos se encuentra extensamente glucosilado, bien por un proceso enzimático (65) o no-enzimático (66). Aunque el colágeno del tipo IV es por excelencia la proteína colagénica de las láminas basales, se observa la

presencia de otros tipos (tipo V y tipo I) como componentes de la lámina basal de los capilares retinianos (8,67,68). Resta confirmar si la reacción, mediada o no por enzimas, es la responsable de una alteración colagénica estructural de origen bioquímico, o contribuye de forma directa al engrosamiento de la lámina basal de los capilares retinianos de forma idéntica al tejido renal.

Estudios realizados en animales galactosémicos (5,69) han resultado importantes para la comprensión del comportamiento celular y de las alteraciones bioquímicas en la retinopatía diabética. Se ha podido mimetizar el engrosamiento de las láminas basales, la pérdida selectiva de pericitos, la formación de microaneurismas, y la presencia de capilares acelulares en animales no diabéticos sometidos a una dieta enriquecida con galactosa por un largo período de tiempo (70). Estos resultados evidencian un excelente modelo de experimentación para el estudio de la susceptibilidad celular bajo los posibles efectos metabólicos de la ***vía del sorbitol*** (51). Esta, corresponde a la secuencia de reacciones que involucran a dos enzimas celulares: la *aldosa reductasa* y la *sorbitol deshidrogenasa*. La primera reduce varios azúcares hacia sus respectivas formas alcohólicas para, después, bajo la acción de la segunda enzima, ser oxidadas hacia cetosas. Así, la glucosa se reduce rápidamente en sorbitol y, más lentamente, se oxida a fructosa, lo que implica un acúmulo intracelular de sorbitol. Por un mecanismo de constantes de concentración de la glucosa, la aldosa reductasa no es operativa excepto cuando se impone un estado mantenido de altos niveles de glucosa, tal como se observa en las hiperglucemias incontroladas, con la saturación de las vías normales del metabolismo de los hidratos de carbono. En base a estos hechos, confirmados por medio de técnicas inmunocitoquímicas (71), se ha propuesto una teoría bioquímica para la explicación de la pérdida selectiva de los pericitos en la enfermedad: la actividad de la aldosa reductasa es especifica en los pericitos de los capilares retinianos en especímenes humanos, pero no ha sido evidenciada en las células endoteliales. Por otro lado, investigaciones complementarias evidencian la actividad de la enzima en células endoteliales y pericitos de capilares retinianos en cultivo (72).

FACTORES MITOGENICOS Y ANGIOGENICOS

La neovascularización es el proceso por el cual se originan vasos de neoformación. Varios factores y condiciones patológicas pueden determinar el desarrollo de este proceso y, como hemos descrito, el ambiente hipóxico, con la consiguiente isquémia, es condición fundamental para la aparición de la retinopatía. Estructuralmente, la neovascularización aparece tras una serie de estados secuenciales donde las células endoteliales tienen un papel determinante. Los capilares de neoformación aparecen principalmente a partir de áreas de degradación de la lámina basal de las células endoteliales de los capilares. Estas emiten expansiones citoplásmicas a través de brechas formadas en la lámina, elongándose y alineándose unas con otras bajo la acción de factores quimiotácticos, para formar "gérmenes" (o brotes) de capilares endoteliales. Las mitosis de las células endoteliales extienden los brotes vasculares, donde se observa un pequeño lumen que aparecerá como consecuencia de la curvatura interna de cada célula endotelial. Dos brotes, con sus respectivas cavidades luminares, pueden eventualmente sufrir una anastomosis formando una asa, tras el cual se observa el inicio de una microcirculación. Los pericitos migran a través de estas nuevas estructuras, y establecen una interacción con el medio extracelular, completando el proceso de neovascularización (73). Así, para comprender los estadíos de la neoformación vascular bajo un punto de vista estructural, y considerando los fenómenos celulares en el proceso, se pueden observar cuatro etapas principales: 1) degradación enzimática de la lámina basal alterada; 2) migración de las células endoteliales bajo la influencia de factores quimiotácticos; 3) proliferación de las células endoteliales; 4) interacción de éstas con los pericitos y la matriz extracelular.

La hipótesis de que un "factor angiogénico" se produce a partir de las células retinianas en los territorios anóxicos, que implica a la formación de neovasos en la retina, ha sido la idea original aceptada desde hace mucho tiempo para la explicación de la neovascularización en la retinopatía diabética proliferativa (74,75). Recientemente se ha demostrado *in vitro* la proliferación de las células

endoteliales con el estímulo y el crecimiento de vasos de neoformación, cuando entran en contacto con el fluido intraocular aspirado de ojos humanos que presentaban un proceso de neovascularización. Cuando el experimento se repitió en presencia de aspirados vítreos que provenian de ojos normales, se observó una actividad angiogénica tanto estimuladora como inhibidora, sugiriendo que la posible causa de la neoformación vascular en los pacientes diabéticos es un desequilibrio entre estímulo e inhibición (76).

Actualmente se conocen una serie de sustancias con características angiogénicas aisladas esencialmente a partir de capilares tumorales de neoformación (77). Estos elementos incluyen sustancias de muy bajo peso molecular (100 D), conocidas como las prostaglandinas (PGE_1, PGE_2), y péptidos y proteínas entre 2 kD y 30 kD, denominados Factores de Crecimiento (73).

Entre el grupo de los primeros polipéptidos reconocidos como potentes mitógenos de las células endoteliales, destacamos la familia de los ***Factores de Crecimiento con Afinidad por la Heparina*** (HBGF) (78). Tras haber sido extensamente analizados y caracterizados (columna de afinidad, secuencia proteica, etc.), los HBGF fueron divididos en dos clases, que han dado origen a los ***Factores de Crecimiento Fibrobrástico ácido y básico*** (FGFa, FGFb). El primero aislado exclusivamente en el tejido neural, como el cerebro y retina (79), el segundo, además, en ciertos tumores y otros tejidos, incluyendo los cartílagos (80). En estudios realizados en cultivos de células endoteliales derivadas de vasos retinianos, y otros *in vivo*, se evidenciaron las características mitogénicas, quimotácticas y reguladoras de la neovascularización de los FGF (81,82). Se ha descrito el papel angiogénico del FGFb en ojos humanos portadores de una enfermedad proliferativa intraocular y sometidos a vitrectomía; mediante análisis de muestras vítreas por medio de un ensayo ELISA cuantitativo, se observaron niveles altos del péptido en la mayoría de los pacientes que presentaban retinopatía diabética proliferativa con neovascularización activa (83).

El ***Factor de Crecimiento "Insulin-like"*** (IGF) es un péptido con secuencia similar a la pro-insulina, en sus subgrupos I y II (IGF-I o somatomedina C y IGF-II) (84). El IGF-I promueve aumentos significativos en la quimiotaxis de las células endoteliales de los capilares retinianos humanos y bovinos, como también en las de la aorta fetal bovina bajo un patrón dosis dependiente (85). Asimismo, estimula la liberación del activador del plasminógeno a partir de las células endoteliales retinianas derivadas de pacientes diabéticos, pero no a partir de las células retinianas originarias de pacientes sin la enfermedad; contribuye además a la secreción de proteasas y en la proliferación de las células endoteliales (86), estimulando además la quimotaxis, la proliferación y la desdiferenciación fibroblástica de las células del epitelio pigmentario de la retina (87). Evidencias adicionales del papel importante que desempeña el IGF-I en la neovascularización han sido aportadas por un ensayo clínico; en pacientes portadores de una retinopatía diabética no proliferativa y que, más tarde, desarrollaban la forma proliferativa de la enfermedad, presentaban aumentos considerables de los niveles plasmáticos del péptido en el momento de la aparición de los vasos retinianos de neoformación, cuando se comparan con los niveles plasmáticos de los meses anteriores del inicio de la aparición de la proliferación vascular (88). Por su bajo peso molecular (7 kD), y su presencia en la circulación, podría constituir fácilmente un factor angiogénico local, alcanzando la cavidad vítrea a partir de las alteraciones de la barrera hemato-retiniana. Las concentraciones de IGF-II en muestras vítreas de pacientes diabéticos no cambian de manera significativa con relación a las muestras normales (89).

El ***Factor de Crecimiento Epidérmico*** (EGF) y el ***Factor de Crecimiento Transformante Alfa*** (TGF-α) son miembros de otra familia de potentes mitógenos para una variedad de diferentes tipos de células *in vitro*, incluyendo queratinocitos y fibroblastos (90,91). Ambos péptidos poseen acción angiogénica importante siendo que sus receptores fueron identificados en vasos retinianos bovinos, así como también la proteína ligante del TFG-α y su RNA*m* (92). Todavía permanece desconocido el posible papel de ambos en las enfermedades vasculares proliferativas en ojos humanos.

El ***Factor de Crecimiento Transformante Beta*** (TGF-β) se encuentra en varios tipos de tumores y tejidos normales incluyendo el riñón, la placenta y las plaquetas sanguíneas (73). Actualmente se conocen varios subgrupos y, de entre ellos, tres humanos (β1, β2, β3); los genes que codifican sus respectivas proteínas estan estrechamente relacionados entre si, aunque determinan acciones biológicas distintas (93). En estudios experimentales con ratones se ha observado su potente acción estimuladora sobre la población local de macrófagos y fibroblastos, así como una sobre producción de colágeno y la formación de capilares (94). En contraste, el TGF-β1 inhibe de manera importante la proliferación y la motilidad de las células endoteliales cardíacas bovinas *in vitro*, previamente inducidas por el FGF básico (95). Este efecto paradójico y bifuncional en la proliferación celular podría estar modulado por factores presentes en el proceso inflamatorio, pero ausentes en condiciones de cultivo. Con base a estos hechos, y con referencia a la neoformación vascular, se ha postulado que, bajo condiciones normales, el TGF-β en un estado latente producido por pericitos parece ser activado por células endoteliales, produciendose una inhibición de la mitosis de las últimas. Cuando el estrecho contacto pericito-célula endotelial se pierde (desaparición de los pericitos), la activación del TGF-β latente también se perderá; factores de crecimiento como el FGF básico, producido por células endoteliales, podría actuar como estimulador de la proliferación, migración, y neoformación vascular (27,96).

CONCLUSIONES

Hemos intentado establecer, de manera sucinta, algunos aspectos celulares relacionados con los fenómenos de más trascendencia en la fisiopatología de la retinopatía diabética en sus fases pre y proliferativa, así como en la neoformación vascular.

Tomando la retinopatía diabética como un modelo proliferativo y, al tiempo, como un ejemplo patológico de neoformación capilar, queda claro que los procesos bioquímicos fundamentales se inician tempranamente en el curso de la enfermedad diabética, evolucionando lentamente hasta generar alteraciones funcionales y estructurales en el tejido retiniano y en la hemorreología de la microcirculación de la retina que, con el paso del tiempo, lleva a muchos de los pacientes a la neovascularización retiniana y consecuente formación de un tejido fibrogliovascular, que caracteriza los estadíos más avanzados de la enfermedad intraocular. Los estados prolongados de hiperglucemia alteran el metabolismo normal de la glucosa, con la aparición de vías metabólicas coadyuvantes y compensatorias que implican disturbios metabólicos tóxicos en la intimidad celular. Asimismo, promueven cambios anatómicos estructurales, como el engrosamiento de las láminas basales y cambios de glucosilación de proteínas de la matriz extracelular con su acúmulo secundario. Los pericitos de los capilares retinianos se pierden selectivamente, y las células endoteliales se deterioran bajo un nuevo ambiente nocivo, a lo cual se añaden las alteraciones de la reología sanguínea impuestas por altos niveles plasmáticos de determinadas proteínas. Estos cambios en la matriz extracelular, en los elementos de la sangre, en los pericitos y en las células endoteliales se combinan para formar un entorno de hipoperfusión con un aumento de la permeabilidad capilar, motivados por profundos cambios de la barrera hemato-retiniana. Todo ello determina un estado hipóxico, con la consiguiente isquémia retiniana, permitiendo el paso de proteínas y factores del plasma hacia ella y al vítreo. El tejido retiniano parece responder a esta agresión con una neoformación vascular, con el objeto de restablecer posiblemente una perfusión sanguínea compatible con las necesidades normales. Los vasos de neoformación, sobre la retina o dentro de la cavidad vítrea, tienen una evolución dependiente de múltiples factores así como de la idiosincrasia de cada paciente. Una vez establecida, se acompaña inevitablemente de la aparición de un tejido fibroglial adyacente, con graves consecuencias para la arquitectura intraocular.

Con el avance de las técnicas de biología celular y molecular, se han visto ampliadas las posibilidades de interpretación de ciertos procesos claves en la fisiopatología de las complicaciones oculares de la diabetes. Hechos importantes se añaden cada día que corroboran para la mejor

comprensión de los mecanismos involucrados en la proliferación endotelial y en la patogénesis de la neoformación vascular.

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OBJETIVOS

OBJETIVOS

La Vitreorretinopatía Proliferativa (VRP) y la **Retinopatía Diabética Proliferativa** (RDP), son graves cuadros clínicos intraoculares caracterizados por una proliferación celular activa sobre la superficie retiniana y/o en la cavidad vítrea; la primera se trata de una complicación nefasta del desprendimiento de retina, sea de origen regmatógeno o traumático (con o sin perforación ocular), pero frecuentemente observada como causa principal de los fracasos post-operatorios de la cirugía reparadora del desprendimiento de retina; la segunda, se trata de una complicación de las manifestaciones oculares de un proceso más generalizado que afecta al organismo como un todo. Sus mecanismos fisiopatogénicos son distintos; la primera se presenta como un proceso aislado y localizado de la economía ocular, fruto de una agresión mecánica que implica la pérdida brusca de la arquitectura e integridad vitreorretinal, mientras que la segunda siempre conlleva fenómenos complejos de angiogénesis donde el régimen de isquemia retiniana tiene un papel importante. No obstante, ***ambas son consecuencia de una agresión, constituyen entidades patológicas de patogénesis poco conocidas, y ambas se caracterizan, en sus respectivos mecanismos fisiopatológicos, por fenómenos de migración, adhesión y proliferación celulares*** que anteceden a los estadíos accesibles al diagnóstico clínico por el oftalmólogo.

Las primeras investigaciones llevadas a término con relación a la VRP incluyeron modelos experimentales que intentaron desarrollar la enfermedad en animales, utilizando inyecciones intravítreas de células, partículas o sustancias de variada procedencia, e incluso creando mecánicamente la producción de desprendimientos de retina. Se asemejaban al cuadro clínico de la alteración, pero no reproducían con exactitud los aspectos esenciales de la enfermedad humana. No obstante, constituyeron modelos apropiados para el ensayo terapéutico de fármacos para su tratamiento. Con las técnicas modernas de cirugía de la retina y del vítreo, desarrolladas en las dos últimas décadas, se han realizado distintos estudios con especímenes patológicos humanos, obtenidos a partir de ojos sometidos a cirugía intraocular: las membranas epirretininas y los aspirados vítreos. Estos estudios, al utilizar muestras humanas, permiten acercarse más al mejor entendimiento de su patogénesis.

A diferencia de la problemática de la VRP, la diabetes es un proceso multidisciplinario, en el cual concurren los estudios de muchos especialistas; los experimentos con modelos animales en el intento de reproducir la patología sistémica, las nuevas técnicas de biología celular y molecular y los cultivos celulares y de tejidos, añaden cada día resultados más prometedores para su mejor comprensión. En lo referente a las complicaciones retinianas de la diabetes *mellitus*, las investigaciones son más restringidas, pero los resultados no son menos prometedores y a semejanza de la VRP, los intentos de mimetizar la patología en modelos animales, es tarea difícil, casi imposible.

¿ Qué debemos saber realmente acerca de la VRP y la RDP para comprenderlas mejor y, en última instancia, poder proponer una terapéutica más racional y efectiva ?

Esta ha sido la cuestión preliminar que nos ha orientado inicialmente en el planteamento experimental que se presenta en este trabajo. El tejido fibrocelular de la VRP (membranas epirretinianas, MER) y fibrogliovascular de la RDP (membranas vitreorretinianas, MVR), son la consecuencia de una serie de fenómenos que caracterizan un proceso proliferativo intraocular: *"el final de la cadena"*. Las células que constituyen estos tejidos *reparacionales*, son las células contenidas en la estructuras normales de la intimidad intraocular. Para que éstas, separadas o conjuntamente puedan formar este tejido *reparacional*, debe existir la concurrencia de varios factores que modifican su comportamiento, así como la producción de un ambiente propicio para sus cambios. Para que estos elementos celulares migren, se adhieran, proliferen, y formen un tejido conjuntivo fibroso (MER o MVR), y para que la presencia de vasos de neoformación determinen el estadio proliferativo de la enfermedad diabética intraocular, se puede hipotetizar que ***el papel de la matriz extracelular y los mecanismos de interacción de las células con su substrato, son esenciales y anteriores a la formación del tejido fibroso reparacional.*** Por tanto, creemos que la matriz

extracelular puede constituir uno de los primeros *"eslabones de la cadena"*.

Para que un determinado tipo celular responda a un estímulo específico que induzca su migración, adhesión y ensamblaje con su substrato, y se produzca su proliferación con la consiguiente diferenciación, es necesario el reconocimiento del entorno extracelular por parte de la célula, a través de sus receptores de membrana. La matriz extracelular representa así el substrato imprescindible para que se produzcan éstos fenómenos.

Por otro lado, hasta la fecha no se ha explorado suficientemente el posible papel del vítreo en ambas patologías. Creemos que esta estructura posee un papel coadyuvante, al ofrecerse como *"vehículo"* y *"medio"* por el cual varios fenómenos de señal para la migración y proliferación se procesarían.

Así, por estas razones, nuestro objetivo principal ha sido el estudio del posible **papel de la matriz extracelular y sus componentes** en ambas patologías. Entender la enfermedad proliferativa intraocular como **un modelo humano patológico *in vivo* de migración, adhesión, proliferación y diferenciación celular con el ensamblaje de los componentes celulares y su entorno**, es esencial para comprender los posibles fenómenos que involucran las fases tempranas de membranogénesis de la VRP y la formación neovascular de la RDP.

Para poder alcanzar este objetivo ha sido necesario establecer varios objetivos parciales que nos permitirán acceder de modo gradual al conocimiento del proceso proliferativo. Estos objetivos han sido:

1. Caracterización morfológica y ultraestructural de los elementos celulares y vasculares, así como su relación con la matriz extracelular en las membranas fibrocelulares y fibrogliovasculares de las respectivas patologías.
2. Caracterización de los tipos celulares implicados en el proceso proliferativo intraocular, mediante la aplicación de técnicas inmunocitoquímicas para detección de filamentos intermedios.
3. Establecimiento del posible papel de las glucoproteínas multifuncionales de adhesión de la matriz extracelular (*fibronectina*, *laminina*, y *vitronectina*) en el proceso proliferativo intraocular, analizando:

3.1. Expresión y patrón de distribución en las membranas fibrocelulares de la VRP y en los capilares de neoformación en las membranas fibrogliovasculares de la RDP.

3.2. Expresión y patrón de distribución de sus respectivos receptores de membrana plasmática, los complejos receptores del subgrupo β_1 y de las integrinas $\alpha_v\beta_3$, en las membranas fibrocelulares y en los capilares de neoformación en las membranas fibrogliovasculares.

4. La identificación y el análisis cuantitativo en el vítreo normal y patológico de las mencionadas glucoproteínas, con el objetivo de valorar su contribución en las diferentes fases de ambas patologías.

CAPITULO I

Proliferative Vitreoretinopathy and Proliferative Diabetic Retinopathy: Ultrastructural Characteristics and Stereologic Study on Proliferative Preretinal Membranes.

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Abstract: Proliferative vitreoretinopathy (PVR), macular pucker (MP) and proliferative diabetic retinopathy (PDR) are intraocular disorders characterized by cell migration, adhesion, and proliferation, with contractile preretinal membrane formation. By electron microscopy, preretinal membranes appear as sheets of well-developed connective-like tissue, in which several cell types were observed: 1) differentiated cells such as retinal epithelial cells, glial cells, fibroblasts, plasmatic cells, and also endothelial cells and pericytes of newly-formed capillaries in PDR membranes; 2) undifferentiated cells with fibroblast-like or myofibroblast-like appearance; and 3) macrophage-like cells. By unbiased volume/density estimation (*Vv*) between cell/matrix components on PVR, MP, and PDR membranes, we found: 1) a reduction in cell/matrix ratio for PVR, MP, and PDR respectively ($p \leq 0.05$); 2) *Vv* estimation was a quantitative temporal parameter for evaluation of the proliferative intraocular tissue evolution; and 3) this quantitative parameter allowed us to establish homogeneous and significant different samples, whose components correspond to different clinical groups (PDR, MP, PVR). Although these diseases have different pathogenesis, on the basis of ultrastructural and stereologic data, fibrocellular and fibrogliovascular tissue formation in intraocular proliferative disorders could reflect intraocular wound repair.

Key words: Epiretinal membranes, proliferative intraocular disease, retinal detachment, ultrastructure, stereology.

INTRODUCTION

Preretinal proliferative membranes, which are usually formed after rhegmatogenous retinal detachment, ocular trauma including blunt and penetrating injuries, intraocular inflammation, vitreous haemorrhage, and as a localized process like a macular pucker (MP), have been known to show contraction causing traction and distortion of the underlying retina (1,2). These epiretinal membranes in eyes with proliferative vitreoretinopathy (PVR) are fibrocellular and often avascular. In proliferative diabetic retinopathy (PDR), vascular endothelial cell proliferation originating newly-formed capillaries are predominant, first on the vitreoretinal interface and later in the vitreous cavity (3,4). In this stage glial and fibrous components increase rapidly, and a fibrogliovascular tissue characterizes these vitreoretinal membranes.

These disorders have different pathogenesis which are poorly understood, although several investigations reported a similar wide variety of cell types scattered within the fibrous matricial tissue in membranes, including glial cells, retinal pigment epithelium, fibroblasts and macrophages (1,5-9). Electron microscopy studies (1,2) on epiretinal and vitreoretinal membranes surgically obtained from patients with PVR and PDR, showed cells with myofibroblastic characteristics that are thought to be responsible for contraction strength in membrane. This evidence has been likened to the behaviour of the intraocular proliferative tissue formation in the "scars" of healing wounds. In immunohistochemical studies (10,11), PVR membranes were classified as early or late according to the duration of the illness; cell amount did not differ greatly between early and late membranes, in spite of a tendency for determinate cell types to disappear, while others tended to persist (11). On the other hand, epiretinal membranes are classified into two categories, simple and complex, by their clinical appearance and consequences for the retinal surface (12).

In the present study we have studied ultrastructural cell characteristics and we carried out a stereologic analysis on PVR and PDR membranes to study the behaviour of matrix and cell

elements and discern whether changes in proportionality of cell-matrix ratio could be related to the time of evolution and/or aetiology of proliferative disease.

MATERIAL and METHODS

Twenty-two preretinal membranes were obtained by peeling with appropriate vitreous forceps during pars plana vitrectomy. Twelve membranes were obtained from PVR patients, four from PDR patients and six specimens from patients with MP after retinal detachment surgery. Clinical records of cases were observed with special attention to the onset of intraocular proliferative disease and the time of clinical duration of preretinal membrane. In PVR specimens, the clinical presentation was graded from C1 to D3 following Hilton *et al.* (13). The macular epiretinal membranes were graded in stages 0 to 2 following Gass (14).

The tissue specimens were immediately fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffered saline solution (PBS pH 7.4) and then contrasted in 1% osmium tetroxide for one hour. After standard dehydration in a graded acetone series, specimens were embedded in resin to be polymerized at 60°C. Semithin sections (0.5μm - 0.9μm) were stained with toluidine blue and examined by light and phase contrast microscopy (Polyvar II, Reichert-Jung, Germany). Ultrathin sections (50nm - 70nm) were contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (Hitachi 800 MT, Hitachi Inc., Japan). If the specimen could be removed in a sufficient piece, half of it was cut and processed for routine histopathologic evaluation. After fixation in 4% paraformaldehyde and rinsed in PBS 0.1 M, specimens were immersed in 2.1 M sucrose solution and then placed in embedding compound (OCT Miles Co., USA). The samples were frozen in isopentane and stored at -35°C. Frozen sections (6µm - 9µm) were placed on 0.5% gelatin-coated slides, air-dried at room temperature and then stained with haematoxylin-eosin for light microscopy observation.

For the stereologic study several sections ($n_s > 10$), and fields ($n_f > 3$) in sections, for each specimen, were randomly selected and photographed. We applied a volume density test estimation (*Vv*) to estimate the total volume occupied by cells related to the total volume of membrane tissue (cell and extracellular matrix volume), excluding the volume occupied by capillaries in cases of vitreoretinal membranes from PDR patients. *Vv* was applied according the Delesse principle (15), which allows an unbiased estimation for the amount of volume per volume unit, and in this case, stablishes a relative estimation of the volume occupied by cells in each sample, independent of magnification, section orientation, or method used to prepare samples. The sections photographed were projected and measurements were performed by random superposition of an unbiased test-volume frame (4.23 mm^2/test point). Statistical analysis was performed by a Student-Newman-Keuls (SNK) test for multiple mean comparisons adjusted in $p \leq 0.05$ (16), and an unbalanced analysis of variance (ANOVA) by least-significant difference (LSD in SAS program, 1984) for comparisons within and between clinical groups. We arbitrarily used the term *"cell fraction (CF)"* of the specimen, to designate the relative volume occupied by cells in samples obtained by *Vv* estimation.

RESULTS

Light microscopy and stereologic study

Light and phase-contrast microscopic examination of the cross-sectioned preretinal membranes showed isolated or grouped cells, in cord or nest, surrounded by an extensive amount of dense connective tissue. Within matrix, the cell-collagenic material ratio was extremely variable. In the fibrocellular membranes (PVR and MP) we observed a moderate or scanty connective matrix with abundant cell population which showed intense pleomorphism (Fig. 1A to 1C). Fusiform-like cells were predominant and many other cell types were seen, associated or not with pigment. Long segments of intact internal limiting membrane were present in four MP epiretinal membranes (66%), but less frequently in the PVR samples (16.5%) (Fig. 1B); these segments typically had a smooth inner surface firmly adhered to the epiretinal membrane, while an irregular outer surface faced the retina. Fibrogliovascular membranes (PDR) showed numerous newly-formed capillaries of varying diameters firmly surrounded by an abundant fibrous matrix; some pigmented cells with macrophage morphology, epithelioid-shaped cells, and few plasmatic cells were also seen (Fig. 1D to 1F).

Vv estimation showed significant differences in both statistical analysis carried out.

Independent of clinic or pathological classifications, four distinct groups were disclosed based on SNK test (adjusted to $p=0.05$): (a) the first grouped preretinal membranes with slight CF, had $Vv= 0.098 \pm 0.018$ and were composed by all PDR membranes (n=6) and half of MP epiretinal samples (n=3); (b) with moderate CF, the second group had $Vv= 0.246 \pm 0.024$ composed by another half of MP specimens (n=3); (c) the third grouped membranes that had $Vv= 0.533 \pm 0.028$ with high CF, were constituted by PVR epiretinal membranes; (d) a very high CF group of VRP epiretinal membranes with $Vv= 0.678 \pm 0.026$. Table I showed the different groups of *Vv* estimation classed by SNK test.

Table I: Classification of preretinal membranes by *Vv* estimation.

Vv ratio*	*CF*	pathological group
0.098 ± 0.018	slight	*PDR* and *MP*
0.246 ± 0.024	moderate	*MP*
0.533 ± 0.028	high	*PVR*
0.678 ± 0.026	very high	*PVR*

Vv, volume density test estimation in mean ± S.E; *CF*, cell fraction.
*, differences were determined by a Student-Newman-Keuls (SNK) test adjusted to $p \leq 0.05$.
PDR, proliferative diabetic retinopathy; *MP*, macular pucker; *PVR*, proliferative vitreoretinopathy.

In the second step of the analysis, statistical data were compared by pathological groups to confirm difference obtained in the initial analysis; ANOVA test by least-significant difference for comparisons within and between pathological groups was used.

Difference between pathological groups (PDR, MP, and PVR): Distinct groups according to pathology were considered. The CF in PDR membranes ($Vv= 0.092 \pm 0.019$) showed significant difference ($p=0.05$) when compared to MP membranes ($Vv= 0.182 \pm 0.097$) and to PVR specimens ($Vv= 0.593 \pm 0.072$) ($p=0.001$). Difference between MP and VRP membranes were also noted ($p=0.001$).

Differences within a pathological group (MP and PVR): According to the time of clinical duration, MP membranes were arbitrarily classed into two groups: (a) MP membranes with upto 6 months' evolution; and (b) MP membranes with more than 6 months. Although *Vv* estimation tended to increase with time, no significant difference was found between the first group ($Vv= 0.157 \pm 0.092$) and the second ($Vv= 0.218 \pm 0.091$) group, but when the latter was compared with the PDR samples ($Vv= 0.092 \pm 0.019$), difference was noted ($p=0.05$). Similarly, PVR epiretinal membranes were classed as: (a) PVR membranes with upto 2 months' evolution ($Vv= 0.633 \pm 0.065$) and (b) PVR membranes with more than 2 months ($Vv= 0.588 \pm 0.096$). Although *Vv* estimation tended to decrease with time, no significant difference was found between groups.

Transmission electron microscopy study

By transmission electron microscopy (TEM), eight cell types were distinguishable in preretinal membranes: (a) endothelial cells and pericytes (capillaries) (Fig. 2); (b) fibrous astrocytes (Fig. 3); (c) retinal pigment epithelial cells (Fig. 4); (d) fibroblasts (Fig. 5); (e) macrophages and phagocyte-like cells (Fig. 6); (f) cells with myofibroblastic differentiation (Fig. 7); and (g) plasmatic

cells (not shown). Table II shows the percentage of positive specimens for each cell type observed.

Table II: Ultrastructural features in preretinal membranes

	FA	*RPE*	*F*	*M*	*My*	*ILM*	*Cap*	*Pl*
PVR(n=12)	6(*50*)*	10(*83*)	8(*67*)	6(*50*)	12(*100*)	2(*17*)	1(*8*)	2(*17*)
MP (n=6)	5(*83*)	4(*67*)	3(*50*)	2(*33*)	5(*83*)	4(*67*)	1(*17*)	1(*17*)
PDR(n=4)	4(*100*)	2(*50*)	4(*100*)	4(*100*)	2(*50*)	no	4(*100*)	3(*75*)

* specimen(%)

FA,fibrous astrocyte; *RPE*,retinal pigment epithelial cells; *F*,fibrocyte; *M*,macrophage; *My*,myofibroblastic differentiation; *ILM*,internal limiting membrane; *Cap*,capillaries; *Pl*,plasmatic cells.

PVR, proliferative vitreoretinopathy; *PDR*, proliferative diabetic retinopathy; *MP*, macular pucker.

* *Extracellular matrix:* Specimens were composed of variable amounts of fibrillar collagen randomly arranged in both dense and loose bundles (Fig. 1D to 1F). Collagenic fibrils of varying diameter (100nm to 450nm) usually contained periodic bands. Fibrin aggregates were noted in PDR preretinal membranes. Preserved red blood cells were observed within collagenic tissue and sometimes were incorporated by cells of connective tissue (Fig. 1D and 1F).
* *Endothelial cells and pericytes (capillaries):* Newly-formed capillaries with variable diameters (2μm to 40μm) were a very common feature of PDR membranes (Fig. 2), but absent in MP and rarely observed in PVR specimens (8%) (Fig. 1D). In PDR samples, capillaries presented well-developed pericytic processes. Endothelial cells were plump, with luminal surface smooth, or thin and elongated, presenting cytoplasmic projections toward the lumina. The latter was a common characteristic of wide-diameter vessels (Fig. 2B, 2C, and 2E). Endothelial cells showed irregular-shaped nuclear membranes with numerous pinocytic vesicles, which were localized on both luminal and basal sides in a plasma membrane arrangement (Fig. 2F). Masses of microfilaments were observed in both pericytes and endothelial cells. *Fenestrae* in endothelial cells were noted in wide capillaries; endothelial pores were usually single or grouped in alignment, closed by a one-layered diaphragm (Fig. 2F). Most of newly-formed capillaries presented endothelial cells connected by tight junctions (Fig. 2G). Pericytes were identified sometimes plump or fusiform, closely related with endothelial cells. These cells contained dilated rough endoplasmic reticulum *cisternae*, aggregates of intermediate filament bundles, and occasional lysosomal inclusions. Well-developed basement membranes were observed in monolayer or stratified structures enveloping endothelial cells and adjacent pericytes (Fig. 2B and 2E).
* *Fibrous astrocytes:* This was the most common finding, observed in MP (83%), PDR (100%), and PVR (50%) specimens. They are large and generally spindle-shaped or elongated (Fig. 3) displaying a linear or cluster arrangement. These cells had tendency to polarize, presenting several cytoplasmic processes and variable amounts of well-defined basement membrane deposition. Numerous junctional complexes were observed and well-developed intracytoplasmic organelles and polyribosomes were noted (Fig. 3B to 3E). Fibrous astrocytes showed extensive amounts of both intermediate filaments masses and bundles of microfilaments (Fig 3D and 3E).
* *Retinal pigment epithelial cells (RPE):* RPE cells were identified mainly in PVR (83%) and MP

(66%) membranes. These cells presented atypical morphology when organized in cell layers within collagenic matrix (Fig. 1E), while their typical cuboidal-shape was noted in cells in a rosette-like arrangement (Fig. 4B). RPE cells were polarized, showing numerous cytoplasmic microvillous processes in their apical surface as well as a developed basement membrane (Fig. 4A to 4D). Specialized junctional complexes were noted mainly on the lateral side of cells. Numerous well-defined bounded melanosomes without stratification were observed, wich were sometimes arranged in the apical aspect of cells (Fig. 4A to 4C); melanin pigment was often, but not always, present in cells. In a rosette-like arrangement cells tended to constitute a luminal surface composed by their apical cytoplasmic processes (Fig. 4B). Developed organelles and bundles of microfilaments were seen (Fig. 4D).

* *Fibrocytes:* Fibrocytes were usually fusiform and recognized mainly in PDR (100%) (Fig. 1F), PVR (66%) (Fig. 5), and MP (50%) samples. Cells lack polarity and basement membrane formation. Abundant rough endoplasmic reticulum *cisternae* and Golgi complexes, as well as prominent condensed polyribosomes were often observed (Fig. 5B). Newly-formed collagen fibres were usually noted around these cells (Fig. 5A and 5B). Intracytoplasmic membrane-bounded melanin granules and microfilament aggregates were occasionally seen (Fig. 5A).
* *Macrophages and phagocyte-like cells:* Macrophages were identified by their large size and oval shape, without polarity or basement membrane formation, observed isolated or in a cluster arrangement. They were mainly observed in PDR (100%) (Fig. 2C) and less evident in PVR (50%) membranes (Fig. 6B). Cells contained variable amounts of intracytoplasmic pleomorphic inclusions, with numerous secondary lysosome vesicles and residual bodies. These lysosomes presented both melanin and sometimes haemosiderin in various stages of degradation (Fig. 6C and 6D). Some phagocyte-like cells had a cuboidal-shape with poor-developed endomembranes and cytoplasm processes; these cells occasionally tended to polarize. Several lysosomal vesicles and non-degraded bounded melanin granules were seen (Fig. 6A). In addition, phagocyte-like cells with irregular morphology presenting large amounts of degraded pigment were also noted (Fig. 6C).
* *Myofibroblastic cells:* Myofibloblast-like cells were usually identified by their linear aggregates of intracytoplasmic microfilaments with fusiform dense bodies (Fig. 7); this linear package of microfilaments, often marginally localized, apparently ran the length of the cells. Spindle-shaped morphology, lack of polarity and absence of basement membrane were also observed. Presence of *fibronexus* described in wound healing (17) was not observed. Features of myofibroblastic differentiation were frequently present in MP (83%) and PVR (100%), but occasionally in PDR (50%) samples. Myofibroblast-like cells sometimes showed intracytoplasmic pigment granules, a well-defined basement membrane and a tendency to polarize (Fig. 7B and 7C). Masses of intermediate filaments and well-developed organelles, such as fibrous astrocytes, were occasionally observed. Myofibroblast-like cells often exhibited developed mitochondria with dense matrix.

DISCUSSION

Preretinal membranes consist of fibrocellular (PVR and MP) or fibrogliovascular (PDR) proliferations on the retinal surface or in the vitreous cavity respectively, the formation of which depends on several factors, including aetiology and duration. Our structural analysis of these membranes reveals a heterogeneous cell population with variable cell/matrix ratio. In agreement with previous studies (1-9,23) we recognized several cell types based on specific ultrastructural features: 1) glial cells, RPE cells, and macrophages; 2) cells with fibroblast and myofibroblast appearance, but without definite cytoplasmic characteristics to categorize them as member of specific cell type; 3) pigment-laden cells with phagocytic activity; and 4) plasmatic cells. These findings indicate that cells could be derived from various intraocular sources and a specific CF in membranes may be related with

the development of the pathology that determines the clinical characteristics observed in each disorder.

Cells with myofibroblastic differentiation are observed in all types of preretinal membranes. These cells showed: 1) prominent developed mitochondria with dense matrix, representative of a high energetic activity; 2) some cells showed segments of basement membrane; 3) absence of fibronexi; is a characteristic myofibroblastic structure induced by increased levels of both collagen and fibronectin synthesis (17). Although these structures are absent, variable amounts of fibronectin are present in the matrix (11,12,19,20,21). By immunofluorescence and immunoelectron microscopy, we observed fibronectin as a most representative extracellular component in preretinal membrane matrices, most frequently observed in pericellular arrangement in the earliest membranes (time of evolution $\leq$ 2 months); this pattern evolves to a fibrillar diffuse arrangement in older membranes (unpublished data). Since some myofibroblast-like cells presented well-developed segments of basement membrane, as observed in myoepithelial cells (43), a possible epithelial origin could be proposed. The myofibroblast capacity of contraction is thought to explain clinical consequences such as traction on retinal surface, with similarities to wound healing (28,29). Although, studies on preretinal membrane after contraction represent a limited source of data to understand mechanisms of differentiation and remodelling in retinal surface, our results suggest that cell proliferation begins following intraocular tissue damage, and then cell redifferentiation take place with synthesis and secretion of connective matrix material.

In the present study, and in accordance with previous reports (1,5,6,25), macrophagic activity in preretinal membranes appears to be carried out by at least two cell types. Some evidence was found: 1) red blood cells and plasmatic-like cells were occasionally observed within collagenic material, suggesting direct contact with bloodstream elements; this could explain the presence of macrophages from monocytes which were induced to differentiate by the connective matrix of proliferative tissue; 2) some cells with phagocytic activity tend either to polarize and present non-degraded pigment material or to exhibit atypical macrophage features, with poorly-developed cytoplasmic processes and endomembranes, suggesting a macrophage-like cell type. The pluripotential nature of RPE cells has been reported (27), and thus redifferentiated RPE cells with phagocytic activity may proliferate, and regain or retain their original epithelial characteristics (9,25,26). Another possible source of cells with phagocytic activity may be represented by the hyalocytes of cortical vitreous. Their ultrastructural features, properties of migration and phagocytosis as well as ability to act as macrophage have been described (32-34). In addition, many cells identified as fibroblast and macrophage-like cells closely resemble hyalocytes (see Fig. 1D, 5A, and 6C).

The physiopathologic mechanisms associated with each disorder may be distinct because the angiogenic and vasoproliferative phenomena noted in PDR (18) are absent in PVR and MP; newly-formed capillaries are rare in PVR and MP epiretinal membranes (1,6,10). On the other hand, in these intraocular proliferative diseases, proliferative tissue formations present common events: 1) cell proliferation, migration, adhesion, and finally tissue contraction; 2) processes of extracellular matrix formation and cell-to-matrix interaction as a functional basis for membranogenesis; 3) similarities in cell types and surrounding matrix as membrane components, which have been elucidated by immunochemical studies (10,11,20-22); 4) low mitotic activity observed in specimens, suggesting a stabilized process prior to its definitive organization; and 5) the newly-formed proliferative tissue represents a final histopathologic outcome of a complex proliferative process.

Newly-formed capillaries in PDR membranes showed singular characteristics: 1) most capillary endothelial cells were joined by tight junctions; 2) capillaries often contained *fenestrae* bridged by diaphragms; 3) capillaries were surrounded by stratified basement membranes, and pericyte processes were usually present. Although these findings were in agreement with previous studies (3,4,38), fenestrated capillaries are not a feature of all preretinal membranes. Fenestration in capillaries may occur normally in the eye and other tissues (36,37), nevertheless fenestrated capillaries arising in pathologic conditions have rarely been studied. The function of *fenestrae* in pathologic conditions

is unknown, but could be related to states of increased permeability observed in both non-pathological and repairing processes; for example, normal non-fenestrated muscle capillaries acquire fenestration during wound healing and become more permeable than normal muscle vessels (39). Morever, the rise in permeability is reinforced by frequent intraocular bleeding observed in clinical evolution of disease; this could explain the plasmatic elements often observed within the matrix, as discussed above.

PDR membranes are characterized by a low cell/matrix ratio, presenting a well-developed and stabilized collagenic matrix. This long-standing fibrogliovascular tissue is recognized to exert poor tractional forces on the underlying retinal surface (4,18,31), similar to post-contracted tissue in wound repair. For these reasons we hypothesize that the neovascularization observed in preretinal PDR membranes contributes to early organization of the matrix material, and fenestrated capillaries and bleeding allow the access of cells and substances of plasmatic origin; rapid growth of matrix component is initially observed, and then collagenic organization with functional maintenance of fibrogliovascular tissue. Involutional states in PDR membranes after vascular obliteration support this (40).

Data obtained by electron microscopy showed collagen as the major structural component of the extracellular matrix in all specimens examined; its compact arrangement in parallel fibrillar organization suggests a maturation event in the matrix material. This collagenic component, secreted by cells (41,42), has a leading structural role, not only stabilizing the framework but also providing tensile strength.

Stereologic techniques provides an interesting approach in cell/matrix correlation to characterize specific membrane organization. As described, preretinal membranes may be considered as sheets of conjunctive tissue in which dynamic mechanisms for adaptation and remodelling are present; then, unbiased volume-density (*Vv*) estimation determines quantitative values for cell/matrix ratio, independent of factors such as final magnification and section orientation (24). In PDR, the fibroglial tissue that encloses newly-formed capillaries presents slight CF. In our view, this tissue state is determined by the chronic behaviour of PDR (18), which could be compared to a mature connective tissue with low cell/matrix ratio. On the other hand, a sudden disruption of the vitreoretinal anatomy is the leading feature in cases of retinal detachment complicated by PVR (12,13,19). An arbitrary temporal division of PVR and MP epiretinal membranes according to clinical data suggests progressive stability in the cell/matrix ratio over time, in spite of a slight tendency in decrease the ratio in PVR specimens. Using a subjective scale to judge the proportion of collagen in each sample, Hiscott *et al.* (10) showed that in PVR long standing epiretinal membranes contain more extracellular material but fewer cells than membranes of short clinical duration. Based on our stereologic data, the behaviour of cell/matrix ratio in newly-formed fibrocellular tissue observed in PVR and MP, has several similarities, and could reflect specific intraocular wound repair.

Our stereologic results allowed us to classify preretinal membranes according to a constant and unbiased estimation, and then corroborate data by analysis based on clinical criteria. Some of the evidence is remarkable: 1) *Vv* estimation is a quantitative temporal parameter for proliferative intraocular tissue evolution, which is, in addition, specific for each pathogenesis (*Vv* PVR > *Vv* MP > *Vv* PDR), since a decreasing cell/matrix ratio was observed for PVR, MP, and PDR respectively; 2) this quantitative parameter allows us to establish homogeneous and statistically distinct samples, the components of which correspond to different clinical groups (PDR, MP, PVR). These results allow us to consider *Vv* estimation in preretinal membranes as a parametric index of the histopathologic characterization of the newly-formed tissue in intraocular proliferative disorders.

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Figure 1.

Figure 1. Semithin-sectioned preretinal membranes showed pleomorphic cell population and variable cell fraction (CF). (**A**) A moderate CF in MP specimens was noted; plump and epitheliod-shaped cells with abundant and light cytoplasm were common findings in these membranes. (**B**) High CF characterizes PVR epiretinal membranes; fusiform-like cells and others with variable pleomorphism containing or not pigment, were scattered throughout the matrix. Extensive segments of intact inner limiting membrane (*arrow*) were occasionally noted. (**C**) Some PVR specimens possess a very high CF with a compact arrangement of fusiform and epithelial-shaped cells, which were arranged in cords or clusters. (*Toluidine blue X 650, bar=30μm*).

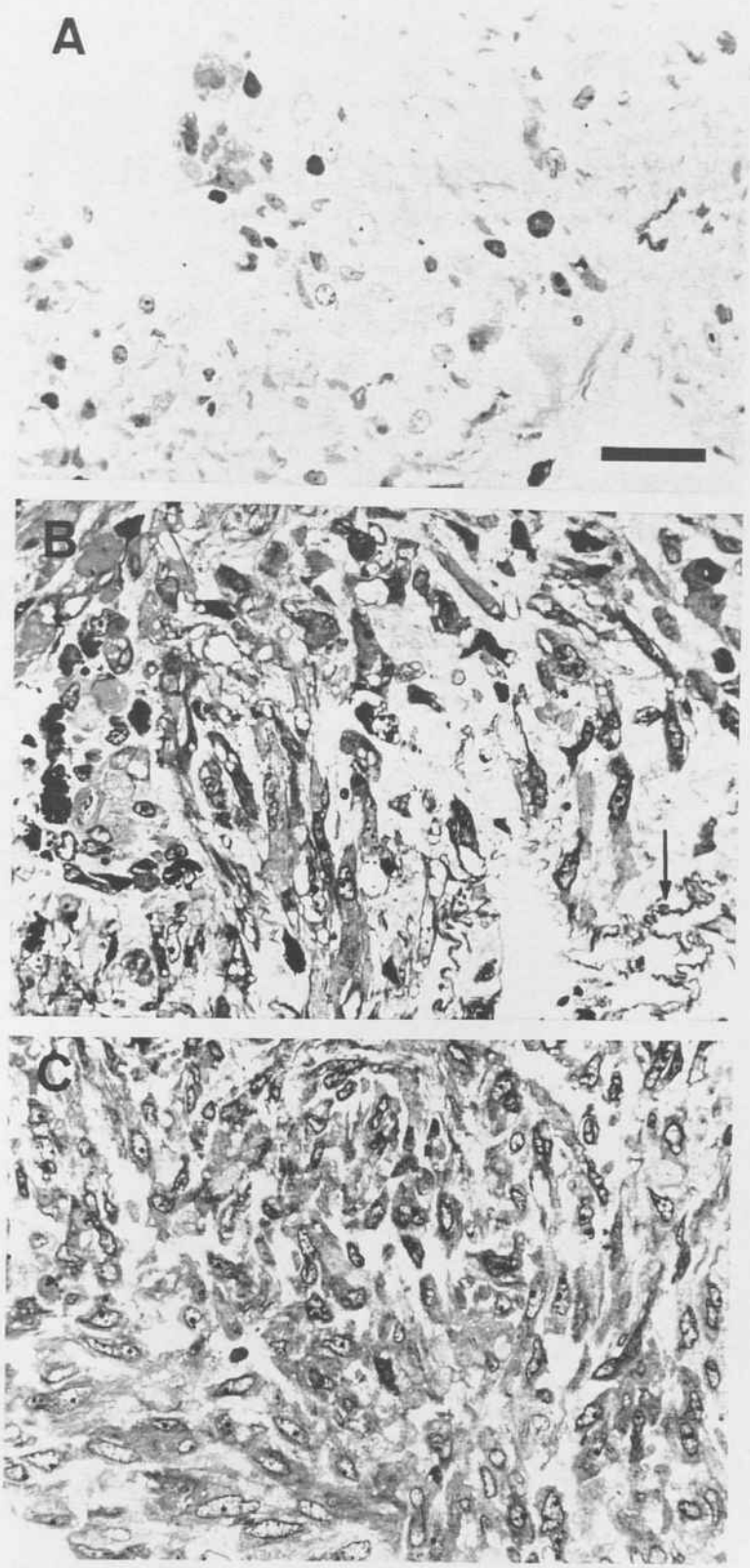
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Figure 1

(continuation).

Figure 1 (continuation). Low magnification (TEM) of ultrathin-sectioned preretinal membranes. (**D**) A very high CF specimen of PVR showed a wide variety of cell morphology types, with or without pigment, distributed in scanty matrix material; Newly-formed capillaries (*asterisk*) were a rare feature in PVR epiretinal membranes (*X 4,000, bar=5μm*). (**E**) Cells with similar intracytoplasmic characteristics but differing in morphology were usually seen; this PVR specimen had high CF with a moderate amount of collagenous matrix surrounding cells in cluster arrangement (*X 2,100, bar=10μm*). (**F**) PDR samples showed grouped cells regularly arranged within large amounts of collagenic extracellular matrix (*ecm*); slight CF was noted (*X 3,600, bar=5μm*).

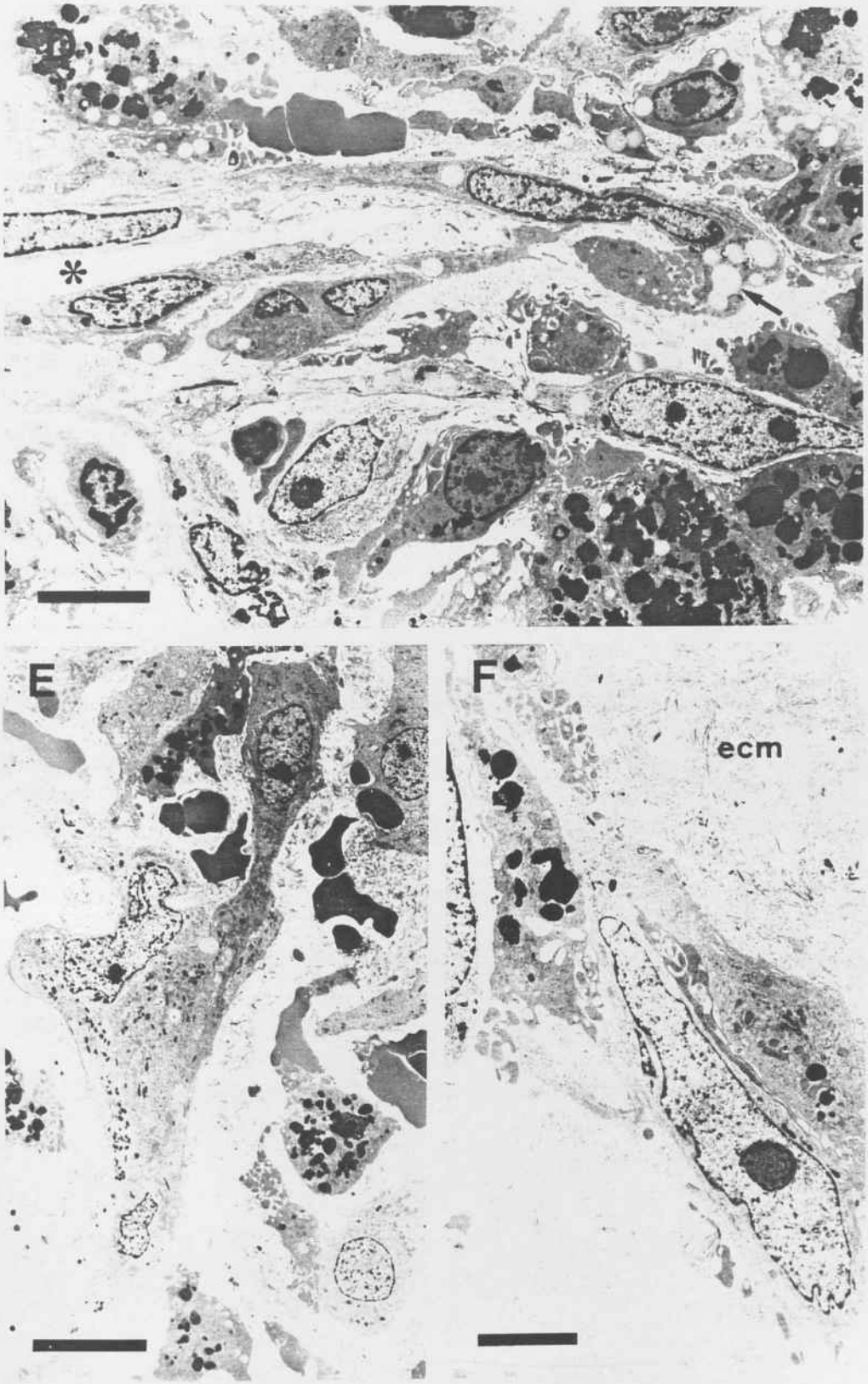
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Figure 2.

Figure 2. Characteristics of newly-formed capillaries in vitreoretinal membranes of PDR. **(A)** Semithin-sectioned specimens stained with toluidine blue showed a well-developed pericytic-endothelial complex as well as capillaries with defined lumina (*L*). Endothelial cells (*ec*) and pericytes (*p*) were found firmly surrounded by dense and abundant extracellular fibrous matrix (*ecm*). Few epithelial and fusiform-shaped cells were linearly arranged as a monolayer at the edge of sample, with their inner aspect attached to the matrix material; microvillous cytoplasmic projections were noted at the outer cell surface (*arrows*), which could represent the vitreal interface of membrane (*X 795, bar=30μm*). **(B)** Ultrathin section (TEM) showed newly-formed capillaries with narrow diameter that often present thicker and cuboidal-shaped endothelial cells (*ec*) with lumen (*L*) surface smooth; an extensive amount of stratified basement membrane (*bm*) was present (*X 4,500, bar=5μm*). **(C)** On the other hand, capillaries with wide luminal diameters showed thin, elongated endothelial cells with numerous cytoplasmatic projections toward luminal surface; pericytes (*p*) were frequently observed. Isolated or grouped pericapillar macrophages (*m*) were usually noted; extracellular collagenic matrix (*ecm*) (*X 2,000, bar=10μm*). **(D)** The superficial monolayer of cells visualized in Figure A was observed at the ultrastructural level. Areas of double-layered cells with fusiform-shaped feature, sometimes with fibrocytic characteristics, were closely adhered at collagenic substance. Microvilli (*arrows*) at the outer surface were also seen (*X 3,300, bar=5μm*).

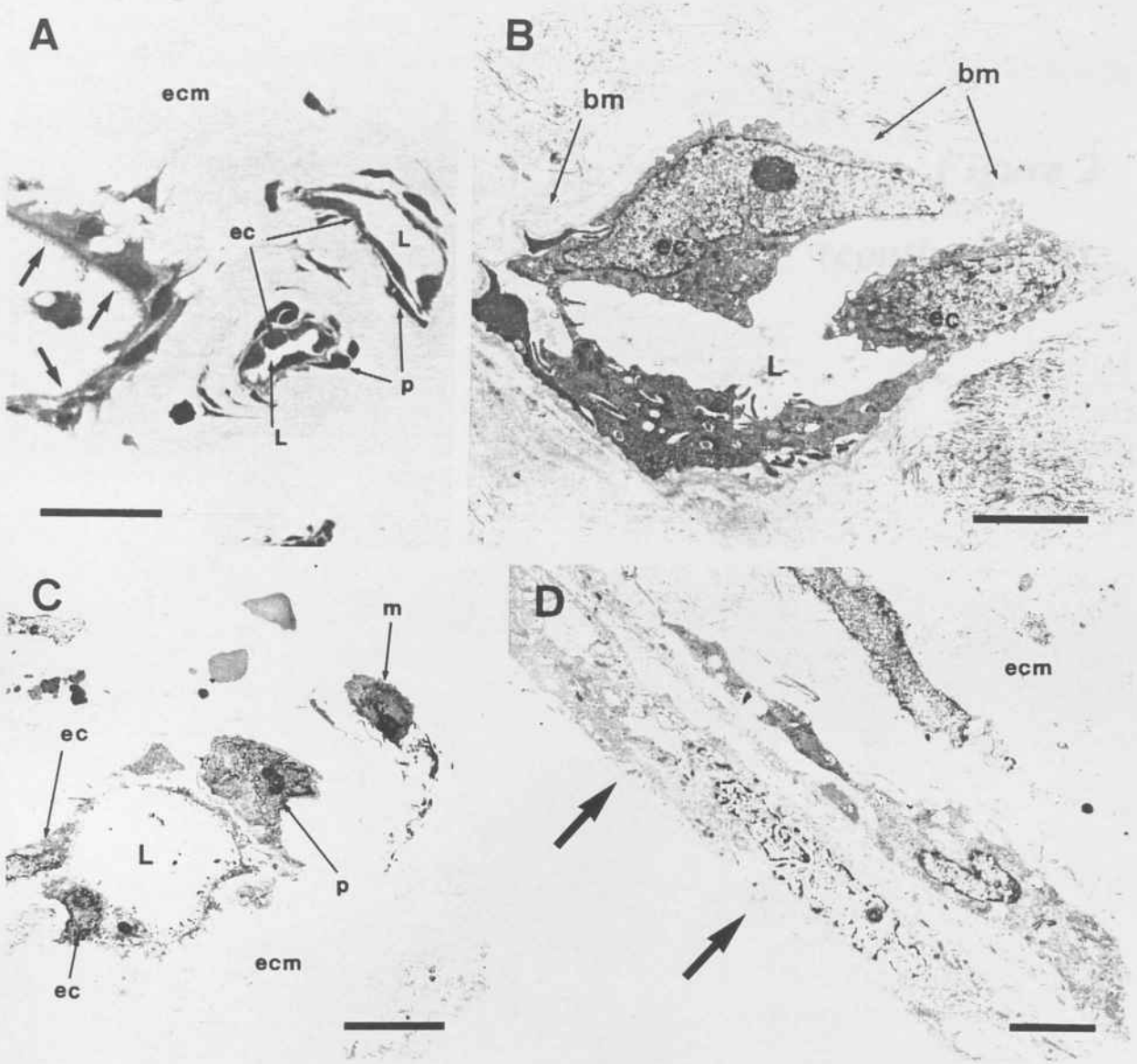
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Figure 2

(continuation).

Figure 2 (continuation). (**E**) High magnification (TEM) of newly-formed capillary with widely luminal (*L*) diameter showed an irregular endothelial cells (*ec*) surface presenting numerous cytoplasmic projections (*arrows*). A unilaminar basement membrane (*bm*) was present (*X 15,600, bar=1μm*). (**F**) Endothelial fenestrations and several pinocytic vesicles (*open arrow*) at the luminal aspect of endothelial cells were seen. *Fenestrae* showed single diaphragm component (*arrows*) (*X 42,000, bar=0.5μm*). (**G**) Endothelial cells were firmly joined by tight junctions (*arrows*) (*X 39,000, bar=0.5μm*).

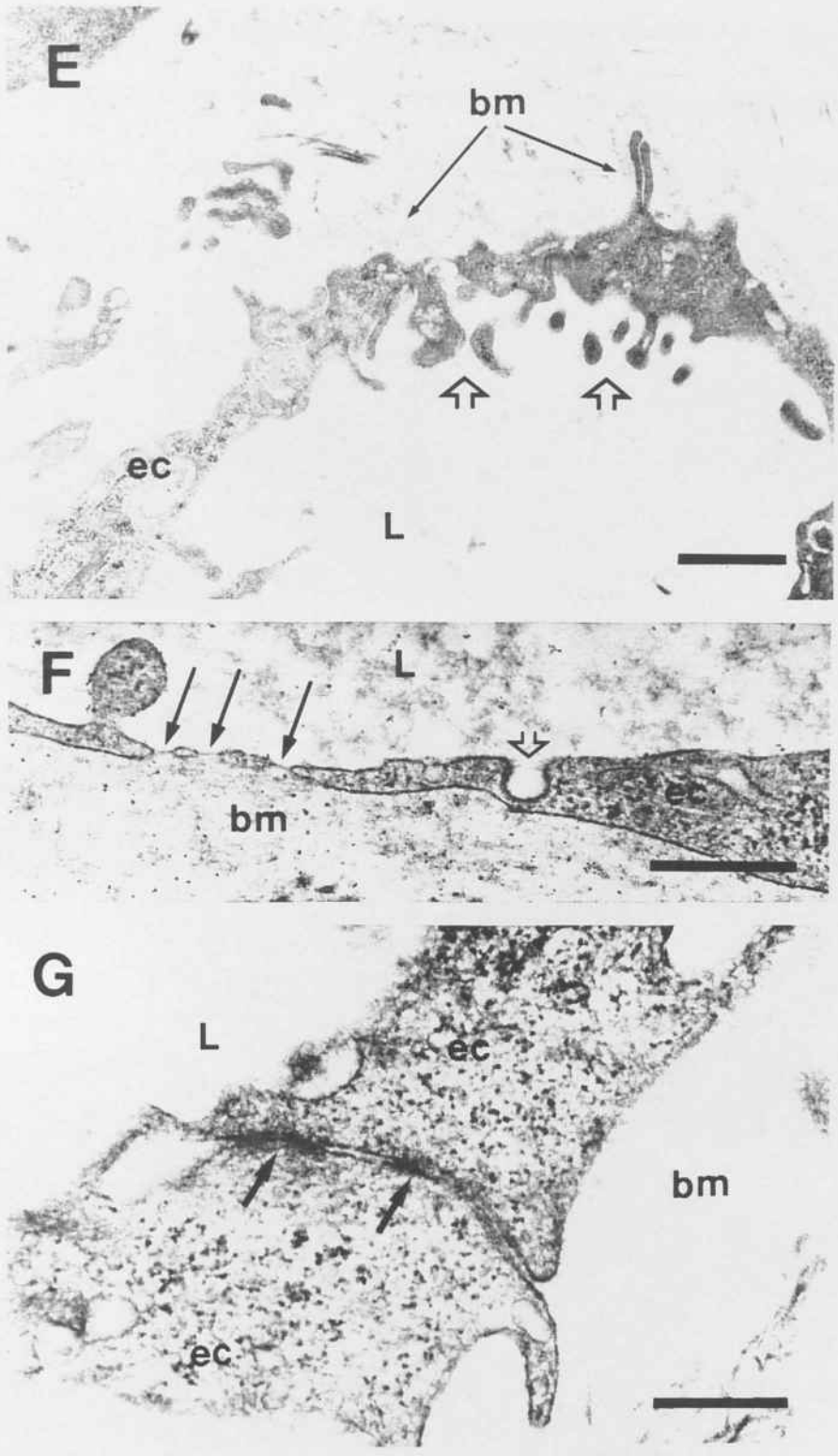
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Figure 3.

Figure 3. Fibrous astrocytes in preretinal membranes. (**A**) Low magnification electron micrograph showing epithelioid-shaped cells arranged at the edge of PDR specimen; well-developed organelles, aggregates of microfilaments (*arrowhead*), tendency to polarize, and segments of basement membrane (*arrows*) were noted (*X 5,200, bar=3μm*). (**B**) High magnification of bracketed area in Figure A, illustres microfilaments aggregates (*arrows*) and well-developed *cisternae* surfaces of rough endoplasmic reticulum (*asterisk*) (*X 21,000, bar=1μm*). (**C**) Fine junctional complexes (*arrows*), regular arranged, joined the lateral aspect of cells (*X 17,000, bar=1μm*). (**D**) Great amounts of intermediate filaments bundles (*arrows*) and numerous rough cytoplasmic processes (*cp*) were noted (*X 26,000, bar=1μm*). (**E**) Variable amounts of polyribossomic complexes (*asterisk*) suggest increased synthetic activity of cells. Segments of basement membrane (*bm*) were seen; microfilament aggregates (*arrows*) (*X 21,000, bar=1μm*).

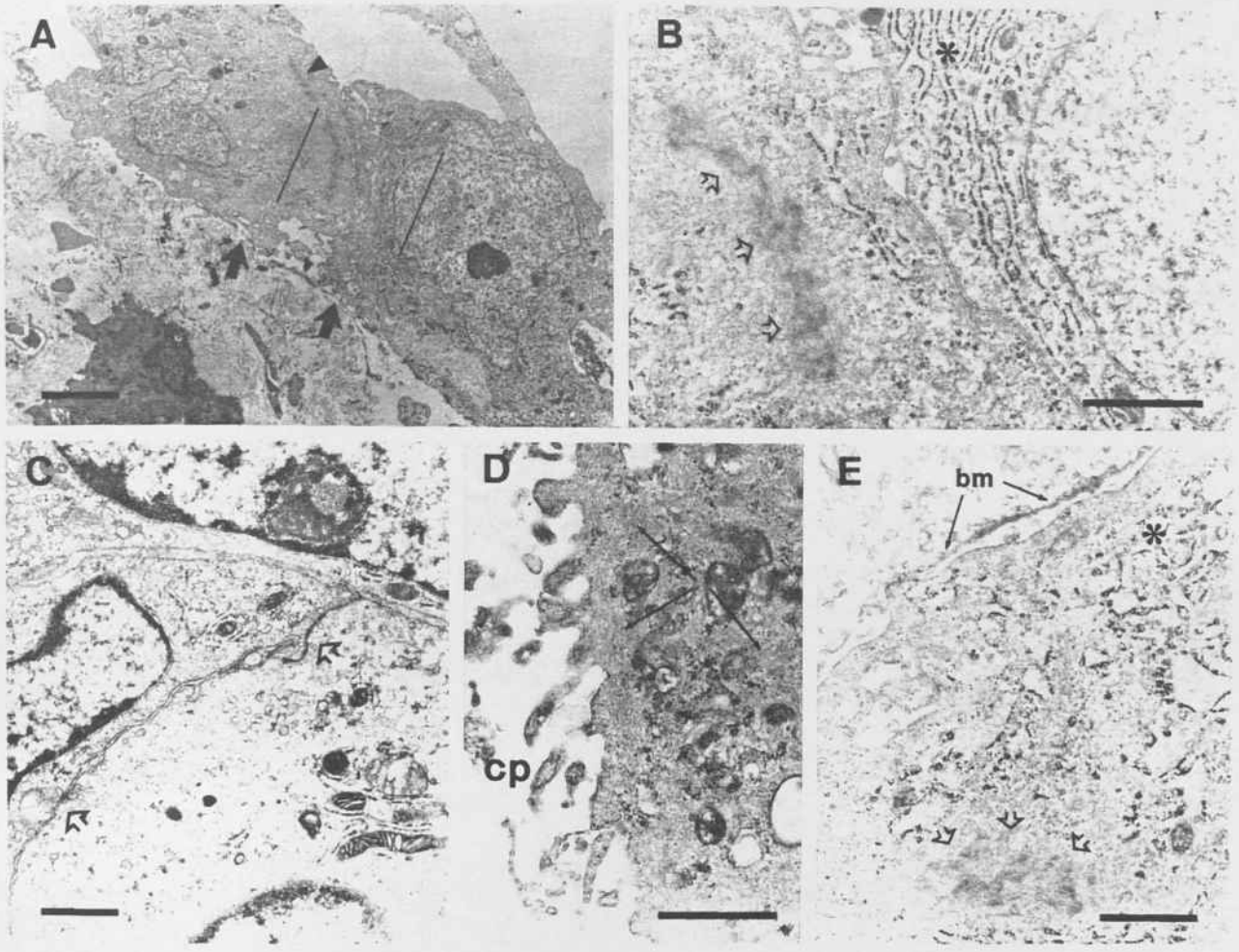
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Figure 4.

Figure 4. Retinal pigment epithelial cells (RPE) in preretinal membranes. (**A**) Transmission electron micrograph showing numerous fine cytoplasmic processes (*cp*) at the apical domain of polarized RPE cell; intracytoplasmic bounded melanin granules (*m*) were often noted (*X 20,800, bar=1μm*). (**B**) Epithelial morphology was observed in RPE cells joined by junctional complex (*arrows*), which were mainly observed at the lateral aspect of cells. In this adhered arrangement, RPE cells form a false lumen with their apical processes facing it (*asterisk*) (*X 14,000, bar=2μm*). (**C**) Junctional complexes (*arrows*) in regular arrangement were localized on the opposite side of apical cytoplasmic processes; non-stratified bounded melanin granules (*arrowhead*) (*X 20,800, bar=1μm*). (**C**) A well-developed basement membrane (*bm*) was closely related to the basal side of RPE polarized cells; aggregates of microfilaments centrally or peripherally arranged (*arrow*) were present (*X 26,000, bar=1μm*).

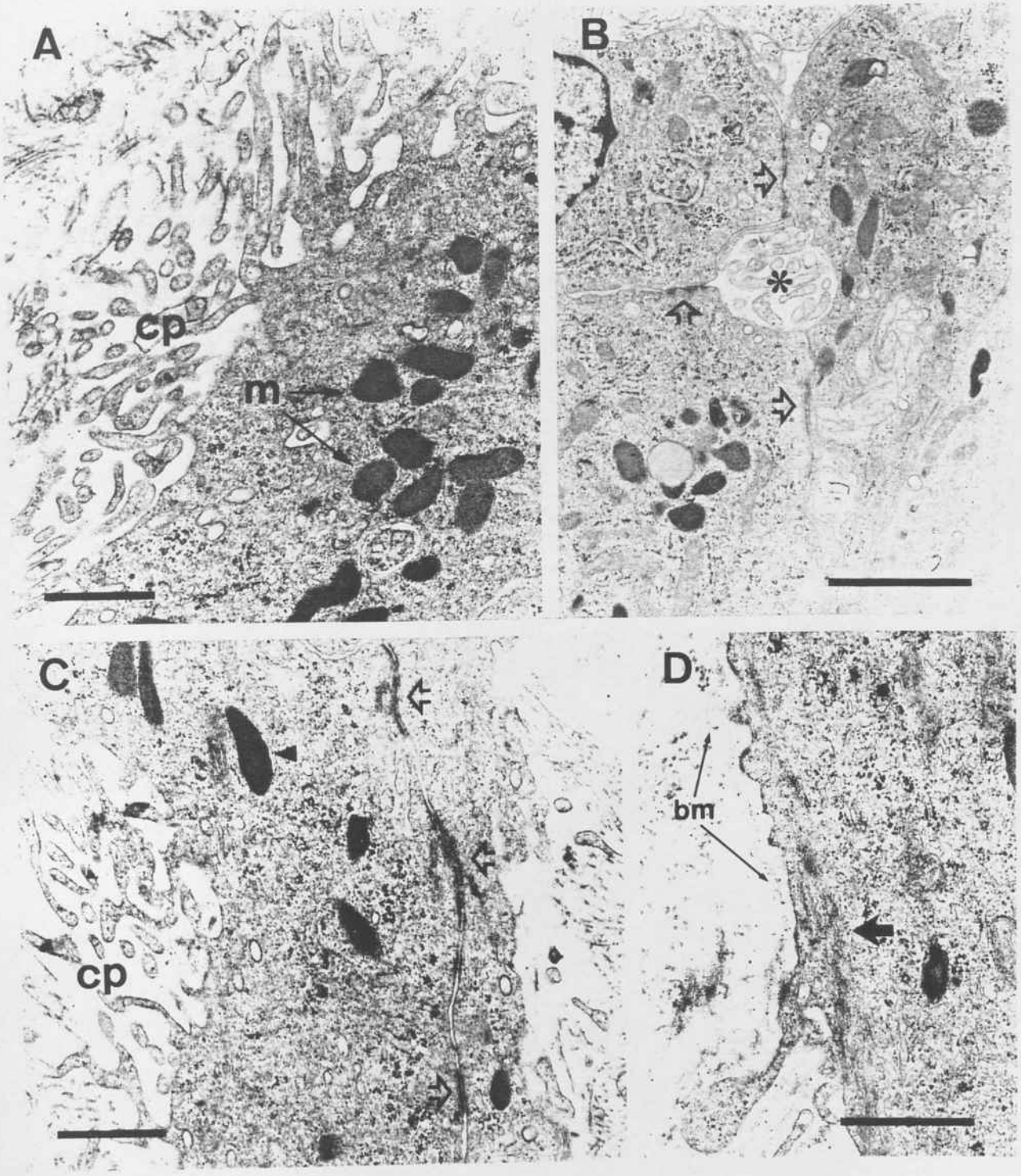
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Figure 5.

Figure 5. Fibroblasts in preretinal membranes. (**A**) Fibroblasts exhibited a fusiform morphology, without polarization or basement membrane formation, associated with adjacent collagen fibres of increased diameter, which represent newly-synthesized collagen (*col*). Intracytoplasmic melanin granules (*arrows*) and bundles of intermediate filaments (*arrowhead*) were occasionally seen (*X 8,600, bar=2μm*). (**B**) High magnification electron micrograph showed numerous well-developed *cisternae* of rough endoplasmic reticulum (*arrows*), and new collagen fibres deposition around cell (*X 12,500, bar=2μm*).

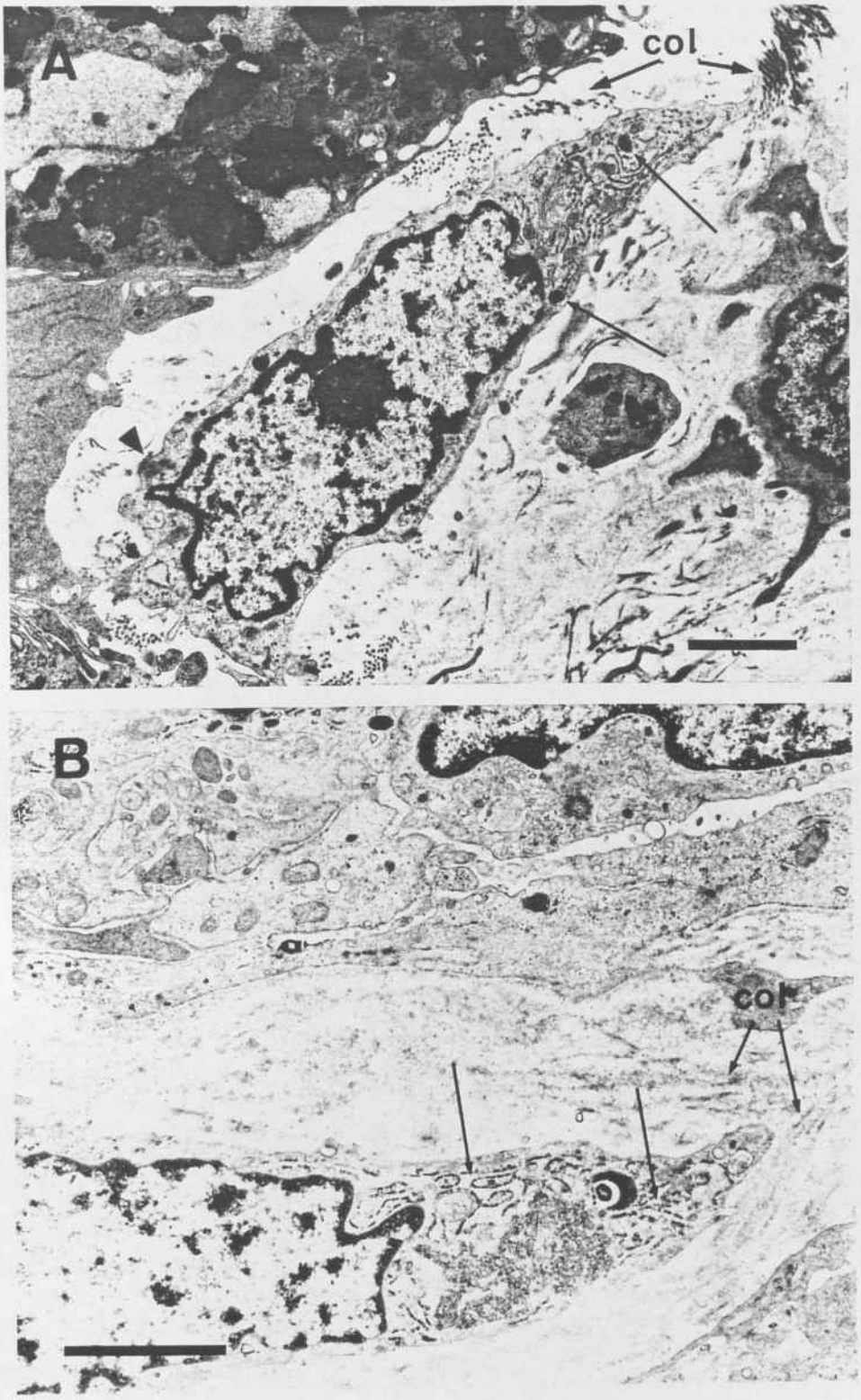
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Figure 6.

Figure 6. Macrophages and phagocyte-like cells in preretinal membranes. (**A**) Large epithelioid-shaped cells, which present numerous cytoplasmic processes and variable amounts of pleomorphic inclusions with pigment degraded or not, could represent RPE cells with phagocytic properties (*X 4,700, bar=3μm*). (**B**) Macrophages were identified by numerous secondary lysosomal vesicles, containing melanin granules in variable stages of degradation (*arrows*), and residual bodies (*X 6,900, bar=3μm*). (**C**) Cells with irregular morphology containing melanin or hemosiderin granules (*arrows*), which were delimited by secondary lysosome membranes, could represent resident hyalocytes with macrophagic function (*X 8,600, bar=2μm*). (**D**) High magnification micrograph showing pigment granules contained into secondary lysosomes (*arrows*) (*X 43,000, bar=0.5μm*).

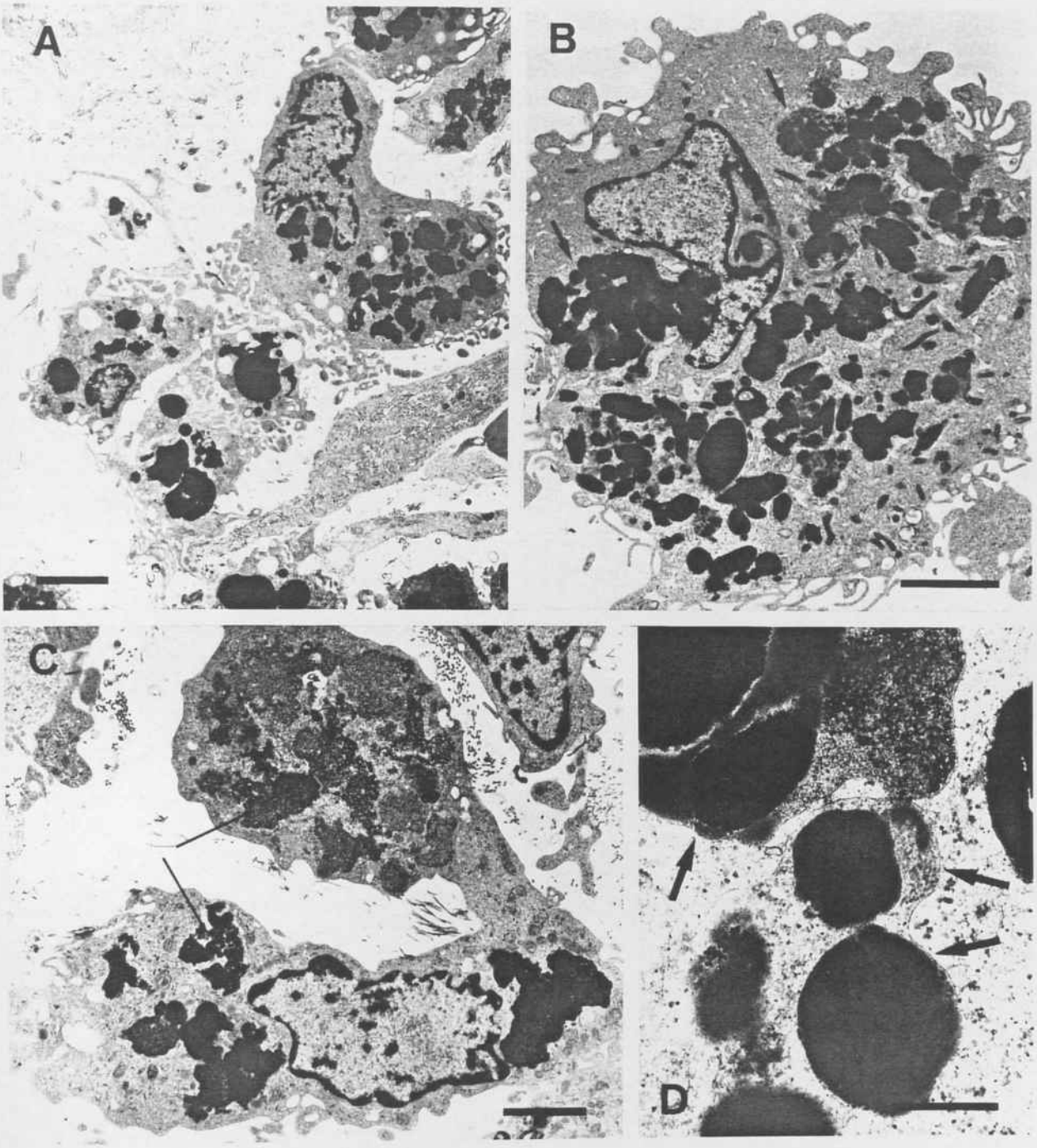
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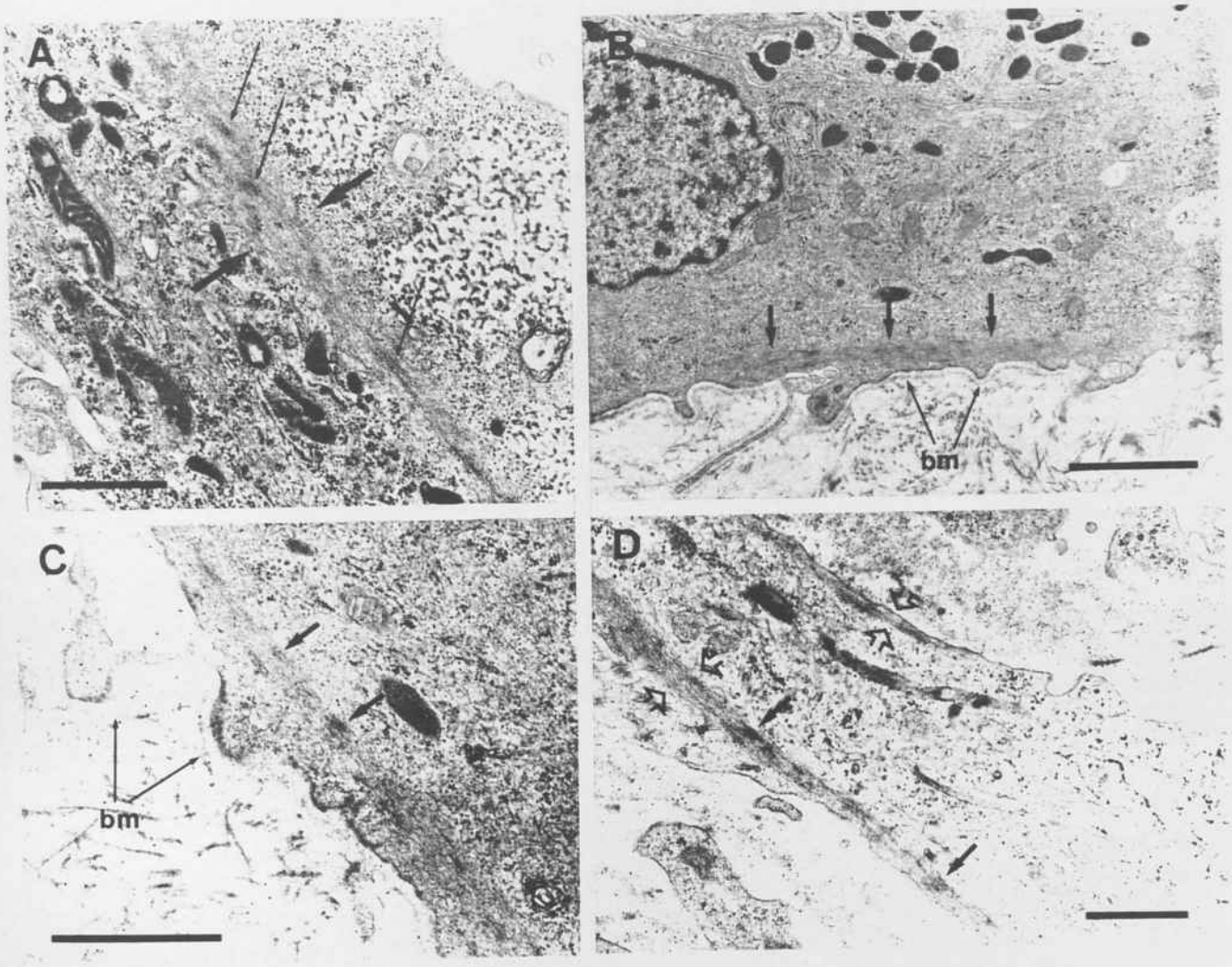
Figure 7.

Figure 7. Myofibroblastic differentiation in preretinal membranes. (**A**) Myofibroblastic differentiation was characterized by linear aggregates of intracytoplasmic microfilaments (*between arrows*) often localized marginally, which ran the length of cell; this microfilament package possesses fusiform densities (*large arrows*) (*X 26,000, bar=1μm*). (**B**) Cells with RPE intracytoplasmic features with a well-developed basement membrane (*bm*) presented myofibroblastic characteristics (*arrows*) (*X 9,000, bar=3μm*). (**C**) High magnification micrograph shows a continuous basement membrane and bundles of microfilaments with fusiform densities (*arrows*) (*X 36,000, bar=1μm*). (**D**) Fibroblast-like cells with myofibroblastic features; aggregates of microfilaments with fusiform densities (*arrows*) were noted in both marginal sides (*between arrows*) of plasma membrane (*X 21,000, bar=1μm*).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CAPITULO II

Electron-Immunocytochemical Characterization of Cell Intermediate Filament Proteins in Epiretinal Membranes.

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Abstract: Immunocytochemical staining methods for intermediate filament (IF) proteins were applied in an attempt to characterize cells involved in epiretinal membranes (ERM) of proliferative vitreoretinopathy (PVR). We used conventional electron microscopy to study morphological features and light and electron immunocytochemistry methods to study cytokeratin (CK), vimentin (V), glial fibrillary acidic protein (GFAP) expression, and anti-macrophage (Mac) as specific internal markers to identify different cell types in ERM. Indirect immunofluorescence on consecutive serial sections showed groups of cells localized in similar regions of the same specimen labelled for different IF markers (CK or V; GFAP or V). Electron-immunocytochemistry revealed that: 1) Cells with epithelioid-shaped features with or without pigment, resembling RPE cells in their morphology and/or arrangement, can express CK or V. 2) Cells with glial ultrastructural features have positive labelling for GFAP or V. 3) Pigment-laden cells with macrophage-like ultrastructural characteristics, whether or not positive for specific mAbs anti-Mac, can express V, but rarely CK. 4) Myofibroblast-like cells and cells with fibroblast shape may be V-positive or negative for all IF markers tested. Our data suggest that some cell types in ERM may express at least two different IF proteins, indicating an active dedifferentiation process in PVR.

Key words: Proliferative vitreoretinopathy, retinal detachment, immunocytochemistry, cytokeratin, vimentin, glial fibrillary acidic protein, macrophage.

INTRODUCTION

Epiretinal membranes (ERM) are characterized by a cellular proliferation and connective tissue formation on the surface of the retina. This proliferative process can occur in several ocular disorders (1,3), but is typically the leading histopathologic attribute of proliferative vitreoretinopathy (PVR), which sometimes complicates the natural history or surgical treatment of rhegmatogenous retinal detachment, and is the major reason of failure after surgery. During the evolution of PVR, the contraction of ERM leads a marked distortion of the retinal structure, resulting in a complex tractional retinal detachment which is difficult to repair.

Although the pathogenesis of ERM formation is not completely understood, attempts have been made to determine the cell origin in membranes based on ultrastructural (1-8) and light microscopy immunocytochemical (9-13) criteria. These studies have shown several types of cell, including retinal pigment epithelial cells (RPE), glial cells, fibroblasts, and cells with myofibloblast transformation, which have been implicated in contractile cell phenomena observed in PVR process. In vitreous and/or standard cultures, most of these cell types may undergo phenotypic changes, and thus no longer resemble the normal cell populations from which they are derived (14-16). There is also evidence to suggest that several peptides and serum proteins may stimulate cell migration, adhesion and proliferation, and tissue contraction (17-20).

Several immunocytochemical studies on preretinal membranes have been reported without an ultrastructural evaluation. They have provided useful criteria, although they were only partially successful, first since there were some discordances between immunocytochemical results and ultrastructural studies in many ERM series (1,7-10). Second, some cell types that proliferate in ERM showed few distinguishing characteristics; they are usually described as fibroblast-like cells (1,3,6,7) or as a presumed or indeterminate cellular type (1-8), suggesting loss or modification of cellular

characteristics during proliferative process. Finally, dedifferentiation and adaptation phenomena in cells are commonly observed in cultures and several pathological processes (21,22).

We performed optical and electron immunocytochemical studies to determinate intermediate filament (IF) expression in cells of ERM obtained from patients with PVR. We used ultrathin sections by cryoultramicrotomy that preserves both good tissue antigenicity and ultrastructure for IF and phenotypic evaluation respectively. At the same time a conventional ultrastructural study was performed to corroborate morphological data.

MATERIAL and METHODS

Human ERM (n=29) were removed by appropriate retinal peeling from patients undergoing intraocular surgery for retinal reattachment. Specimens were immediately fixed in 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) solution, pH 7.4, for at least 12 hours at 4°C. They were then rinsed in PBS 0.1 M pH 7.4 and when there was sufficient tissue (n=11), fixed membranes were divided in two under a dissecting microscope. One half was placed in 4% paraformaldehyde in PBS 0.1 M and stored at 4°C for conventional ultrastructural microscopy. The remainder specimens were immersed in 2.1 M sucrose in PBS for 30 min, mounted on a metal stub for cryoultramicrotom, rapidly frozen in liquid nitrogen and maintained at -196°C before cryoultramicrotomy.

For electron microscopy, specimens (n=11) were abundantly rinsed in PBS 0.1 M pH 7,4, post-fixed in 1% osmium tetroxide in phosphate buffered solution for one hour, dehydrated in increasing graded concentrations of acetone, and then embedded progressively in resin (Spurr technique) for polymerization at 60°C. Ultrathin sections (50nm - 75nm) were obtained by conventional ultramicrotomy (OmU2, Reichert-Jung, Germany), placed on copper grids (200 mesh), and then stained with uranyl acetate and lead citrate solution for conventional transmission electronic microscopy (TEM) (Hitachi 800 MT, Hitachi INC., Japan).

For immunocytochemical procedures consecutive serial semithin sections (0.3μm - 0.4μm) at -70°C, and consecutive serial ultrathin sections (90nm - 0.1μm) at -105°C, were obtained by cryoultramicrotomy (Ultracut FC4D, Reichert-Jung, Germany). Semithin frozen sections were placed on 0.5% gelatin-coated slides in a humidified chamber at 4°C before optical immunocytochemical detection; some sections of each specimen were controlled by toluidine blue staining. Ultrathin frozen sections were placed on gold grids (200 mesh) formvar-coated for TEM and then maintained in PBS 0.1 M (pH 7.4) at 4°C before the electron immunocytochemical study.

Several primary monoclonal or polyclonal antibodies for IF detection were used: glial fibrillary acid protein (GFAP), cytokeratin (CK), vimentin (V) and macrophages (Mac). We used a rabbit anti-bovine GFAP antiserum (Dakoppats, Denmark) at 1:200 dilution, a mouse IgG directed against vimentin (clone V9; Dakopatts, Denmark), mouse IgG anti-CK (clone K8.13, I.C.N., U.S.A.), and mouse IgG anti-Mac (clone 25F9; Boehringer, Germany), all at 1:30 dilution. Fluorescein (FITC)-conjugated goat anti rabbit immunoglobulin antisera (Boehringer, Germany) for the primary polyclonal antiserum and TRITC (rhodamine) or FITC-conjugated anti mouse immunoglobulin antisera (Dakopatts, Denmark) for all mouse mAbs were used at 1:25 dilution for secondary fluorescent detection. For electron immunocytochemistry, protein A/colloidal gold 16nm (pA-Au 16nm), produced in our laboratory, and rabbit anti-mouse IgG-Au 10nm (Amersham, U.K.) were used at 1:50 dilution for respective species primary antibodies. Both primary and secondary reagents were diluted in 1% ovalbumin in PBS-Glycine 0.1 M (pH 7.4). A rabbit preimmune serum (Sera-Lab, Sussex, England) in the same primary antibody dilutions was used for negative control.

Indirect immunofluorescence was performed as described previously (23,24). In brief, semithin frozen sections were air-dried at room temperature, washed (3 X 5 min) in PBS-Glycine 0.1 M, and blocked (10 min) 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 then rinsed with PBS-Glycine 0.1 M and the secondary conjugated antibodies (IgG-FITC or IgG-TRITC) were applied for one hour at room temperature in the dark. After the last incubation, slides were washed and mounted with 70% glycerol in 5% *n*-propyl galleate buffered mounting medium. Negative control sections were prepared by substitution of the primary antibody by preimmune rabbit normal serum in corresponding dilution. Immunostaining was visualized with an epifluorescence microscope (Polyvar II, Reichert-Jung, Germany).

For electron immunocytochemical staining, grids were pre-treated in ammonium chloride in

PBS 0.1 M solution (10 min) to eliminate non-specific radicals, rinsed in PBS-Glycine 0.1 M solution (4 X 2 min), and then blocked in 0.5% ovalbumin in PBS-Glycine 0.1 M solution (10 min) at room temperature. Primary antibodies were incubated for 30 min at the dilutions mentioned above and washed in PBS-Glycine 0.1 M. Secondary anti-mouse IgG-Au 10nm or pA-Au 16nm was then applied for 20 min. After successive washes, first in PBS 0.1 M solution (4 X 2 min) and then in bidistilled water (6 X 2 min), grids were contrasted in 0.03% uranyl acetate solution (10 min) and a thin surface membrane of methyl-cellulose was applied. Negative control sections were performed by omission of the primary antibodies. Results were observed in conventional TEM (Hitachi 600 AB, Hitachi Inc., Japan).

RESULTS

Indirect Immunofluorescence

Observation of ERM stained by toluidine blue showed cells of heterogeneous population with widely variable morphology, which were scattered in the matrix (not shown). Moderate amounts of extracellular connective tissue embedding various cell types, such as epithelioid or fusiform-shaped cells in cord or cluster arrangement were noted. Numerous large, rounded cells with light cytoplasm were seen. Some cells with or without pigment were observed. Within the matrix the ratio between cells and collagenous material was variable. Several specimens were densely pigmented.

Analysis of 29 ERM sections treated with antibodies directed against IF (GFAP, V, CK) and Mac revealed distinct patterns of immunostaining (Fig. 1). Positive-stained cells for V were observed in 27 specimens (93%). These cells varied in morphology, including fusiform, rounded and cuboidal-shaped cells, which were arranged in clusters scattered in the matrix (Fig. 1A) or cords disposed in layers (Fig. 1C); occasionally V-positive cells were observed in evident peripheral arrangement in samples (Fig. 1B). GFAP-positive cells were observed in 26 specimens (89.6%) in round elongated or spindle-shaped morphology. The cells were usually arranged in peripheral layers in samples (Fig. 1F), or seen as isolated cells within the matrix (Fig. 1G). Label for CK was noted in 18 ERM specimens (63%). Isolated cells were clearly stained in the periphery of samples (Fig. 1E), while large cluster or cords of cells were also observed arranged in layers (Fig. 1D). These cells sometimes differed in aspect, with epithelioid characteristics, plump or fusiform-shaped cells set in connective tissue. Cells positive for anti-Mac, usually arranged in foci or as a isolated cells associated with pigment and granules (see below), were only noted in 7 samples (24%).

Comparing consecutive specimen sections, which were labelled by different markers, cells positive for different IF proteins were seen in the same area and arrangement across the sample. Cells positive for both V and CK were observed in a similar arrangement and aspect when several consecutive sections were analyzed (Fig. 1C and 1D). In addition, some samples displayed V and GFAP-positive cells in peripheral arrangement (not shown).

Ultrastructural and Immunoelectron Microscopy

Cryoultramicrotomy ultrathin sections provided an acceptable morphologic resolution to compare with samples prepared by conventional ultrastructural microscopy.

By electron microscopy, retinal pigment epithelial cells (RPE) were identified by their polarity with numerous cytoplasmic and microvillous processes as well as intracytoplasmic bounded melanin granules; cells were occasionally arranged in rosette-like configuration forming a false lumen, in which microvilli facing it (Fig. 2A and 2E). These cells were frequently, but not always, CK-positive (Fig. 2B to 2D) and negative for GFAP or anti-Mac. Cells with similar arrangement and characteristics expressed V with variable degree of labelling (Fig. 2F to 2I). This suggests that cells can express, lose or acquire these proteins during the proliferative process.

Cells presenting glial ultrastructural features were often elongated or spindle-shaped with a tendency to polarize, showing microvillous-like processes. Masses of microfilaments and IF associated with well-developed organelles, such as rough endoplasmic reticulum were usually noted.

In these cells, GFAP and V were usually expressed, but not CK or anti-Mac (not shown).

Macrophages were recognized by their large epithelioid or rounded morphology, with pleomorphic cytoplasmic contents and lysosomal vesicles with residual bodies (Fig. 3B and 3E); cells sometimes contained non-degraded bounded pigment (Fig. 3A). These cells were not always positive for the specific IgG-anti Mac but frequently expressed V (Fig. 3C and 3D). CK-positive cells with these characteristics were rarely seen, but negative labelling for GFAP were observed.

Fibroblasts or fibroblast-like cells were usually fusiform and lack polarization, surrounded by large amounts of collagen bundles (Fig. 4). Well-developed rough endoplasmic reticulum with numerous *cisternae* were frequently observed; sometimes intracytoplasmic pigment was present. In some cases V-positive labelling was observed (Fig. 4C and 4D), but generally these cells were negative for all IF markers.

Myofibroblast-like cells presented bundles of microfilaments often localized marginally with a subplasmalemic arrangement, running the length of the cell (Fig. 5A). These cells usually possessed a spindle or epithelioid-shaped morphology with undifferentiated features, or both glial and RPE intracytoplasmic characteristics. Myofibroblast-like cells frequently expressed V in variable intensity of labelling (Fig. 5B to 5D), but not GFAP, CK or anti-Mac proteins.

DISCUSSION

Much significant progress has been made in elucidating physiopathogenic mechanisms of cell migration, adhesion and proliferation that characterize PVR and contribute to ERM formation. However, the initial events and cell types that lead to ERM development remain unresolved. Immunocytochemical procedures on light and electron microscope suggested that cells in ERM show an active dedifferentiation process, which makes elucidation of their origin difficult.

Several studies (1-8) have attempted to determine the ultrastructural features of cells in ERM, obtained by intraocular surgery from PVR patients, or *in vitro* or *in vivo*. Our conventional ultrastructural data are in agreement with those previously reported. However, since dedifferentiation phenomena usually occur in active processes of cell migration and proliferation (21,22), cell origin identification based only on ultrastructural criteria is imprecise and remains to be established by other approaches.

Immunocytochemistry for IF proteins was initially applied in ERM (9-13,25) in an attempt to elucidate the possible origin of the cells. We used IF expression as specific internal markers in immunoelectron microscopy and we also correlated cell-type identification with conventional ultrastructural morphological features. Several circumstances were observed: 1) Cells with epithelioid-shaped features, with or without pigment, resembling RPE cells in their morphology and/or arrangement, can express CK or V. 2) Cells with glial ultrastructural features with positive labelling for GFAP or V. 3) Pigment-laden cells with macrophage-like ultrastructural characteristics, whether or not positive for specific mAbs anti-Mac, can express V, but rarely CK. 4) Myofibroblast-like cells and cells with fibroblast-shaped morphology may be V-positive cells, or negative for all IF markers tested.

Pre-embedding electron-immunocytochemistry in preretinal membranes (27) showed cells with variable morphological characteristics such as: (a) polarized cells with microvilli and foot processes, (b) spindle-shaped fibroblast-like cells, (c) undifferentiated, pigment-laden cells, and (d) small, round, mononuclear cells with short processes, did not express any marker (GFAP, V, or CK) as internal protein. In addition, poorly differentiated cells with numerous mitochondria and others with lightly stained nuclei with scanty cytoplasm can express any of the above IF proteins. The present study showed expression of different intermediate filament proteins in cells that presented similarities in their ultrastructural features. By cryoultramicrotomy techniques, tissue antigenicity and morphologic characteristics can be sufficiently preserved to compare with conventional ultrastructural related methods, but was not definitive to identify cell origin.

Results of CK immunostaining on ERM indicated this procedure as a valuable means to determine the contribution and distribution of RPE cells in membranes (10). Nevertheless, we found cells whith ultrastructural features of RPE cells that also labelled for V. Absence of CK expression in RPE-like cells suggests an adaptative state in cell events observed in ERM development. Immunoblot analizes on bovine retina (30), showed RPE cells contained both CK and V filament proteins. Immunofluorescence experiments on bovine RPE cells in culture (28), showed interruption of CK synthesis while cells continued (or began) to synthesize V soon after the onset of the culture. These results agree with studies on cultured human RPE cells, wich reported large amounts of V by electrophoretic assays (31). In addition, changes of IF protein expression with V synthesis were reported in other types of cultured epithelial cells (32,33). Human RPE cells in vitreous culture were recently found to undergo morphologic changes, become undifferentiated, or polarized, or show a fibroblastic appearance, while expressing CK and V in high percentage wich diminished with time (16). Thus, it seems possible that some cells in ERM may lose, change, gain or regain their IF protein expression behaviour, depending on the stage of development.

ERM often present cells with glial features (1-4), which in culture may adopt an even great variety of phenotypes, including spindle-shaped forms. Nevertheless, cultured glial cells retain their GFAP content whatever their morphology (26). We observed cells with glial ultrastructural features labelling for GFAP or V, thus indicating that both proteins could be expressed by glial cells. In various species Müller cells express GFAP under normal conditions and synthesize V in response to retinal injury or degeneration (35-37), confirmed by double-labelling experiments on electron microscopy (38). Similar transition of IF proteins from V to V/GFAP has been reported in the development of retinal astrocytes and radial glia in rats (37,39,40), considered as a specific marker for glial differentiation. Furthermore, human retinal glia grown on vitreous culture showed a time-dependent decrease in the number of cells expressing GFAP, with a corresponding increase in cells expressing V (16). By other post-embedding electron-immunocytochemical approach, Vinores et al. (41) observed a co-expression of keratin-GFAP by some ERM cells; it was not possible to elucidate wether these cells represented glial cells that were induced to express keratin, RPE cells expressing GFAP, or both. Results suggest that an adaptative process with differents IF proteins co-expression could be a generalized event in ERM formation.

During the development of fibrocontractive disorders, fibroblasts acquire contractile features and myofibroblasts have the ability to generate connections on the surrounding extracellular matrix (42,43). Based on immunochemical criteria (44,45), actin expression was involved in preretinal membrane formation and contraction. In addition, V has been described as the most representative IF protein in the wound repair myofibroblasts (46). We identified myofibroblast-like cells as a frequent cell type present in most of ERM specimens analyzed, which, ultrastructurally, presented typical features, such extensive bundles of microfilaments with fusiform densities, localized marginally underneath the plasma membrane. By electron-immunocytochemistry these cells labelled for V in sites corresponding to microfilaments. This suggests that V may act with actin filaments in cell contraction phenomena observed in PVR clinical evolution.

As previously described (1-3,5,8), we identified cells that, ultrastructurally, presented macrophagic features; RPE transformed cells and/or cells of vitreous cortex were described as possible sources of cells with phagocytic properties, although a plasmatic origin was not ruled out (1,2,5). By electron immunocytochemistry we observed that this kind of cell may label for specific antibody anti-Mac, may occasionally label for V but exceptionally for CK. IF expression noted in macrophage-like cells suggests phagocytic activity in ERM from at least two cell types.

For the numerous reasons explained and based on our findings, we recognize that IF expression of cells involved in PVR is far from an accurate understanding. Moreover, differences in cell morphology and metabolic events on internal protein expression may be related to changes in local environment and particular conditions regulating functional characteristics of cells (14-16,26,32-

35,40,43). V expression seems to be usualy related to cell adaptative mechanisms in PVR, then losing its value regarding the cell origin in ERM formation. In PVR, which is characterized by poorly understood multifactorial events, the interaction between cells and substratum is a outstanding phenomenon before, during, and after ERM formation; cell metabolism and protein expression could certainly always respond to specific intraocular condition and modifications. Results obtained in the present work do not determine cell origin in ERM, but suggest that some cell types can express at least two IF proteins, in agreement with previous studies (27,41). Cells with similar morphological features expressing different IF proteins indicate that dedifferentiation and adaptation cellular phenomena, which were observed in cultured cells (47,48), may characterize the complex functional and metabolic changes in growing cells during PVR.

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Figure 1.

Figure 1. Indirect immunofluorescence for IF proteins in ERM semithin sections. (**A**) V was noted in cells with a wide variety of morphologic aspects (*X 290*). (**B**) Clusters of cells localized at the edges of specimens occasionally showed immunoreactivity for V (*X 580*). (**C**) V-positive cells with epithelioid-shaped characteristics and grouped in cords arrangement were seen (*X 335*); (**D**) Consecutive section of the specimen observed in C incubated with anti-CK mAbs; positive labelling was noted in cells with similar morphology and apparently localized in similar areas (*X 550*). (**E**) Isolated fusiform-like cells CK-positive were usually observed in samples (*X 680*). (**F**) GFAP-positive cells were frequently noted in cluster with peripheral arrangement in samples (*X 680*), or (**G**) as a isolated cells (*X 900*). *Bar=10μm.*

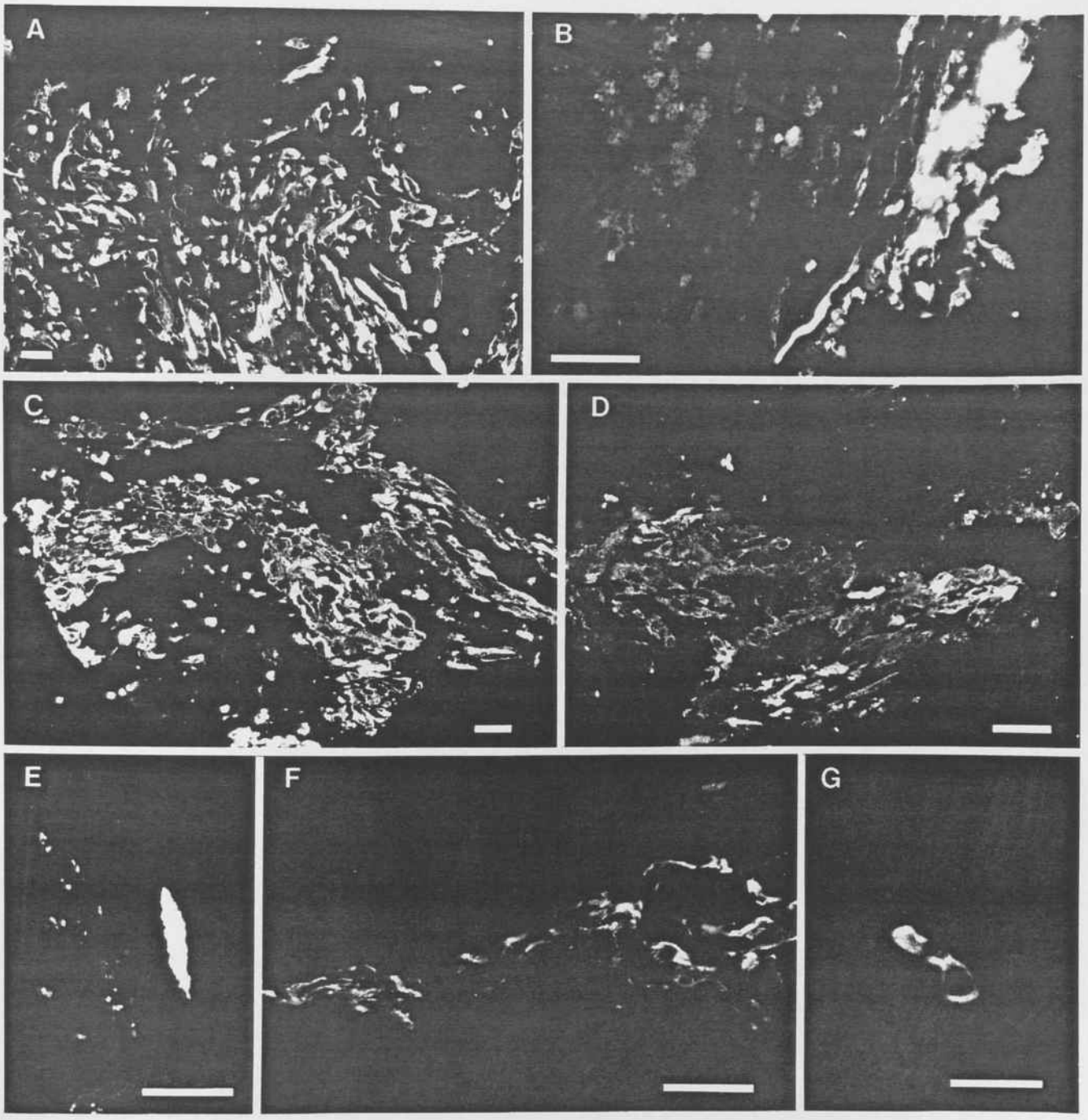

Figure 2.

Figure 2. Ultrathin sections of ERM from PVR. Ultrastructure of retinal pigment epithelial (RPE) cells and immunoreactivity characteristics of CK-positive cells. (**A**) Conventional ultrathin sections; (**B**, **C**, and **D**) Cryoultrathin sections. (**A**) RPE cells showed polarization with cytoplasmic processes (microvillous processes) in their apical domain (*asterisk*), intracytoplasmic bounded melanin granules (*arrow*). They were arranged in clusters, which cells were joined by junctional complex (*arrowheads*) (*X 5,200, bar=5μm*). (**B**) Sections of the same sample were incubated with specific mAbs directed against CK; cells with similar characteristics labelled for immunogold particles (10nm) (*arrows*) (*X 9,600, bar=2μm*). (**C**) Low magnification micrograph showed a pigment-laden cell (*arrowhead*) immunoreactive for CK (*arrows*) (*X 13,200, bar=2μm*). (**D**) High magnification micrograph of bracketed area observed in figure C, showed immunogold labelling (*arrows*) and intracytoplasmic bounded melanin granule (*arrowhead*) (*X 48,000, bar=0.5μm*).

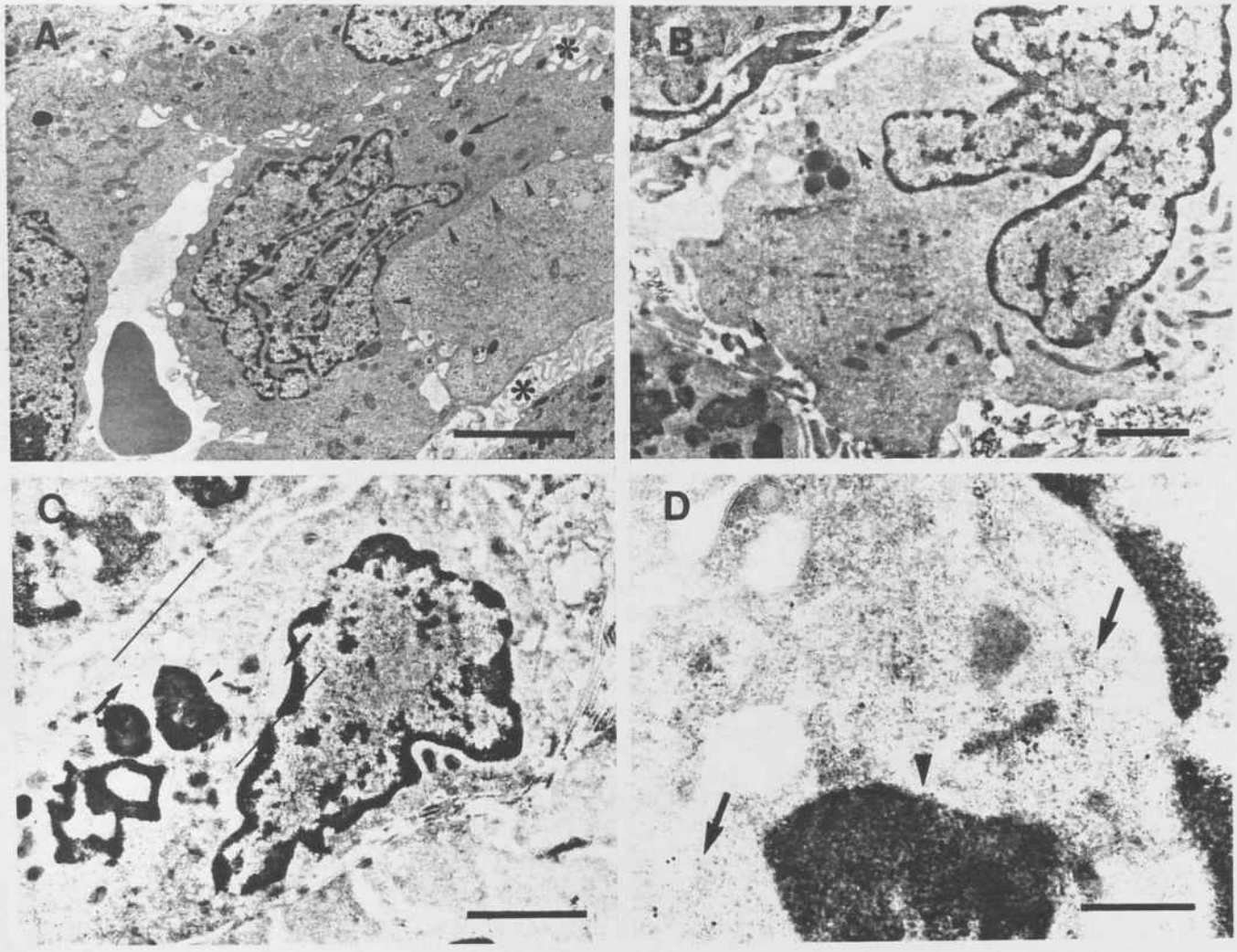
A
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Figure 2

(continuation).

Figure 2 (continuation). Ultrastructure of retinal pigment epithelial (RPE) cells and immunoreactivity characteristic of V-positive cells. (**E**) Conventional ultrathin section; (**F, G, H,** and **I**) Cryoultrathin sections. (**E**) RPE cells were recognized by their cuboidal shape and polarity; these cells were sometimes noted in a rosette-like arrangement with microvilli facing a false lumen (*asterisks*) (*X 2,800, bar=5μm*). (**F**) Polarized epithelial-like cells contained melanin granules and apical cytoplasmic processes (*asterisk*),presented a generalized labelling for V (*arrow*) (*X 13,200, bar=2μm*). (**G**) Cuboidal-shaped cells in a similar rosette-like arrangement showed false lumen structures (*asterisk*) observed in Figure E, and presented immunogold labelling for V (*X 9,600, bar=2μm*). (**H**) High magnification micrograph of bracketed area in Figure G, showed a generalized immunoreactivity for V (*X 24,000, bar=1μm*). (**I**) High magnification of bracketed area seen in figure F, showed labelling (*arrow*) for V (*X 32,000, bar=0.5μm*).

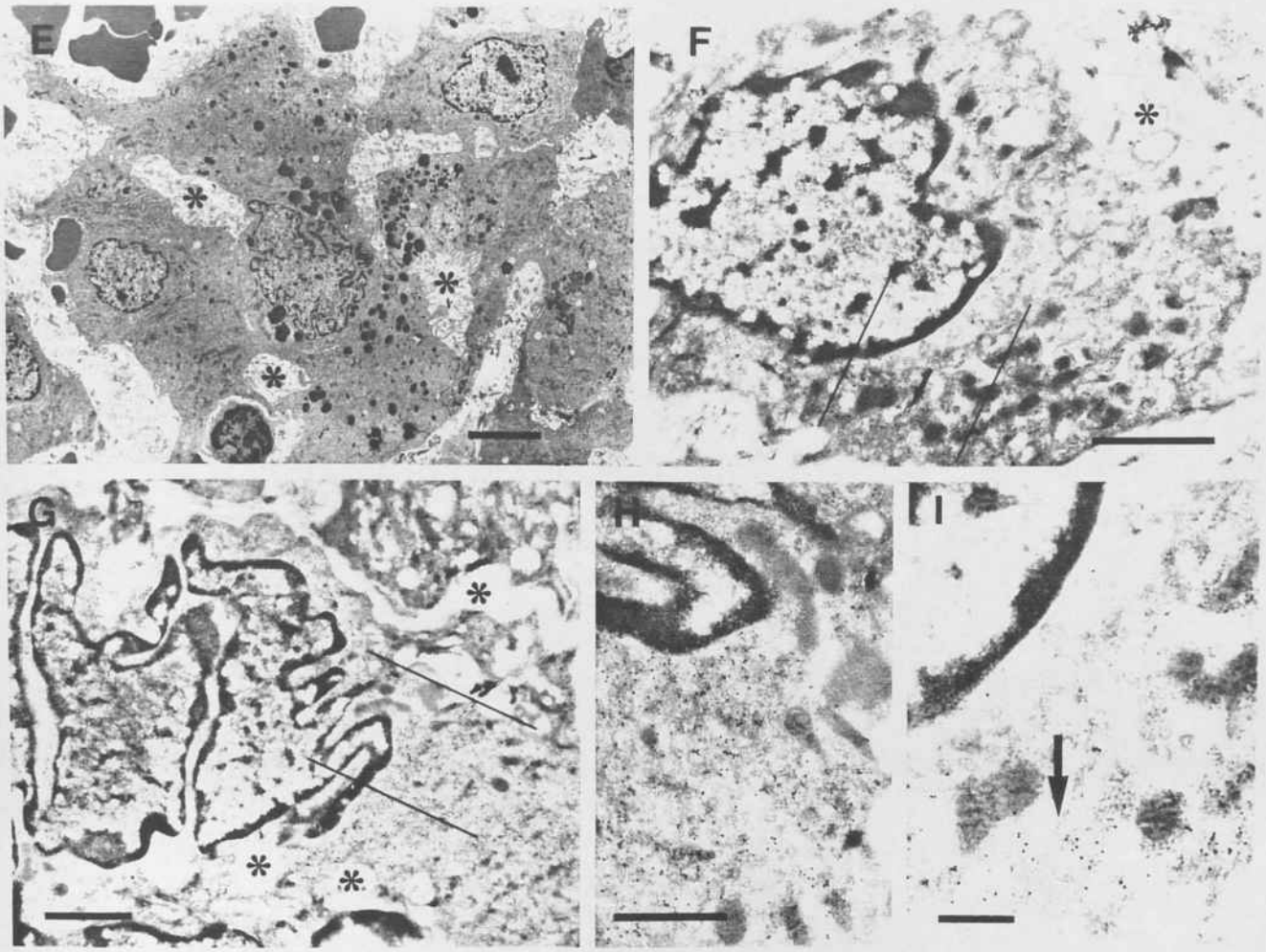
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Figure 3.

Figure 3. Ultrastructure and immunoreactivity characteristics of macrophages and phagocytic-like cells in ERM. (**A** and **B**) Conventional ultrathin sections; (**C** and **D**) Cryoultrathin sections; (**E** to **G**) Cryosemithin sections. (**A**) Cells with cuboidal appearance and cytoplasmic processes exhibited intracytoplasmic melanin granules in various stages of degradation. (*X 4,200, bar=3μm*). (**B**) Degraded haemosiderin and melanin were observed into secondary lysosomal vesicles (*asterisk*); residual bodies were usually present (*X 14,300, bar=1μm*). (**C**) Pigment-laden cells with macrophagic characteristics occasionally showed immunoreactivity for V (*arrow*) (*X 10,400, bar=3μm*). (**D**) High magnification micrograph of bracketed area in Figure C showed moderate immunogold (10nm) label (*arrow*) for V; non-degraded melanin granule (*upper side*) and degraded pigment vesicle (*asterisk*) were found (*X 30,000, bar=1μm*). By indirect immunofluorescence, specific staining for mAbs anti-Mac was observed. (**E**) Interferencial contrast microscopy showed cells with a cuboidal morphology (*arrows*) included numerous intracytoplasmic pleomorphic contents; these cells (**F**) were positive for IgG anti-Mac (*X 910, bar=20μm*). (**G**) Furthermore, large oval cells with intracellular pleomorphic bodies were also seen positive for anti-Mac (*X 450, bar=40μm*).

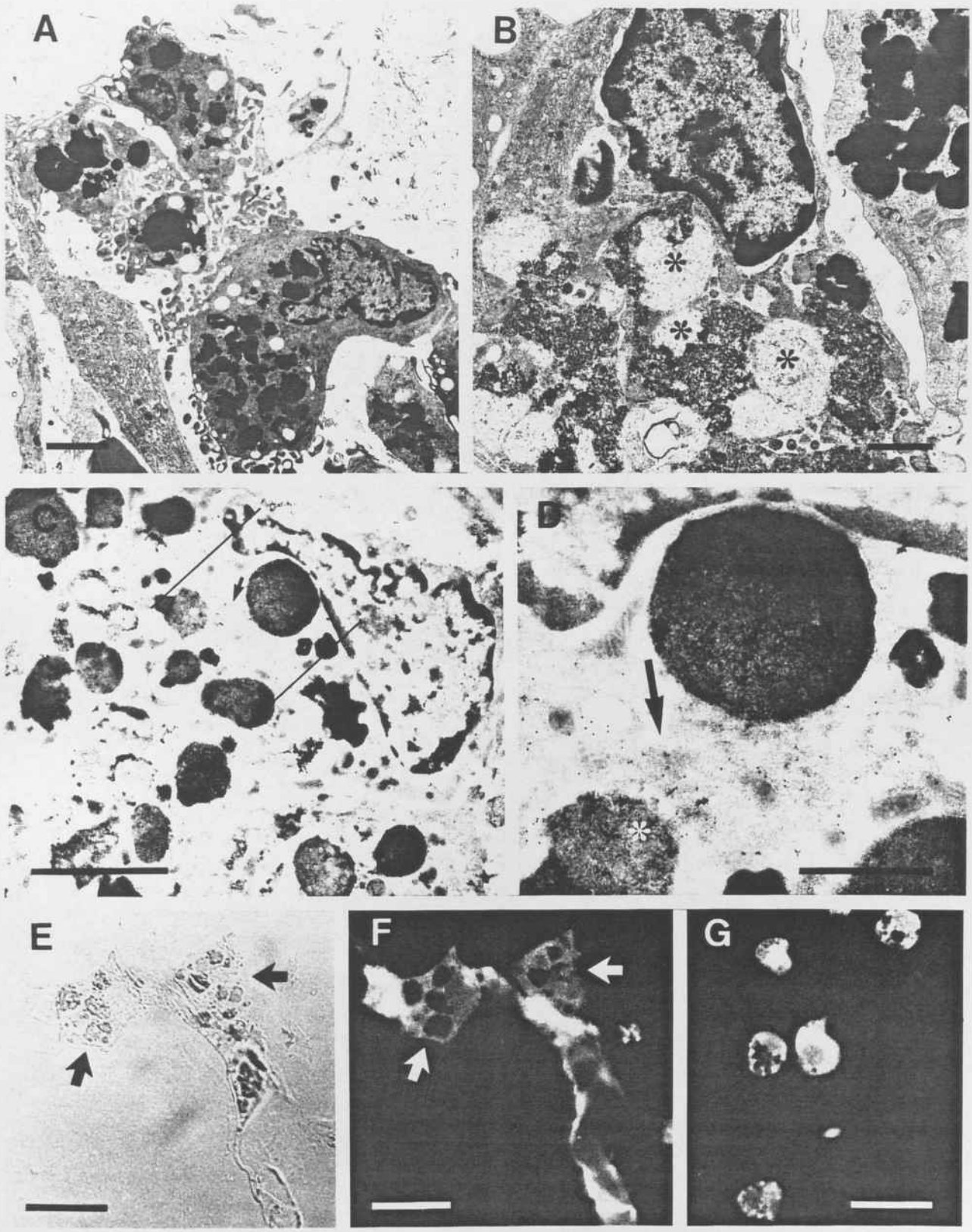
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Figure 4.

Figure 4. Immunoreactivity characteristics of fibroblast-like cells in ERM. (**A**) Conventional ultrathin section; (**B**) Cryosemithin section (**C** and **D**) Cryoultrathin sections. (**A**) Fibroblast-like cells were found in a fusiform morphology and were surrounded by abundant collagenic matrix (*X 5,200, bar=3μm*). (**B**) Indirect immunofluorescence showed V-positive cells with typical fibroblastic morphology (*arrow*) (*X 580, bar=30μm*). (**C**) Fibroblast-like cells occasionally showed positive label for V (*arrow*) (*X 8,400, bar=2μm*). (**D**) High magnification micrograph of bracketed area in figure C, showed immunogold (10nm) labelling for V (*arrow*) (*X 24,000, bar=1μm*).

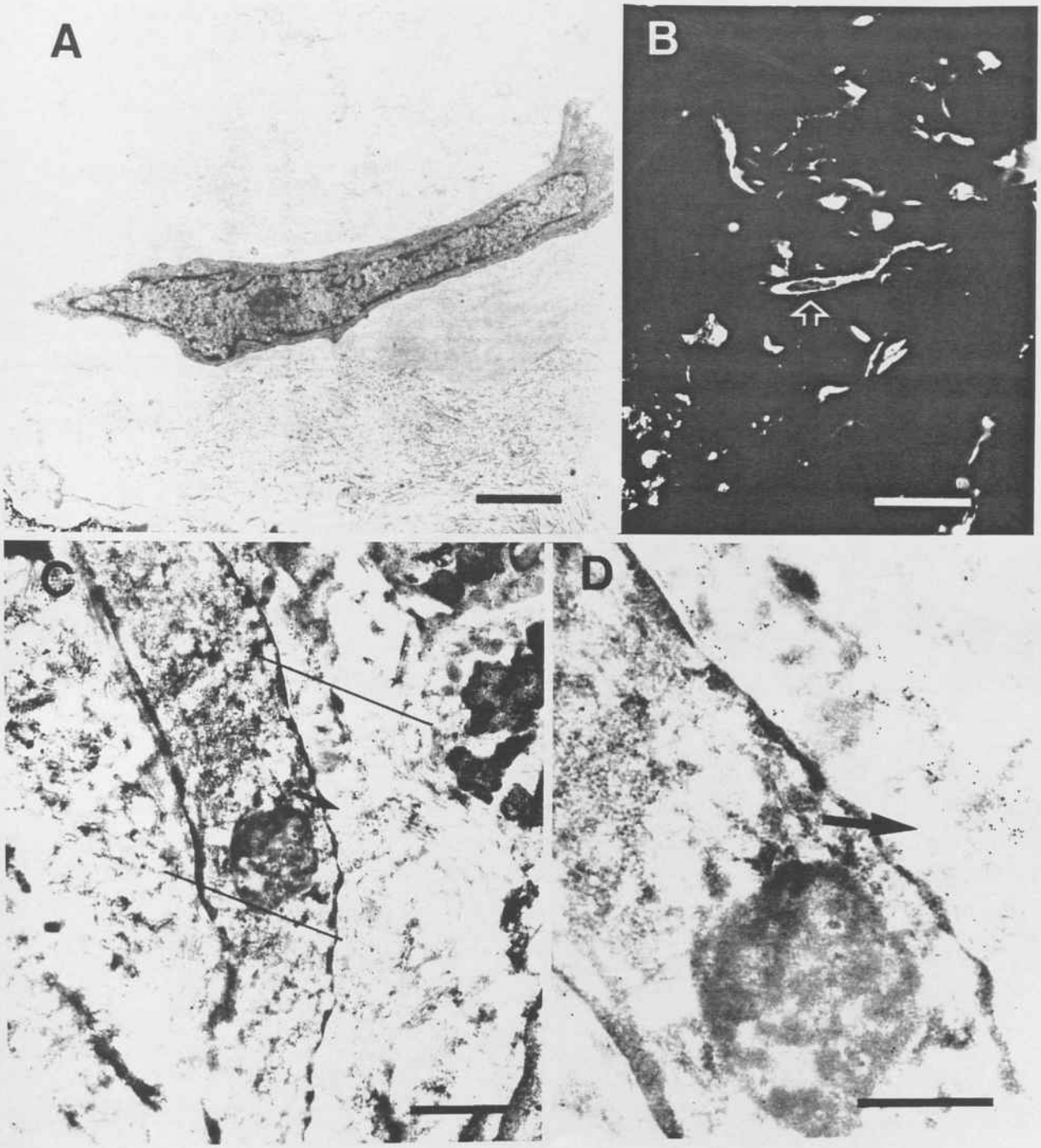
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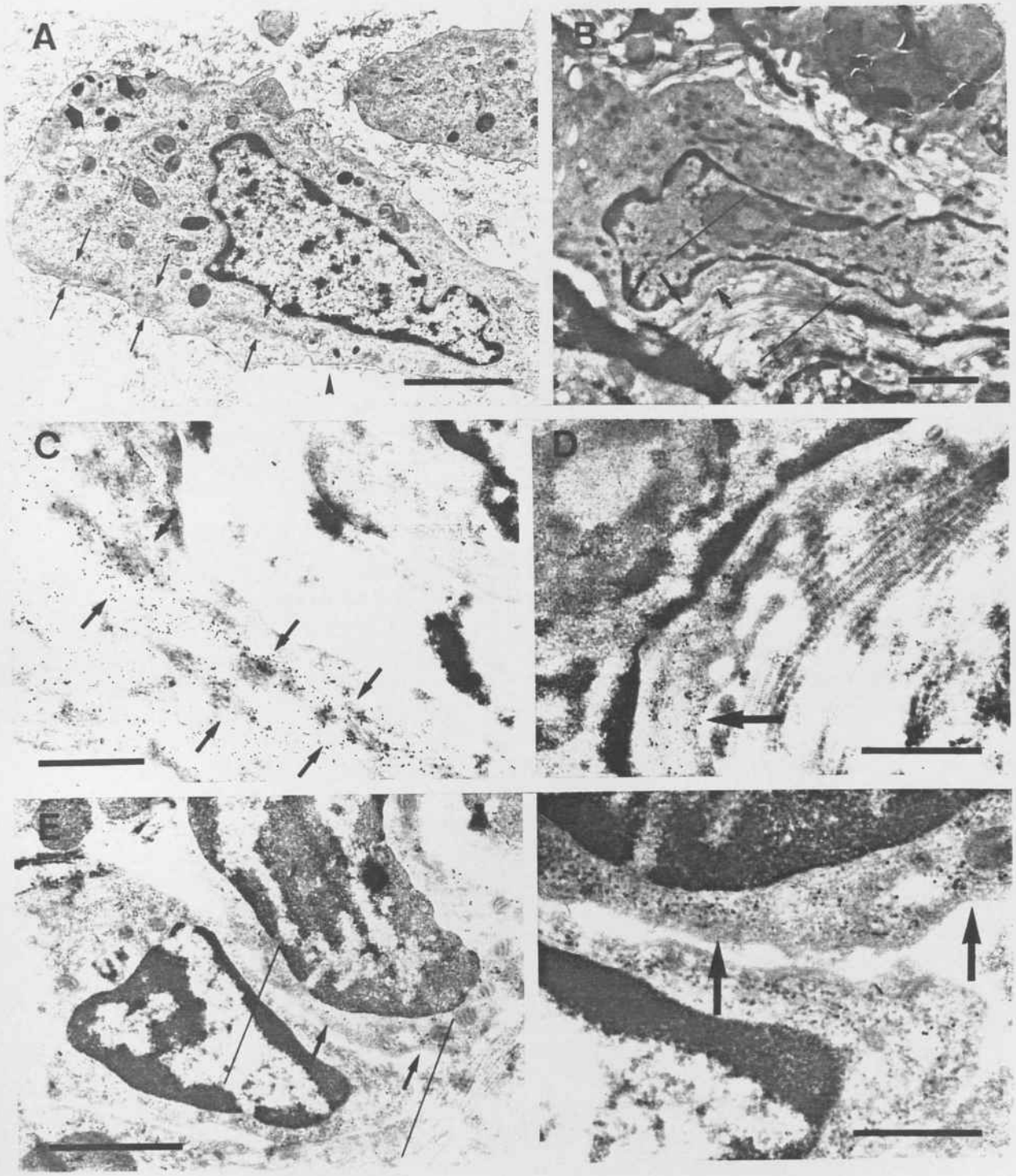
Figure 5.

Figure 5. Ultrastructural and immunoreactivity characteristics of myofibroblast-like cells in ERM. (**A**) Conventional ultrathin section; (**B** to **F**) Cryoultrathin sections. (**A**) Myofibroblastic transformation was characterized by intracytoplasmic bundles of microfilaments with fusiform densities. Bundles running on the cell length axis and were localized at the marginal side of plasma membrane (*between arrows*). Some cells showed well-developed organelles and segments of basement membrane deposition (*arrowhead*). Intracytoplasmic bounded pigment granules (*width arrows*) were occasionally seen (*X 10,200, bar=2μm*). (**B**) Myofibroblast-like cells frequently positive for V, and the peripheral pattern of immunogold (10nm) labelling usually correspond to the marginal arrangement of microfilaments bundles (*arrows*) (*X 8,000, bar=2μm*). (**C**) Immunoreactive V was found in aggregates of filaments arranged in bundles and localized at the periphery of cytoplasm (*between arrows*) (*X 24,000, bar=1μm*). (**D**) High magnification micrograph of bracketed area of Figure B showed immunogold labelling (*arrow*) marginally distributed on cell cytoplasm (*X 27,600, bar=1μm*). (**E**) Undifferentiated cells were occasionally positive for V (*arrows*) (*X 15,600, bar=2μm*). (**F**) High magnification micrograph of bracketed area observed in figure E, showed a scanty labelling peripherally distributed on cytoplasm (*arrow*) (*X 30,000, bar=1μm*).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CAPITULO III

Fibronectin, laminin, vitronectin and their receptors at newly-formed capillaries in proliferative diabetic retinopathy.

Ricardo P. Casaroli Marano, Klaus T. Preissner and Senén Vilaró

submitted to: ***Experimental Eye Research (1994)***

Abstract: Proliferative diabetic retinopathy (PDR) is characterized by intraocular fibrogliovascularized tissue formation in which active vasoproliferative mechanisms are involved. By immunocytochemistry we have studied changes in the distribution pattern of fibronectin (FN), laminin (LN), vitronectin (VN) and their receptors in the newly-formed capillaries of PDR. In intraocular fibrogliovascular tissue of PDR patients, FN was present on both luminal and basal surfaces of endothelial cells, and diffusely distributed in the intrapericytical space. LN was related mainly to the basal surface of endothelial cells and less evident on the pericyte side. VN was occasionally detected on the luminal capillary side, but frequently in the basal aspect of endothelium in the intrapericytical space, where it was colocalized with FN. Beta-1 subunit complex receptors were detected on the luminal side, while $\alpha_v\beta_3$ integrin was identified on both, but more in the luminal than the basal endothelial domain. By slot-blotting techniques and densitometrical analysis, increased concentration of intravitreous FN was observed in PDR, while intravitreous VN levels presented slight variations compared with normal samples. These results suggest that FN, VN, and LN have a key role in the structural arrangement of newly formed capillaries in PDR, and receptor expression could be involved in events of endothelial cell adhesion and proliferation.
Key words: Angiogenesis, neovascularization, glycoproteins, integrins, immunocytochemistry.

INTRODUCTION

Angiogenesis - new blood vessels formation - is a complex process involving several soluble and insoluble components (1-5), occuring during development and in both physiological and pathological processes in which neovascularization itself contributes to the evolution of disease (6,7). Structurally, new capillaries develop as sequential steps, originating mainly at sites of local degradation of the basement membrane which surrounds the microvascular endothelial cells (8). Endothelial cells then migrate toward chemoattractant factors, proliferate, and differentiate to form a capillary sprout. In the later phase, endothelial cells interact with the substrate, such as extracellular or basement membrane proteins (9,10).

A number of highly specialized non-collagenous proteins in the extracellular matrix (ECM), blood plasma and cellular surface membranes, such as fibronectin (FN), laminin (LN) and vitronectin (VN), have an important role in many cell surface interactions. For example, FN and LN help to mediate both cell-to-cell and cell-to-ECM adhesion, promote cell differentiation and proliferation, embryonic cell migration, and are basement membrane compounds (11-13). Recently, VN was reported to exert several regulatory functions in haemostasis, emerging as an adhesive component suitable for multiple interactions at blood-vessel wall interface or other pericellular sites (14). To participate in these functions, each of these glycoproteins could use specialized multidomain structures with a repertoire of various ligand-binding sites or peptide recognition sequences for binding to specific cell surface receptor or other molecules; integrins are transmembrane heterodimeric glycoprotein receptors characterized by α and β associated subunits that mediated this action. The spectrum of ligands bound to each receptor is delivered by the specific subunit ($\alpha\beta$) pairing of the heterodimer complex (15-17). Several integrins which share the β_1 subunit are recognized to be receptors for both FN and LN; for example, endothelial cells are known to express the $\alpha_5\beta_1$, $\alpha_3\beta_1$, and $\alpha_2\beta_1$ integrins (18,19). The $\alpha_5\beta_1$ integrin is an FN-specific receptor (20); $\alpha_3\beta_1$ is a receptor for both

FN and LN (21), while $\alpha_2\beta_1$ has previously been identified as an endothelial cell LN receptor (22). In addition, endothelial cells also express the VN receptor $\alpha_v\beta_3$ integrin, which also binds FN with high affinity (23,24). Then, two major subunit integrins (β_1 and β_3) are expressed by endothelial cells *in vitro*, and these cells can recognize and adhere to basement membrane and matrix protein components.

In proliferative diabetic retinopathy (PDR) - the later isquemical and vasoproliferative stage of this human intraocular disorder - new vessels and sheets of fibrogliovascular connective tissue (vitreoretinal membranes, VRM) proliferate on the surface of the retina or optic nerve head and into the vitreous cavity. It is a leading cause of visual loss in diabetic patients (25,26). Ultrastructurally, the earliest stages of retinopathy involve alterations in basement membranes, selective loss of pericytes and structural changes at the vessel walls. Later, in the vasoproliferative stage, migration, adhesion, and proliferation of endothelial cells are observed with newly formed capillaries surrounded by abundant connective tissue (26). Mechanisms of pathogenesis are poorly understood, but several biochemical and pathophysiological hypotheses have been proposed (27,28).

Since extracellular glycoproteins and their receptors could have an important role in the angiogenic mechanisms of PDR, we studied the pattern of distribution of FN, VN, and LN and β_1 and $\alpha_v\beta_3$ receptors in the newly-formed capillaries of surgically excised human intraocular tissue. In PDR capillaries, a colocalization of FN/VN in intrapericytical space, corroborated by the presence of $\alpha_v\beta_3$ integrin, suggest a role in capillary-ECM adhesion. Increased concentration of FN was also observed in the pathological vitreous of diabetic patients.

MATERIALS and METHODS

Human tissue and samples

Human VRM (n=25) were dissected and removed by appropriate intraocular vitreous forceps from diabetic patients undergoing intraocular surgery for PDR. Specimens were immediately fixed in 1% glutaraldehyde and 2% 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 30 min, mounted on a metal stub, rapidly frozen in liquid nitrogen and maintained at -196°C before sectioning by cryoultramicrotomy. For conventional ultrastructural microscopy, VRM (n=8) were fixed as above, placed in 4% paraformaldehyde in PBS 0.1 M and stored at 4°C. Pathological vitreous (n=7) was obtained during surgery under visual control by aspirating liquified vitreous from the center of the vitreous cavity, with a tuberculin syringe, before the vitrectomy infusion was opened. Samples (200μl - 500μl) were centrifuged (13,500 x g for 8 min at room temperature), devided into aliquots, and then stored at -35°C. Control samples (n=5) were obtained from normal human eyes donated for corneal transplant (Eye Bank, Barraquer Ophthalmological Centre, Barcelona, Spain), as described above.

Antibodies

For ECM glycoproteins a rabbit antiserum against human fibronectin (Dakopatts, Denmark) and a rabbit antiserum against murine LN (Sigma Chemical, USA) were both used at 1:200 dilution. A mouse monoclonal antibody directed against human VN (mAbs VN7) was used at 1:150 dilution. A polyclonal rabbit anti-murine β_1 integrin subunit (anti-ASβ_1) was provided by Dr. Carles Enrich *et al.* (Department of Cell Biology, School of Medicine, University of Barcelona, Spain), used at 1:50 dilution. The $\alpha_v\beta_3$ monoclonal LM609 prepared as described (29) was a kind gift from Dr. David Cheresh (Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA, USA), and used at 1:30 dilution. Rhodamine (TRITC)-conjugated anti-rabbit immunoglobulin antisera (Dakopatts, Denmark) for polyclonal antibodies and FITC (fluorescein) or TRITC-conjugated anti-mouse immunoglobulin antisera (Boehringer, Germany) for mAbs were used at 1:25 dilution for optical immunochemical detection. For electron microscopic immunocytochemistry, protein A/colloidal gold 16nm (pA-Au 16nm), produced in our laboratory, and rabbit anti-mouse immunoglobulin-gold (IgG-Au 10nm)(Amersham, U.K.) were used at 1:50 dilution. In several optical and electron immunocytochemical experiments we used rabbit anti-mouse secondary immunoglobulin (Dakopatts, Denmark) at 1:75 dilution, to amplify the monoclonal detection. Both primary and secondary reagents were diluted in 1%

ovalbumin in PBS-Glycine 0.1 M (pH 7.4).

Indirect Immunofluorescence Procedure

For immunocytochemistry VRM were obtained by consecutive serial semithin sections (0.3μm - 0.4μm) at -70°C (Ultracut FC4D, Reichert-Jung, Germany), prepared on 0.5% gelatin-coated slides and placed in a humidified chamber at 4°C. Indirect immunofluorescence was performed according to a modified technique described previously (30). Semithin frozen sections were air-dried at room temperature, washed (3 X 5 min) in PBS-Glycine 0.1 M and blocked (10 min) 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 one hour at room temperature in darkness. After the last incubation, slides were washed and mounted with 70% glycerol in 5% *n*-propyl galleate-buffered mounting medium. Antibody specificity was tested by western blotting and also negative control sections were prepared by omission of the primary antibodies. Immunostaining was visualized with an epifluorescence microscope (Polyvar II, Reichert-Jung, Germany).

Ultrastructure and Electron-immunocytochemical Procedure

For conventional electron microscopy, specimens (n=11) were abundantly rinsed with PBS 0.1 M pH 7.4, post-fixed in 1% osmium tetroxide phosphate buffered solution for one hour, dehydrated in a graded acetone series, and then embedded in resin to be polymerized at 60°C. Ultrathin sections (50nm - 75nm) obtained by conventional ultramicrotomy (OmU2, Reichert-Jung, Germany) were placed on copper grids (200 mesh), and contrasted with uranyl acetate and lead citrate solution for conventional transmission electronic microscopy (TEM) (Hitachi 800 MT, Hitachi INC., Japan).

For electron-immunocytochemical staining, consecutive serial ultrathin sections (90nm - 0.1μm) at -105°C were obtained by cryoultramicrotomy (Ultracut FC4D, Reichert-Jung, Germany), 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-immunocyttochemical study. The grids were pre-treated in ammonium chloride in PBS 0.1 M solution (10 min) to eliminate non-specific radicals, rinsed in PBS-Glycine 0.1 M solution (4 x 2 min), and then blocked in 0.5% ovalbumin in PBS-Glycine 0.1 M solution (10 min) at room temperature. Primary antibodies were incubated for 30 min at the dilutions mentioned above and washed in PBS-Glycine 0.1 M. Secondary anti-mouse IgG-Au 10nm or pA-Au 16nm was then applied for 20 min. After sucessive washes, first in PBS 0.1 M (4 x 2 min) and then in bidistilled water (6 x 2 min), grids were contrasted in 0.03% uranyl acetate solution (10 min) and a thin surface membrane of methyl-cellulose was applied. Double-staining procedures for VN/FN was carried out in a two-step method. First mAbs VN7 was applied as described, then grids were fixed in 2% paraformaldehyde in PBS 10 mM pH 7.4 (20 min) 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 INC., Japan).

Electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (31). Briefly, normal and pathological vitreous and subretinal fluid samples were mixed in a 1:1 (*vol/vol*) electrophoresis sample buffer (1% sodium dodecyl sulphate/ 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 min, and electrophoresed (Mini-Protean II 200/2.0 Electrophoresis Apparatus, Bio-Rad, USA) for 2.5-4 hours at 50-60 V, on a 7.5% 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 (32). SDS-PAGE proteins were transferred (Trans-Blot 200/2.0 Transfer Apparatus, Bio-Rad, USA) at 20 V, overnight at 4°C onto a nitrocellulose sheet (HyBond-c, Amersham, U.K.). Primary antibodies were incubated overnight at 4°C in 1% non-fat dry milk in 50 mM Tris-HCl (pH 7.4) at 1:1000 dilution. Secondary anti-rabbit or anti-mouse peroxidase-conjugated immunoglobulin (Dakopatts, Denmark) diluted 1:2000 in 0.05% Tween 20 (Sigma Chem., USA) in 50 mM Tris-HCl (pH 7.4) was applied for 3-4 hours at room temperature. The nitrocellulose strips were stained with diaminobenzidine (0.1% DAB in 50 mM Tris-HCl pH 7.4). Protein concentration of all samples was determinated by protein-dye binding (33).

Slot-blotting and Densitometrical Quantification

In order to quantify FN and VN we used a slot blotting method (Bio-Dot SF Microfiltration Apparatus, Bio-Rad Lab., USA) on nitrocellulose sheets for each dilution bank of normal and pathological vitreous samples (32). Nitrocellulose sheets were incubated with primary and secondary antibodies, and stained using the DAB technique described above. A direct densitometrical reading were performed by digitalized imagines of nitrocellulose sheet (IBAS II, Interactive Analysis System, Kontron, Germany). Standard concentrations of purified VN and FN (0.2 $\mu g/\mu l$; Boehringer, Germany) were analyzed simultaneously with each sample.

RESULTS

Ultrastructure, immunofluorescence and electron-immunocytochemistry in newly-formed retinal capillaries

Newly-formed capillaries were a very common finding in preretinal membranes of PDR patients. By electron microscopy, they were seen to have variable diameter (2μm to 40μm) and well-developed pericytic processes (Fig. 1A). Endothelial cells were plump, with luminal surface smooth or thin and elongated presenting cytoplasmic projections toward the lumina (Fig. 1B). The latter was a common characteristic in vessels with wide diameter. Endothelial cells of most capillaries were connected by an adherent junction system showing a regular periodic fusion between their plasma membranes, as observed in tight junctions (Fig. 1D). The pericyte was sometimes plump or fusiform with irregular nucleus membrane. Well-developed basement membranes were noted in monolayer or stratified structure enveloping endothelial cells and adjacent pericytes (Fig. 1B and 1C).

All glycoproteins (FN, LN, and VN) and both $\alpha\beta_3$ and β_1 subunit integrins were identifiable by indirect immunofluorescence (Fig. 2 to 5). Narrow and wide diameter capillaries in VRM showed large amounts of FN (Fig 2). Both basal and luminal sides of endothelial cells displayed immunofluorescence labelling for this protein (Fig. 2B and 2D). Electron-immunocytochemistry corroborated FN luminal presence, often related to cytoplasmic expansions toward capillary lumen (Fig. 2E and 2F). Extensive FN was detected in the intrapericytic space, between endothelial cells and pericytes (Fig. 2G). Large segments of basement membrane were labelled for FN (Fig. 2I).

Positive stain for LN was detected in well-developed capillaries, always in the basal aspect of endothelium (Fig. 3B); some vessels were non-reactive. By electron-microscope, anti-LN label was localized in the intrapericytic space (Fig. 3A and 3C), preferentially on the basal side of endothelial cells rather than in pericyte (Fig. 3C). In some capillaries, the inner aspect of basal lamina was strongly reactive (Fig. 3D).

Fluorescent label for β_1 subunit complex integrin was usually but not always noted in the luminal surface of endothelial cells (Fig. 4A) of narrow and wide capillaries. Electron-immunocytochemistry revealed β_1 subunit restricted to the smooth luminal aspect of endothelial cells and intraluminal cytoplasmic expansions (Fig. 4B); basal laminas were not immunoreactive for this complex. VN immunofluorescence staining was observed in characteristic linear pattern at the basal aspect of capillaries, most evident when longitudinal sections were obtained (Fig. 5B). Double-immunogold labelling experiments showed VN and FN colocalized in the intrapericytic space and basement membranes (Fig. 5A). Predominant binding for FN was noted, while VN showed slight immunoreactivity. Immunogold binding for luminal VN was occasionally observed (not shown). By optical and electron-immunocytochemical techniques, $\alpha\beta_3$ integrin was identified in variable amounts on both luminal and basal endothelium (Fig. 5C and 5D).

Electrophoresis, immunoblotting and densitometrical quantification for normal and pathological vitreous samples

Protein concentration of pathologic PDR vitreous (16.6 ± 8.3 $\mu g/\mu l$) was substantially higher than normal vitreous samples (0.506 ± 0.04 $\mu g/\mu l$) ($p<0.001$, by unbalanced ANOVA test). Normal vitreous, separated by 7.5% and 10% SDS-PAGE under reducing conditions and then stained

by Coomassie Blue or silver staining, revealed a large amount of protein in the band, with a molecular mass around 66 kD. Extensive amounts of smaller proteins (≤ 66 kD) were separated in pathological samples, similar to electrophoretic band patterns of human plasma. Proteins with molecular mass higher than 66 kD were less evident (Fig. 6a).

Western blotting analysis detected FN and VN, but not LN in all pathological samples. Slight immunoreactivity in normal vitreous samples was observed, suggesting both glycoproteins in non-pathological state (Fig. 6b and 6c). Immunoreactive bands were identified in parallel experiments using purified protein and human plasma. Several pathological samples showed small degraded fragments of FN and VN. Direct densitometry, showed differences for both glycoprotein concentrations present in pathological intraocular environment. As previously stated, total protein levels increase significantly in PDR vitreous; total FN concentration also increase in PDR (15.9 ± 1.13 ng/μl) compared with normal samples (0.06 ± 0.002 ng/μl). Nevertheless, a rise in VN concentration (2.09 ± 0.11 ng/μl) was noted, although it was not significant compared with normal vitreous (0.09 ± 0.003 ng/μl). No significant variation for relative concentration of intravitreal VN was observed when compared with normal samples, while vitreous FN levels in pathological samples were highly significant ($p<0.001$, by unbalanced ANOVA test). Table I provides quantitative measurements of total proteins, FN, and VN in normal and pathological samples.

Table I: Concentrations of total proteins, FN and VN in normal and PDR vitreous samples.

	normal (n=5)	PDR (n=7)
Total proteins (μg/μl)	0.506 ± 0.04#	16.6 ± 8.3*
FN (ngr/μg protein)	0.129 ± 0.03	0.958 ± 0.2*
VN (ngr/μg protein)	0.195 ± 0.05	0.126 ± 0.07

PDR, proliferative diabetic retinopathy.
#, means ± S.E.; *, statistical significance of difference from normal samples ($p<0.001$).

DISCUSSION

It has previously been shown that in diabetes, events involving endothelial and other vascular cells were governed by complex metabolic conditions that affect microvessels functionally and structurally (25,28). Endothelial cells require an extracellular signal in order to switch from quiescence to growth and back to differentiation during new blood vessel formation. The extracellular matrix acts locally to modulate the response of endothelial cells to external factors. Vascular permeability, basement membrane compounds, and diabetic condition are involved in these phenomena (3,5,34). We report evidence that extracellular glycoproteins and their ligands are involved in the definitive structure of newly-formed capillaries in human PDR. In addition FN is present at altered levels in intraocular diabetic milieu.

Electron microscopy and immunogold post-embedding demonstrated collagen types IV and V, LN, and heparan sulfate proteoglycans in basement membranes of normal human retinal capillaries; FN was not identified (35). Although previous results obtained by immunofluorescence, reported FN in normal capillaries (36) and retinal vessels of diabetic patients (37), to our knowledge there are few studies on pathologic human specimens focusing on the extracellular matrix in vessels. Our results indicate that FN is frequently found in newly-formed capillary structures.

Cryoultramicrotomy and post-embedding electron-immunocytochemistry provide good ultrastructural and tissue antigenicity preservation to identify this glycoprotein on both sides of the endothelium.

Thickening of basement membranes is a well-recognized structural alteration in diabetic microangiopathy (12,25,27,28); increased synthesis of macromolecular components has been found, including a high-glucose inducing overexpression of collagen type IV, LN, and FN (38). We found considerable amounts of both glycoproteins in intrapericytic space and their close relationship with large segments of basement membrane. FN in intrapericytic space could originate from the bloodstream as a diffusion process, since increased vascular permeability in diabetic retinopathy was observed, by alteration in the barrier function of normal retinal capillaries, which prevents transendothelial transport of proteins from plasma (39). This may also explain the presence of VN and its colocalization with FN. A mechanism for exogenous VN binding could occur analogous to FN, which both are incorporated into the extracellular matrix by an active process (40,41).

VN was identified in areas of pericytic matrices in arterial blood vessels (42), but unlike FN, VN was absent from basement membranes of most tissues studied, except pathologic kidney (43). We have presented evidence that VN is a component of basement membrane in PDR capillaries and colocalizes with FN in the basal aspect of endothelial cells. In addition, moderate immunoreactivity for VN-receptor was also detected in similar regions.

The tissue (insoluble) and plasmatic (soluble) forms of FN are completely cross-reactive in the same species, and therefore immunologically indistinguishable when the usual type of antibody is employed (44). In addition, FN was frequently identified on the luminal side of the endothelium while VN was only occasionally found. Both glycoproteins are present in similarly high concentrations in plasma (11) which suggests a possible plasmatic origin, although evidence indicate that FN and VN could be cell-related. First, FN immunostaining can be demonstrated in tissues that have been exhaustively perfused (45); second, FN was frequently, but not always, observed in all capillaries of the same sample examined by any technique; third, immunoreactivity for FN was clearly higher than VN and if FN and VN were in similar concentration in plasma (11,14), then dense VN labelling might be seen, since we used specific antibodies for both and negative controls indicated the quality of labelling; and finally, both $\alpha_v\beta_3$ and β_1-subunit integrins were identified in the luminal and basal aspects of endothelium related with their glycoprotein.

In cultured fibroblasts, FN was initially synthesized and later secreted and partially incorporated within the matrix following association with the cell surface (46). Recently observed (47), endothelial cells from aortic tissue showed an active capacity for FN biosynthesis, which differed from that described on cultured fibroblasts; the solubility of newly-synthesized FN was related to an intracellular or cell surface-associated form prior to matrix incorporation. For these reasons, the FN found at the luminal side of endothelium in PDR capillaries could represent a synthetic product, although a cellular captation from bloodstream could not be refused. Moreover, FN appears to control endothelial cell growth by direct and active chemical signalling pathways; its ability to promote cell growth and spreading have been demonstrated by mechanisms of cell surface Na^+/H^+ exchange and rise in intracellular pH (10).

Finally, by electron-immunocytochemistry we were not able to identify β_1-subunit related to LN or FN at the basal side of endothelial cells. Although basement membrane formation may be explained as a self-assembly process, the role of integrins, which are able to recognize LN, FN, and type IV collagen has been considered (12,13). Several members of the β_1-subunit complex family were involved as an LN receptor (11,15,17,22). By immunofluorescence experiments, both β_1 and β_3 complexes were identified in vinculin-positive focal adhesion plaques at the basal surface of human microvascular endothelial cells. These receptors bind LN-coated substrate and promote cell adhesion to LN and basement membranes; using blocking mAbs to $\alpha_v\beta_3$ integrin their role in initial cell attachment to LN was observed (48,49).

In normal conditions, intraocular environment is protected from systemic circulation

by the blood retinal barrier which may be damaged in several intraocular disorders; breakdown of this barrier was estimated in the earliest stages of diabetic retinopathy (39,50). We noted a significant rise in intravitreous protein levels and a specific increase in vitreous FN concentration compared with normal samples. Albumin and iron-binding proteins, such a lactoferrin and transferrin, are major components of the soluble proteins in vitreous and were observed in high relative concentration in vitreoretinal disorder compared with normal serum (51,52); authors concluded that these proteins may have a protective role in intraocular disorders. In physiological conditions intravitreal protein concentrations are regulated by factors that adjust vascular permeability and composition of serum ultrafiltrate in the extravascular compartments (53). In PDR, retinal microvessels present defects in vascular permeability, barrier function of capillary endothelium is altered, and newly-formed capillaries may bleed. These factors may undoubtedly contribute to the increase in the concentration of proteins described here.

The rise in intravitreous FN concentration seems to be specific, since the total amount of FN was higher than the relative increase in total proteins in pathological samples. Although the possible plasmatic origin of intravitreous FN cannot be ruled out, our results regarding its immunocytochemical detection and its increased levels detected by immunoblotting techniques indicate that fibronectin may have an important functional and structural role in development of PDR capillaries and in the maintenance of the diabetic environment.

We observed a slight decrease in relative concentration of intravitreal VN compared to normal samples. An absolute rise in intravitreous VN appears to be related to a plasmatic contribution. One important physiological role of VN is its multifunctionality in the haemostatic system at the blood-vessel wall interphase, and its functional repertoire also depends on the respective site of action and on its molecular form, isolated or associated with other components (14). For these reasons, we consider the decrease in intraocular VN levels to be involved in structural vascular alterations to the capillary retinal system and in haemorreological disturbances observed in diabetic patients. Under normal tissue-culture conditions VN and FN act upon the attachment and growth of bovine aortal endothelial cells; VN can be adsorbed from the medium in high enough concentration to mediate cell adhesion, spreading and growth, while FN showed low adsorption onto the substrate (54). These data may provide an explanation for particular changes observed in the VN and FN intravitreal concentrations in PDR. Further studies with PDR pre-proliferative retinal capillaries and angiogenic models *in vitro* could provide additional data to elucidate these questions.

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Figure 1.

Figure 1. Ultrastructure of newly-formed capillaries in PDR preretinal membranes. (**A**) A well-developed pericyte processes (*p*) and basement membrane (*bm*) surround newly-formed vessels with variable lumen (*L*) diameters. Large amounts of extracellular collagenic matrix (*col*) firmly attach endothelial cells (*ec*) and pericytes. (*X 2,800, bar=5μm*). (**B**) Higher magnification of bracketed area in Figure A shows cytoplasmic projections (*arrows*) toward capillary lumina as a common finding in vessels of widely varying diameter. Extensive segments of continuous unilaminar basement membrane were closely opposed to basal side of cell (*X 15,600, bar=1μm*). (**C**) Stratified basement membrane was also seen surrounding endothelial cell (*X 43,000, bar=0.5μm*). (**D**) Intercellular connections (*arrows*) present regular points of fusion between adjacent plasma membranes. Extensive amounts of basement membrane deposition and collagenic material were always present (*X 19,500, bar=1μm*).

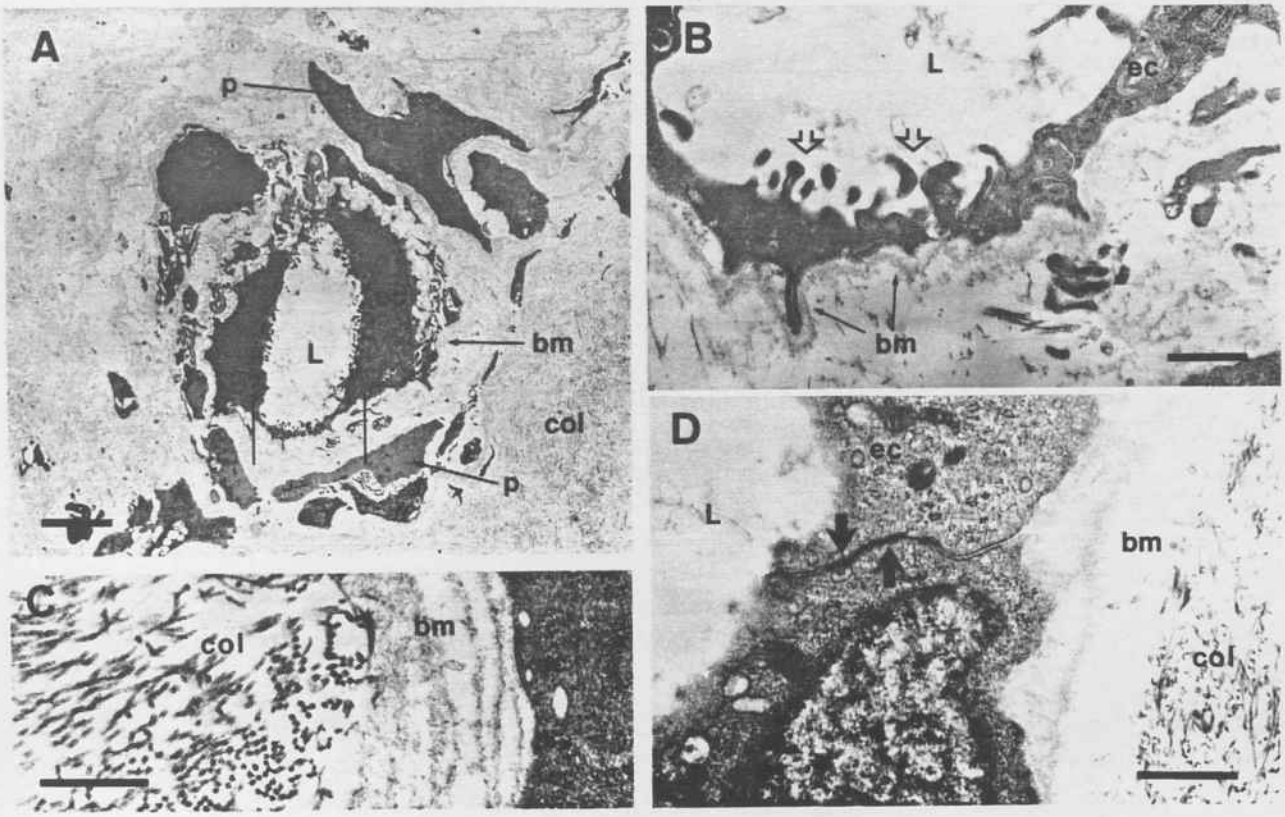
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Figure 2.

Figure 2. Indirect immunofluorescence for FN in newly-formed capillaries of PDR preretinal membranes. (**A**) Interference contrast microscopy in cryostat section showed well-developed newly-formed vessel (*arrow*) surrounded by a compact fibrous matrix; lumen, (*L*) (*X 360, bar=30μm*). (**B**) Fluorescent ring label delineates endothelial cells (*ec*) on the luminal side in large and small capillaries (*X 490, bar=30μm*). (**C**) Interference contrast microscopy in semithin cryomicrotomy section resolves both luminal and basal (*arrow*) aspects of capillary (*X 1,130, bar=10μm*); and (**D**) defined fluorescence label on the basal aspect of endothelial cells that corresponds to the basement membrane (*bm*) domain (*X 910, bar=20μm*).

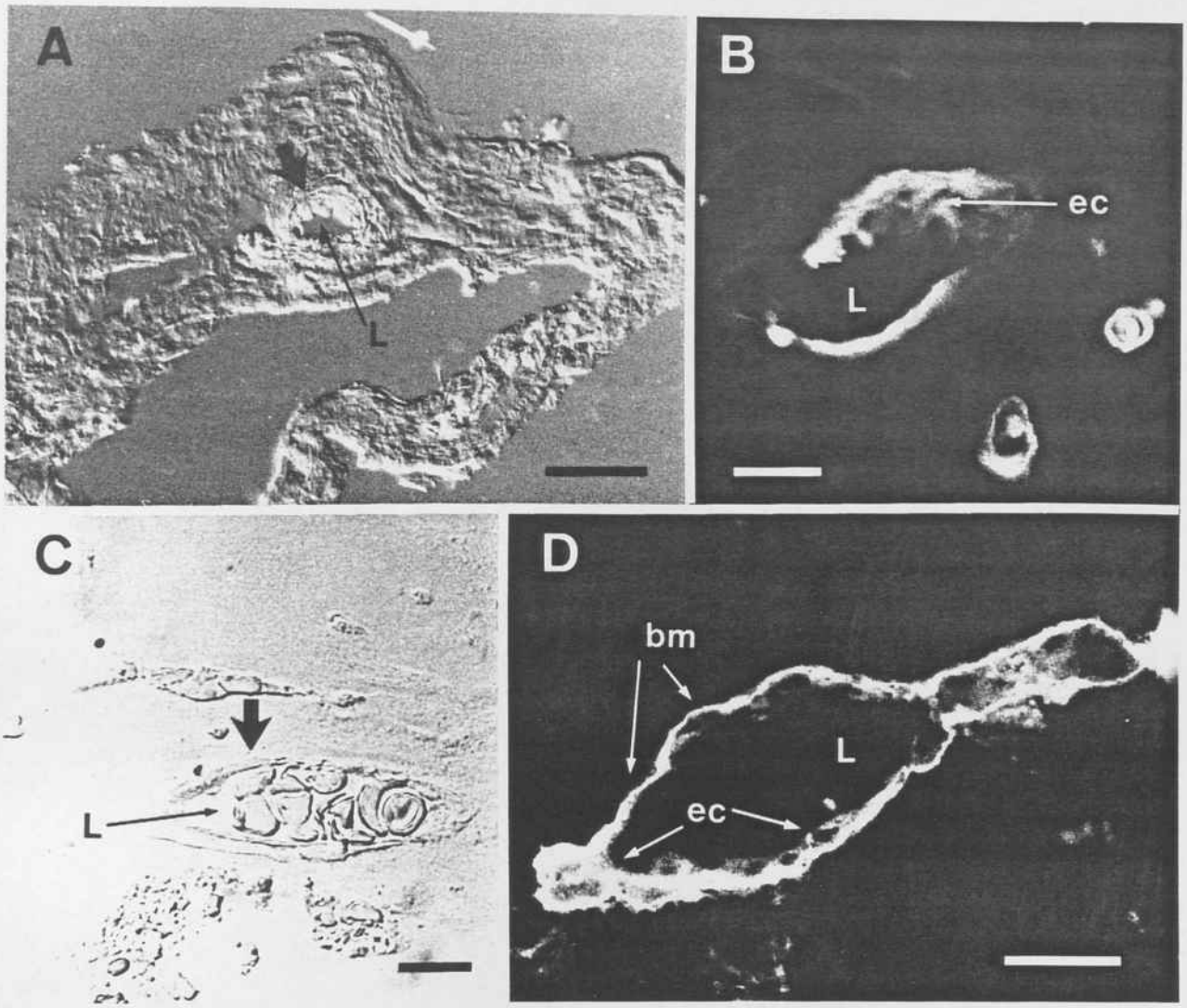
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Figure 2

(continuation).

Figure 2 (continuation). Electron-immunocytochemistry in ultrathin cryoultramicrotomy sections by immunogold labelling for FN in newly-formed capillaries in PDR preretinal membranes. Large amounts of reactive 16nm immunogold labelling for FN were present in both (**E**) on the luminal (**L**) surface of endothelial cells (*ec*)(*arrows*), and (**G**) in the intrapericytic space, between pericytes (*p*) and endothelial cells (*X 13,200, bar=2μm*). (**F**) Immunoreactive FN appears in intraluminal projections (*arrows*) of endothelial cells (*X 27,500, bar=1μm*). (**H**) Immunogold label on the basal side seems related to the plasma membrane (*arrowheads*) (*X 19,200, bar=1μm*). (**I**) Extensive segments of basement membrane (*bm*) label for FN (*X 48,000, bar=0.5μm*).

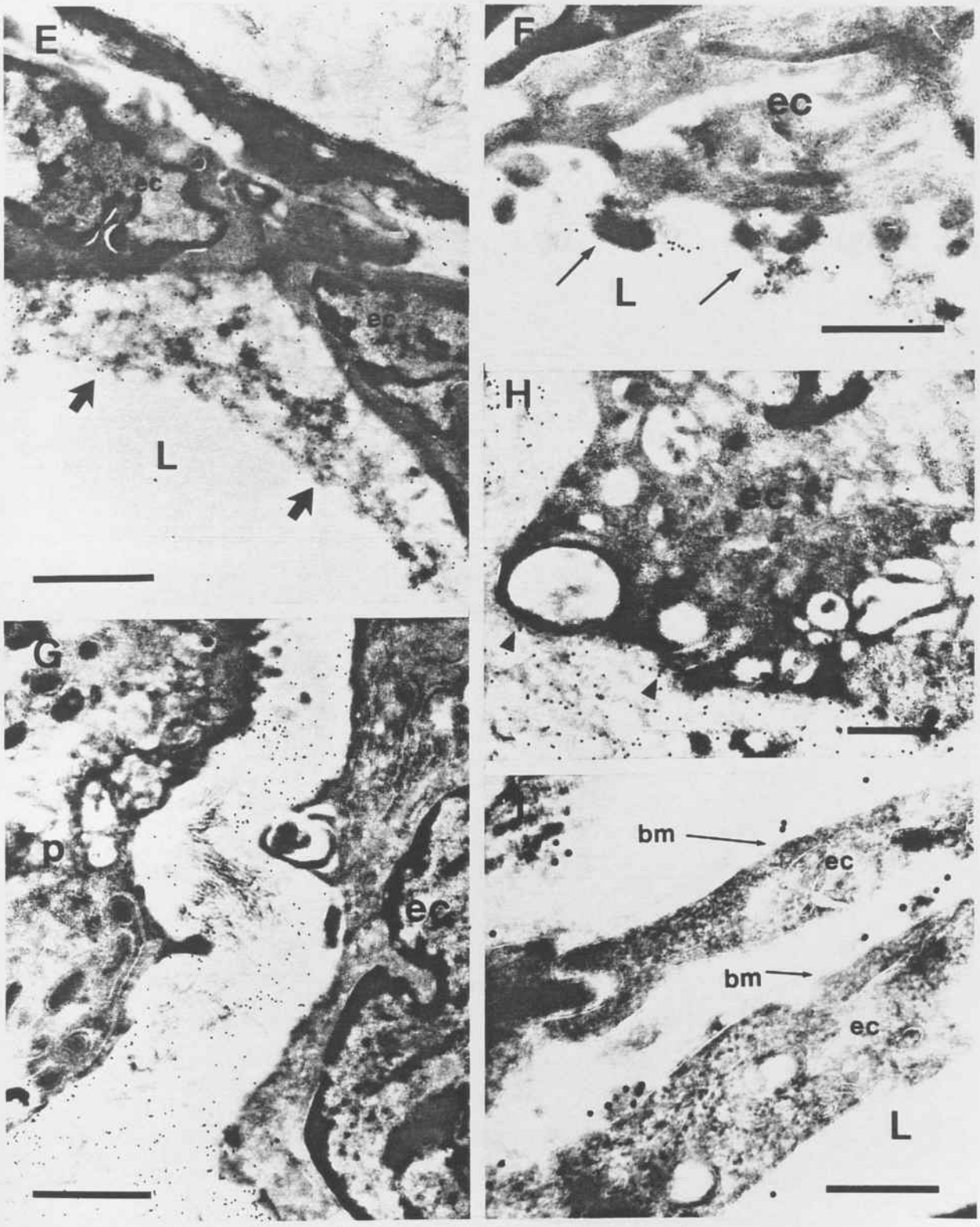
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Figure 3.

Figure 3. Immunoreactivity for LN in newly-formed capillaries of PDR preretinal membranes. (**A**) Ultrathin sectioned capillaries showed moderate immunogold labelling (16nm) in intrapericytic space; pericyte (*p*), endothelial cell (*ec*) (*X 15,600, bar=1μm*). (**B**) Semithin sections examined by indirect immunofluorescence confirmed continuous linear pattern of binding on the basal aspect of endothelial cells in oblique, transversal and longitudinal sectioned vessels (*arrows*) (*X 730, bar=20μm*). (**C**) In intrapericytic space LN binds preferably the basal side of endothelial cells (*arrows*), while pericyte side labels moderate amounts (*X 15.600, bar=1μm*). (**D**) Inner laminas of basement membranes (*bm*) showed preferential labelling for LN (*X 24,000, bar=1μm*).

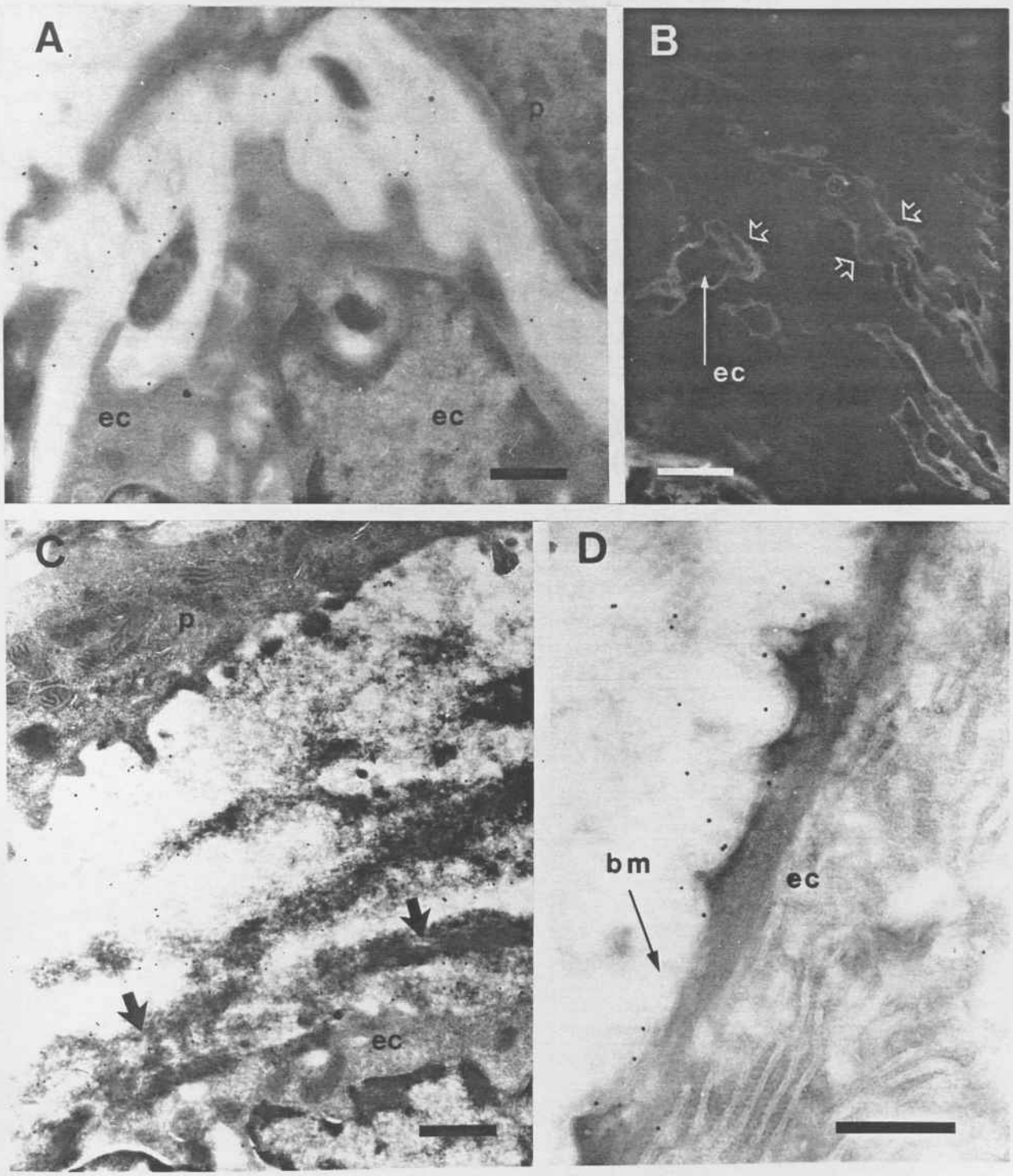
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Figure 4.

Figure 4. The β_1-subunit integrin complex in newly-formed capillaries of PDR preretinal membranes. (**A**) Indirect immunofluorescence showed this receptor in endothelial cells (*ec*) and on its luminal aspect (*L*) (*X 670, bar=30μm*). (**B**) Electron-immunocytochemistry on ultrathin sectioned vessels showed immunogold label on the luminal surface (*L*) but not at the basal domain (*X 27,500, bar=1μm*).

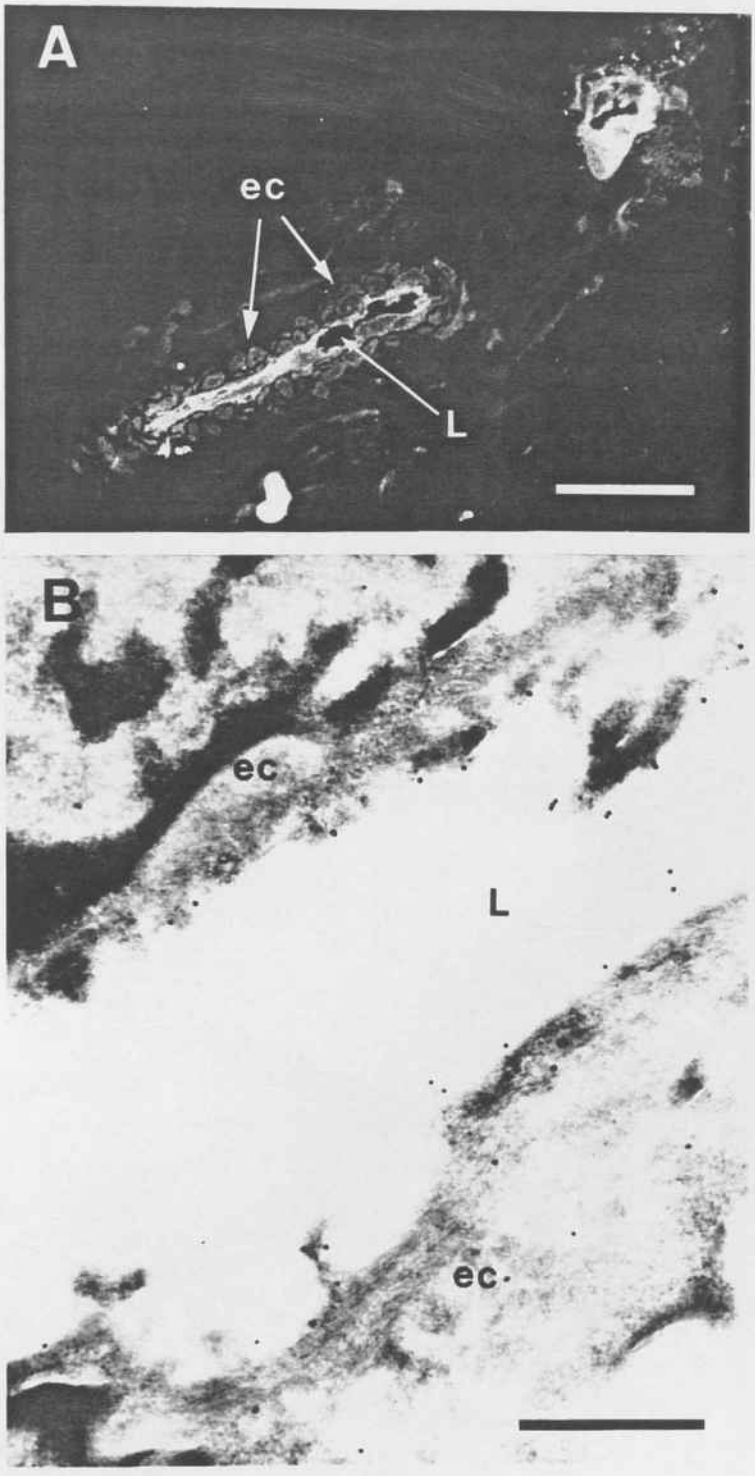
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Figure 5.

Figure 5. Immunoreactivity for VN and its receptor ($\alpha_v\beta_3$) in capillaries of PDR preretinal membranes. (**A**) Electron-immunocytochemistry on ultrathin sectioned capillaries showed a colocalized VN/FN in intramural space; pericyte (*p*), endothelial cell (*ec*). FN immunoreactivity (pA-Au 16nm) (*arrowheads*) was predominant, while reactive VN (IgG-Au 10nm) (*arrows*) was minor component (*X 24,000, bar=1μm*). (**B**) Indirect immunofluorescence for VN resolves label at basal domain of longitudinal capillary sections (*arrows*); lumen (*L*) (*X 730, bar=30μm*). (**C**) Electron-immonocytochemistry with amplification procedures shows $\alpha_v\beta_3$ receptor (pA-Au 16nm) more in lumen (*L*) than on the basement membrane (*bm*) domain (*X 24,000, bar=1μm*). (**D**) Fluorescent staining for $\alpha_v\beta_3$ integrin was observed on the basal (*arrows*) and luminal sides of endothelial cells (*ec*) (*X 730, bar=30μm*).

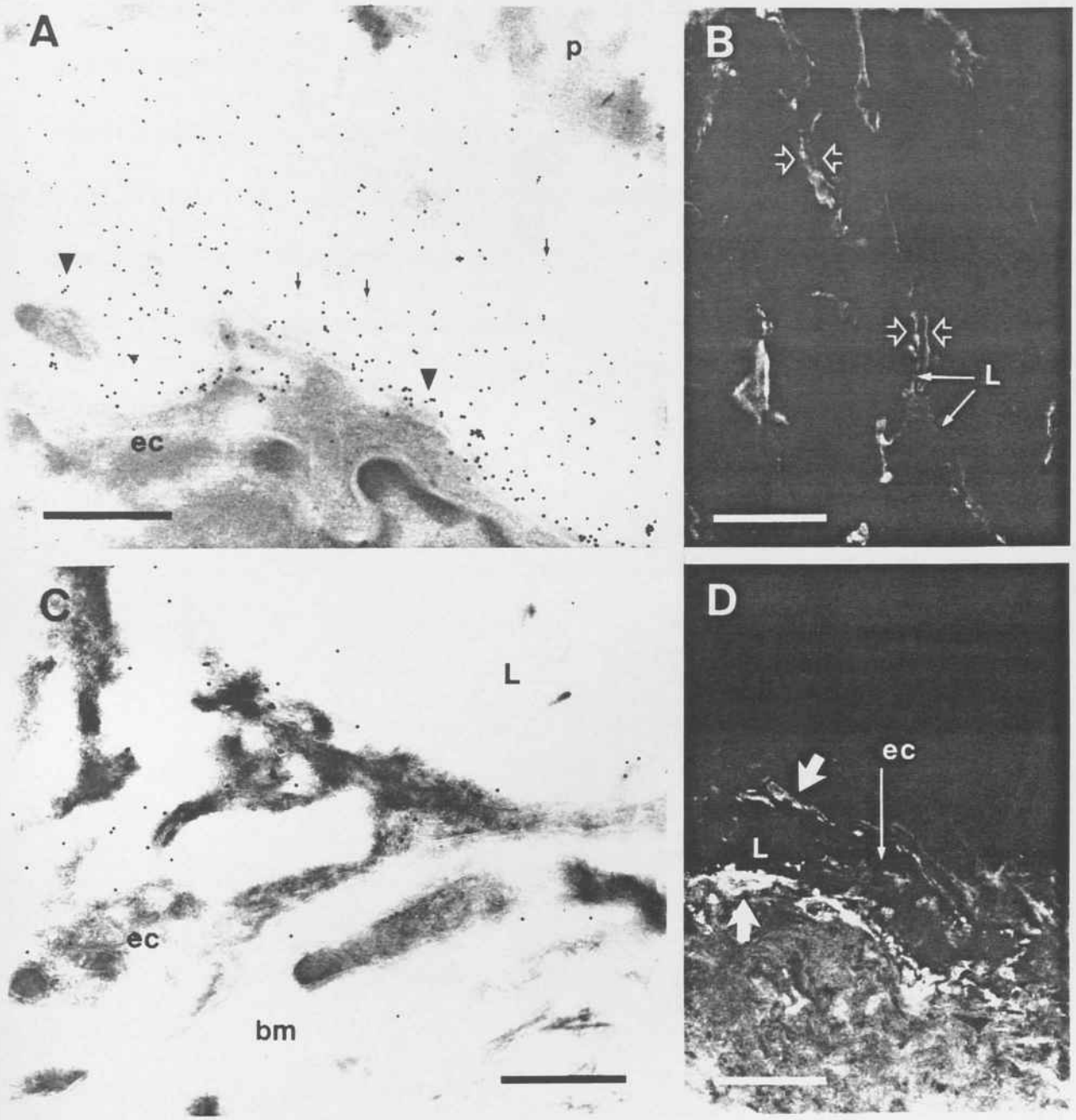
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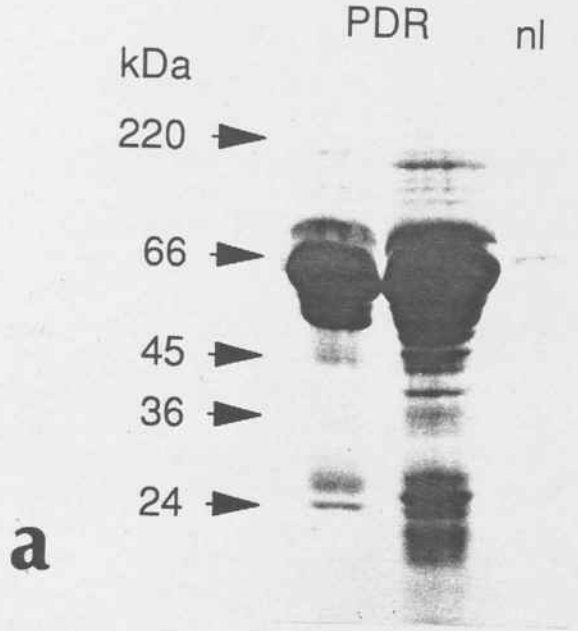
Figure 6.

Figure 6. Immunoblot analysis of proliferative diabetic retinopathy (PDR) and normal (nl) vitreous proteins separated by 7.5% and 10% SDS-PAGE under reducing conditions. **(a)** Electrophoretic profile by 7,5% SDS-PAGE and Coomassie Blue staining revealed a large amount of PDR vitreous protein in band around 66 kD. **(b)** Western blot analysis of normal and pathological vitreous proteins separated on 7.5% SDS-PAGE was positive for FN; **(c)** on 10% SDS-PAGE and incubated with mAbs VN7 directed against VN, 65-75 kD bands confirmed VN detection, and bands of low molecular mass fragments of degraded protein were sometimes seen.



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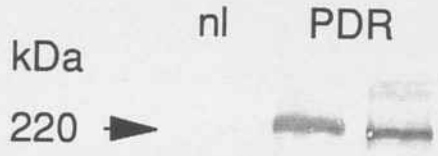

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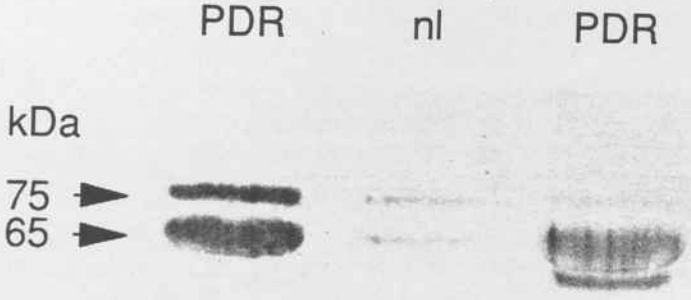

c

CAPITULO IV

The role of fibronectin, laminin, vitronectin and their receptors on cellular adhesion in proliferative vitreoretinopathy.

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Investigative Ophthalmology & Visual Science **in press,1994.**

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 (β_1-subunit complex and $\alpha_v\beta_3$ 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 densitometrical analysis of normal and PVR vitreous were also undertaken. Chi square (x^2) and unbalanced analysis of variance (ANOVA) 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 $\alpha_v\beta_3$ receptors were usually localized in a position that could mediate the interaction of FN, VN or/and LN to the cell plasma membrane. Increased levels of FN concentration were observed in both subretinal fluid and pathological vitreous; intravitreal FN concentration tends to increase with clinical stages of the evolution of PVR, while 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.

key words: Epiretinal membranes, retinal detachment, glycoproteins, integrins, immunocytochemistry.

INTRODUCTION

The biological activities of the extracellular matrix (ECM) reside both in its special components and in its structural integrity (1). 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, tumour-cell invasion and metastasis (2). For example, fibronectin (FN; mol wt 440 kD) is a multifunctional interactive glycoprotein found as soluble plasma protein and an insoluble form organized into extensive ECM, produced and secreted by a wide variety of cell types (3-5). Laminin (LN; mol wt 850 kD) is a major component of basement membranes and possesses multiple functional sites that mediate its interactions with cells and other ECM elements (6-8). Finally, vitronectin (VN; mol wt 65-78 kD) is present in fibrillar pattern in the ECM of a variety of tissues (9,10) and also in circulation with a predominant role in cell adhesion and haemostasis (11,12).

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 (13-17). 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 (18-21). All integrins are α

subunits, noncovalently associated with a β subunit, and can be expressed in a wide variety of cell types (19). Many of the integrins that share the β_1 subunit are known to recognize FN (22,23), LN (24,25), and collagen (26). Cell attachment to VN occurs by any of several β_3 subunit associations, including the classical VN-receptor: the $\alpha_v\beta_3$ integrin (27). These receptors also bind to FN, fibrinogen, von Willebrand factor and collagen (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 (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 (30-32) surrounded by a fibrous matrix with an extensive amount of collagen (30,33). Pathogenesis is poorly understood, especially the mechanisms of cellular migration, adhesion and proliferation. Previous studies have suggested the participation of growth factors (34,35) and serum proteins (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 pathology 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 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 - 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 Lab., USA), frozen in isopentane and stored at -35°C for cryostat sections. Pathological vitreous (n=15) was obtained during surgery under visual control by aspirating liquified 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 (200μl - 500μl) were centrifuged (13,500 x g for 8 min at room temperature), devided 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 D3 according to Hilton *et al.* classification (29). 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 (29).

Antibodies

For immunochemical detection of ECM glycoproteins a rabbit antiserum against human fibronectin (Dakopatts, Denmark) and a rabbit antiserum against murine LN (Sigma Chemical, USA) were used, both at 1:200 dilution. A mouse mAb directed against human VN (mAb VN7) was a generous gift from Dr. Klaus Preissner (Haemostasis Research Unit, Kerckhoff-Klinik, Max-Planck-Gesellschaft, Bad Nauheim, Germany) and was used at 1:150 dilution. A polyclonal rabbit anti-murine β_1 integrin subunit (anti-ASβ_1) was provided by Dr. Carles Enrich *et al.* (Department of Cell Biology, School of Medicine, University of Barcelona, Spain), used at 1:50 dilution. The $\alpha_v\beta_3$ mAb LM609 prepared as described (39) was kind gift from Dr. David Cheresh (Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA, USA), and used at 1:30 dilution. Rhodamine (TRITC) or FITC-conjugated anti-rabbit or anti-mouse immunoglobulin antisera (Dakopatts,

Denmark; Boehringer, Germany) were used for optical immunodetection at 1:25 dilution. For electron microscopic immunocytochemistry, protein A/colloidal gold 16nm (pA-Au 16nm), produced in our laboratory, and rabbit anti-mouse immunoglobulin-gold (IgG-Au 10nm)(Amersham, U.K.) were used at 1:50 dilution. In several electronic immunocytochemical experiments we used rabbit anti-mouse secondary immunoglobulin (Dakopatts, Denmark) at 1:75 dilution 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, Germany) sections (6μm - 10μm) and semithin sections (0.3μm - 0.4μm) at -70°C (Ultracut FC4D, Reichert-Jung, Germany), 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.* (40). Semithin frozen sections were air-dried at room temperature, washed (3 X 5 min) in PBS-glycine 0.1 M and blocked (10 min) 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 one hour at room temperature in darkness. After the last incubation, slides were washed and mounted with 70% glycerol in 5% *n*-propyl galleate-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, Germany).

For each specimen, 9 to 20 whole sections were studied and interpreted at low magnification (X10 - X25) 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 (90nm - 0.1μm) at -105°C were obtained by cryoultramicrotomy (Ultracut FC4D, Reichert-Jung, Germany), 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 pre-treated in ammonium chloride in PBS 0.1 M solution (10 min) to eliminate non-specific radicals, rinsed in PBS-glycine 0.1 M solution (4 x 2 min), and then blocked in 0.5% ovalbumin in PBS-glycine 0.1 M solution (10 min) at room temperature. Primary antibodies were incubated for 30 min at the dilutions mentioned above and washed in PBS-glycine 0.1 M. Secondary anti-mouse IgG-Au 10nm or pA-Au 16nm was then applied for 20 min. After successive washes, first in PBS 0.1 M (4 x 2 min) and then in double distilled water (6 x 2 min), grids were contrasted in 0.03% uranyl acetate solution (10 min) and a thin surface membrane of methyl-cellulose was applied. Double-staining procedures for VN/FN was 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 min) 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 INC., Japan).

Haemoglobin and Protein Sample Measurement

Control for blood plasma contamination in both subretinal fluid aspirates and normal vitreous samples were carried out by haemoglobinometry (41). On the basis of normal vitreous concentrations we excluded all pathological samples that showed haemoglobin levels higher than 0.2 mg/ml (Coulter Counter Model S^5, Beds, England). Protein concentration of all samples was determinated by protein-dye binding (42).

Electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (43). Briefly, normal and pathological 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 min, and electrophoresed (Mini-Protean II 200/2.0 Electrophoresis Apparatus, Bio-Rad, USA) for 2.5-4 hours at 50-60 V, on a 7.5% 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 (44). SDS-PAGE proteins were transferred (Trans-Blot 200/2.0 Transfer Apparatus, Bio-Rad, USA) at 20 V, overnight at 4°C onto a nitrocellulose sheet (HyBond-c, Amersham, U.K.). Primary antibodies were incubated overnight at 4°C in 1% non-fat dry milk in 50 mM Tris-HCl (pH 7.4) at 1:1000 dilution. Secondary anti-rabbit or anti-mouse peroxidase-conjugated immunoglobulin (Dakopatts, Denmark) diluted 1:2000 in 0.05% Tween 20 (Sigma Chem., USA) in 50 mM Tris-HCl (pH 7.4) was applied for 3-4 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

In order to quantify FN and VN we used a slot blotting method (Bio-Dot SF Microfiltration Apparatus, Bio-Rad Lab., USA) on nitrocellulose sheets for each dilution bank of normal and pathological vitreous samples (44). 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, Germany). Standard concentrations of purified VN and FN (0.2 $\mu g/\mu l$; Boehringer, Germany) were analyzed simultaneously with each sample.

Statistical Analysis

Chi square test (x^2) was used to appreciate statistical differences between the variabilities of frequency among groups. To study the variability between means we applied an unbalanced analysis of variance (ANOVA) by least significant difference (L.S.D.).

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 (Fig. 1A and 1B), than in the group of ERM aged between 2 to 6 months ($p<0.005$, by chi square test). Pericellular pattern was rarely observed in the third group of ERM aged over 6 months of evolution, and differences were established with the first group ($p<0.05$, by chi square test). No significant difference was found between the intermediate group and the last. Electron-immunocytochemistry with immunogold labelling 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 (Fig. 1C and 1D); cells with a wide variety of morphology showed large amounts of this glycoprotein.

Dense fibrillar fluorescent labelling was the most frequent finding for FN in matrix material, sometimes localized (Fig. 1I and 1J), sometimes generalized distributed across surface of the sample (Fig. 1E-F and 1G-H). The first group of ERM ($\leq$ 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 test). 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 test).

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 not directly related with it (Fig. 2E). The distribution of LN in collagenic matrix showed a similar pattern to that observed for FN, but restricted in confluent areas of immunogold labelling, 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 mainly for LN (Fig. 3). Areas with pigment-laden cells appeared to show a preference in labelling LN (Fig. 3B and 3C), although there were regions in sections that had significant labelling in the absence of pigmented cells.

VN was demonstrated in 6 (35%) out of 17 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 labelling for VN was distributed around the cell membrane but 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 labelling for $\alpha_v\beta_3$ and β_1 subunit complex integrins was occasionally identified (Fig. 4). The latter was observed more frequently, but less so in ERM with more than 6 months' evolution. By electron microscopy methods, anti-β_1 subunit complex labelling was achieved infrequently, but always correlated with cell cytoplasmic membrane (Fig. 4E); immunolabelled cells did not show any particular morphological characteristic. Alpha-V Beta-3 integrin was infrequently identified, associated with slight immunogold labelling of plasma membrane (not shown).

In collagenous matrix, densely or loosely arranged, variable quantities of FN were also noted (Fig. 5A and 5B). Double-labelling experiments showed a clear colocalization of VN and FN over collagenous bundles (Fig. 5C and 5D) and occasionally in a pericellular pattern, with predominant immunoreactivity for FN (not shown). Electron microscopy controls showed virtually no background of Au labelling.

Electrophoresis and immunoblotting for normal and pathological vitreous, and subretinal fluid samples

Pathological vitreous and subretinal fluid samples of VRP patients were compared with pathological vitreous samples from other intraocular proliferative disorders. Controls were normal human serum and normal vitreous samples obtained from human eyes *post-mortem*. Protein concentration of pathologic VRP vitreous for each stage of disease were substantially increased when compared with normal vitreous samples (0.506 ± 0.04 $\mu g/\mu l$) ($p<0.001$, 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 pathological vitreous and subretinal fluid samples, main bands of low molecular weight ($\leq$ 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 210-230 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 (12). Several pathological samples immunoblotted with antibodies against FN or VN showed low molecular mass bands that could represent degraded fragments for intact FN or VN. LN immunoreactivity was not detected in any subretinal fluid or vitreous sample.

Electrophoresis and immunoblotting for normal and pathological vitreous, and subretinal fluid samples

Pathological vitreous and subretinal fluid samples of PVR patients were compared with pathological vitreous samples from other intraocular proliferative disorders. Controls were normal human serum and normal vitreous samples obtained from human eyes *post-mortem*. Protein concentration of pathologic PVR vitreous for each stage of disease were substantially increased when compared with normal vitreous samples (0.506 ± 0.04 $\mu g/\mu l$) ($p<0.001$, 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 pathological 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 210-230 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 (12). Several pathological 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 pathological samples

Densitometry by IBAS indicated significant differences in the FN concentration among the stages of PVR samples, and other intraocular pathological 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 pathological samples when compared with normal samples levels (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 significant 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 I provides quantitative measurements of total proteins, FN, and VN relative amounts in normal and pathological conditions. The total variation of FN and VN concentrations at each stage of PVR are presented in Figure 8.

Table I: Concentrations of total proteins, FN and VN in normal and pathological vitreous samples.

	Total proteins ($\mu g/\mu l$)$^{\pi}$	Fibronectin (ngr/μg protein)	Vitronectin (ngr/μg protein)
Normal	0.506 ± 0.04	0.129 ± 0.03	0.195 ± 0.05
PVR A$^{\#}$	13.31 ± 3.37**	0.188 ± 0.04	0.144 ± 0.08
PVR B$^{\#}$	15.70 ± 5.10**	0.490 ± 0.08*	0.163 ± 0.08
PVR C	15.13 ± 4.50**	0.523 ± 0.07*	0.093 ±0.04*
PVR D	14.70 ± 2.90**	0.454 ± 0.02*	0.061 ±0.03*
IOFB	20.80 ± 6.00**	1.546 ± 0.50**	0.154 ± 0.09

PVR, proliferative vitreoretinopathy and stages (A,B,C,D); IOFB, intraocular foreign body.
π, means ± S.E.; #, subretinal fluid.
*, statistical significance from normal samples ($p<0.05$); **, statistical significance from normal samples ($p<0.001$).

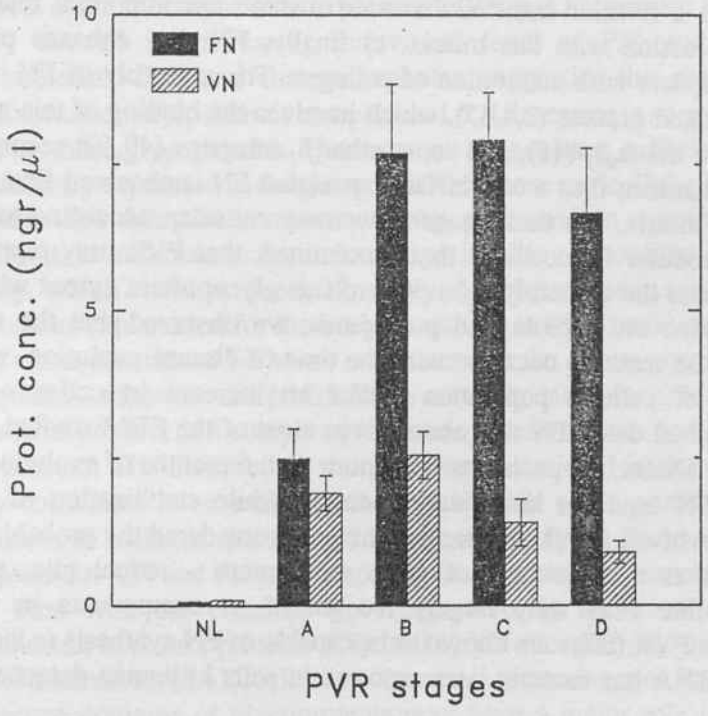

Figure 8. Profile of total concentration of FN and VN in clinical evolutive stages (A, B, C, and D) of PVR. Normal vitreous samples (NL) were also analyzed.

DISCUSSION

Significant progress has been made in elucidating physiopathogenic mechanisms in PVR, although many fundamental question 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 (30-33) and the possible role of soluble and insoluble proteins (35-38) 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 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 labelled 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 generalizedly 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. Analyzing the role of FN in wound repair (45,46) some crucial chronological events were observed: a) during the early stages of wound healing plasmatic-soluble FN stimulates directed cell migration towards the wound, acting as a chemotactic element, and also has a role in cell-to-cell, and cell-to-substrate adhesion; b) later, insoluble FN is secreted by cells to produce a fibrillar extracellular matrix which has important relationships with cellular anchorage and interaction with this matrix; c) 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 (2,4,47), which involves the binding of this glycoprotein by a receptor-like system, while the $\alpha_5\beta_1$ (48), and some other β_1 integrins (49,50) 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 behaviour of FN observed in the intraocular fibrocellular tissue examined, that PVR may represent modified intraocular wound repair, since the assembly behaviour of this glycoprotein agrees with the classical model described above. Based on stereological 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). FN was abundant in most of the ERM studied, being 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 while 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 human PVR (30) - are known to be capable of FN synthesis *in vitro* (51,52), and variable labelling of FN mRNA has recently been reported in cells in human detached retina and in ERM (38).

LN and VN appear to be involved in fibrocellular tissue formation, but only with a secondary role. Their identification was occasionally possible in pericellular and fibrillar patterns. Pericellular LN labelling was noted in this study by optical and electron microscopy immunochemistry 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 (32). In addition, ultrastructural studies found that retinal pigment epithelium and retinal glial cells are

able to constitute basement membranes, rarely observed in fibroblasts or macrophages (30). LN is the major glycoprotein found in basement membranes and acts with collagen type IV as structural proteins. Among several functions, LN promotes epithelial cell adhesion to collagen (53,54), and has the ability to convert embryonic mesenchymal cells into polarized-shaped form (55). These data are sufficient evidence to justify futher doubled-labelling assays to elucidate two major question: 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 (14,56), and its biochemical structure, which includes a domain capable of binding collagen (2,12,57). The cell attachment activity of VN is based on the Arg-Gly-Asp (RGD) sequence (14,16), which is recognized by a wide variety of cell types (16). Properties of VN in haemostasis are extensively described (11,12), but in our view, it is difficult to correlate haemostatic 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-immunochemistry 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 (8,9), 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 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 slight alteration in VN concentration in subretinal fluid and vitreous aspirates, suggesting this process at least for FN.

Expression of β_1-subunit complex and $\alpha_v\beta_3$ integrins is corroborated by the pericellular pattern identified by these glycoproteins. However, we do not have sufficient data to conclude that β_1 complex was preferently expressed in the group of ERM whose pericellular labelling for FN were most evident, or whether this integrin immunoreactivity decreased in specimens with longer clinical evolution. In addition, in immunoelectron microscopy labelling 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 membranogenesis such cellular migration, adhesion and proliferation certainly take place. Moreover surgically excised ERM, represent a final product of a complex proliferative phenomenon, looking like a stabilized "scar" in wound repair process. The distribution and presumed functional interaction of receptors, FN, and LN are generally coincident in several structures (4,19), but some peculiarities are remarkable; for example, a) responses to collagen and FN or LN are commonly mediated by β_1-class integrins, while non-platelet receptors for VN and FN are composed of α_v-subunit in association with β_1, β_2, or β_5 (58-60); b) FN can bind with the classical VN-receptor ($\alpha_v\beta_3$) specifically and with high affinity, supporting cell adhesion to matrix proteins (28); and c) VN-receptor ($\alpha_v\beta_3$) may act as an LN-receptor and can mediate cell adhesion (8,61). All this evidence is indicative that integrins 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, and in this case reflected by a failure of the retinal pigment epithelium function as a barrier (62). High concentrations of proteins in subretinal fluid (early stages) and in pathological vitreous (later stages) characterize the PVR intraocular environment, and 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 1/4 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 dose-dependent manner, and among these serum elements, FN was higly active (36,63). In addition, important alterations in integrity and in permeability of blood-barriers have been found in eyes with diabetic retinopathy (62), and in a transient form in eyes with retinal detachment (64). Haemorrhage may also result in release of plasma or serum components into the vitreous cavity. and these are thought to be associated with the increased incidence of PVR after penetrating ocular injuries (65), with or without intraocular foreign bodies (IOFB).

Our findings showed increased levels of intravitreal FN and protein in traumatic PVR with IOFB, associated with accepted haemoglobin concentrations ($\leq$ 0.2 mg/ml) in samples. Breakdown of blood-retinal barriers may explain the changes in proteins and FN levels observed in pathological intraocular milieu. Intraocular FN levels vary significantty in the earliest stages of PVR (stages A, and B) when we could only analyze subretinal fluid, since 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 (29). 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 (29). Increase in FN levels could not be exclusively explained by plasmatic origin, since the protein concentration remains unchanged. An attractive hypothesis is that early cell production of FN at stage A is responsible of 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, since plasmatic concentration of FN (0.3 $\mu g/\mu l$) and VN (0.2 - 0.4 $\mu g/\mu l$) are similar (2,5,11,12), and 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* (3,66,67). 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* (9,10,45,68) supports this hypothesis.

Finally, it has recently been described that chronic wound fluid samples, examined by immunoblotting and cell adhesion assays, showed a marked, and sometimes complete, degradation of VN, and a less evident degradation of FN, which alters the cell adhesion necessary for normal wound closure (46). 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 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.

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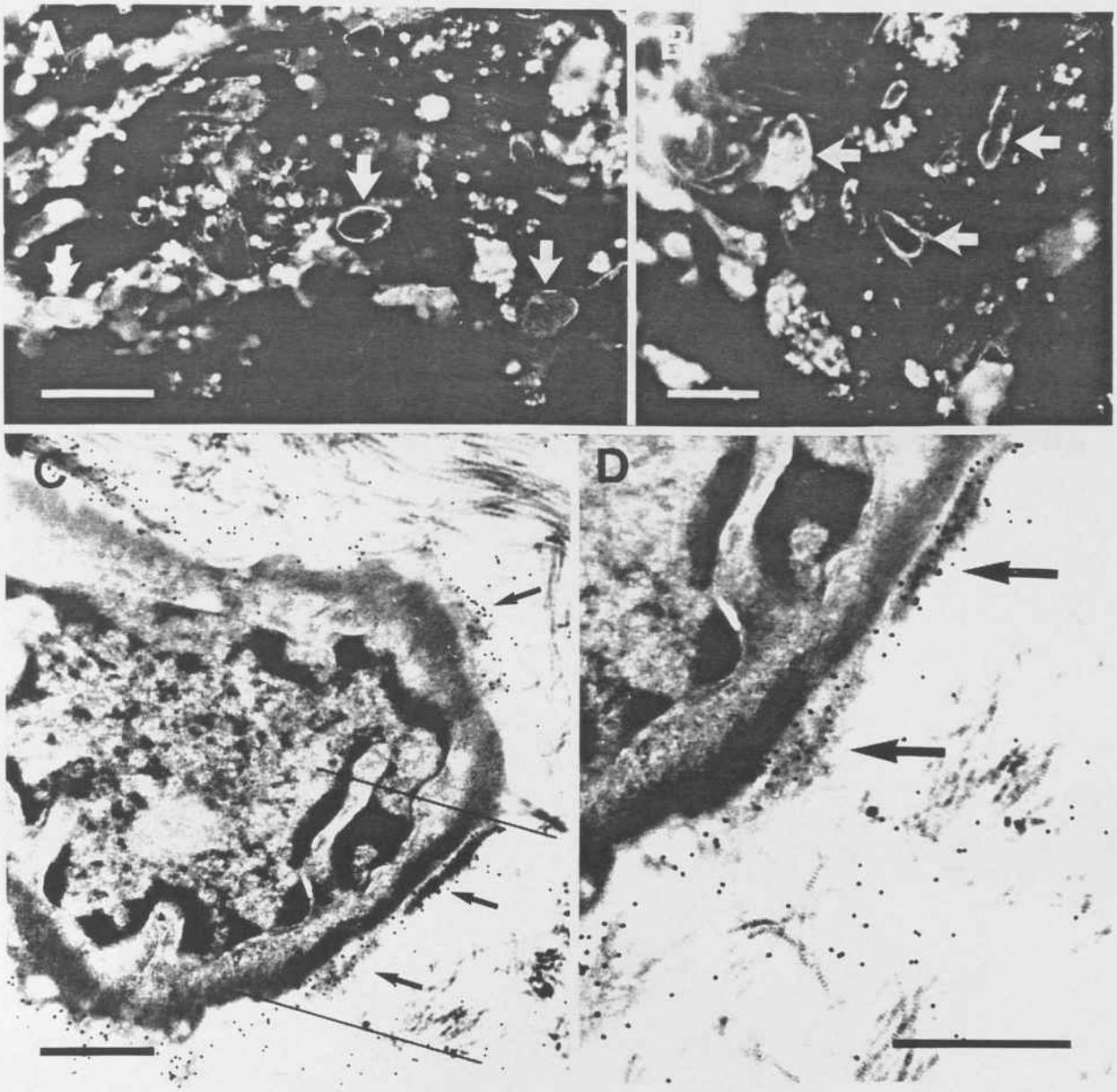
Figure 1.

Figure 1. Fibronectin expression in ERM. **(A)** Semithin sections prepared by indirect immunofluorescence stain showed moderate amounts of pericellular FN (*arrows*) (*X 990, bar=20μm*); **(B)** a wide variety of morphological cell types presented immunoreactive FN (*arrows*) (*X 800, bar=20μm*). (C) Immunoelectron microscopy showed labelling (16nm gold) (*arrows*) closely related with plasma membrane (*X 19,200, bar=1μm*). **(D)** Higher magnification of bracketed area in Figure C, immunoreactive FN binds a peripheral electrodense material (*arrows*) related with cell membrane observed in some cell types; collagenic matrix composed by fibers with clear periodicity present variable amounts of immunogold label (*X 32,500, bar=1μm*).


A
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D

Figure 1

(continuation).

Figure 1 (continuation). **(E)** Cryostat sectioned specimens analyzed by interference contrast microscopy revealed abundant and dense fibrillar matrix in regular arrangement (*X 390*). **(F)** By immunofluorescence, abundant amounts for this protein in generalized and regular thicker fibrillar pattern across the specimen surface were seen (*X 390*). **(G)** Generalized and fine fibrillar FN irregular distributed in matrix material were also common pattern of labelling (*X 315*). **(H)** The same section examined by interference contrast microscopy reveals areas of regular and random collagenous fiber arrangement (*X 390*). **(I)** Localized areas of regular pattern of immunofluorescence stain (*X 390*), contrast with **(J)** areas of irregular fibrillar distribution in the same specimen (*X 315*); (*bar=30μm*).

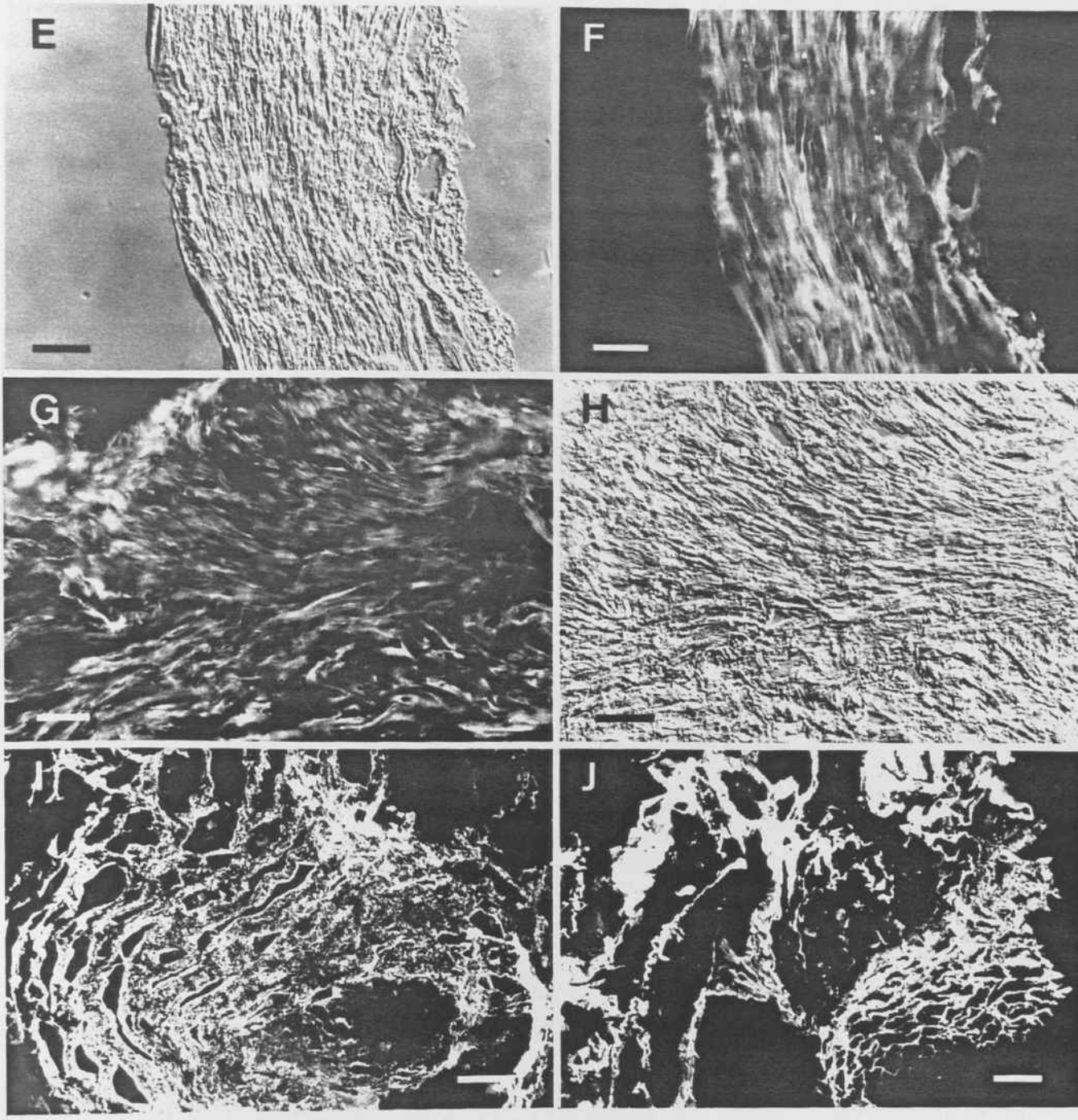
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Figure 2.

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*) (*X 900, bar=20μm*). **(C)** Slight labelling in a fine fibrillar pattern of distribution **(D)** in small localized areas in the same sectioned specimen examined by interference contrast microscopy (*X 360, bar=40μm*). **(E)** By immunoelectron microscopy, pA-Au (16nm) particles were noted both related to the plasma membrane (*arrow*) and the extracellular material surrounding cells (*X 19,200, bar=1μm*). **(F)** Collagenic fibers with periodicity presented immunoreactivity for this glycoprotein (*X 39,500, bar=0.5μm*). **(G)** Scanty immunoreactivity for LN was common feature in matrix (*19,200, bar=1μm*).

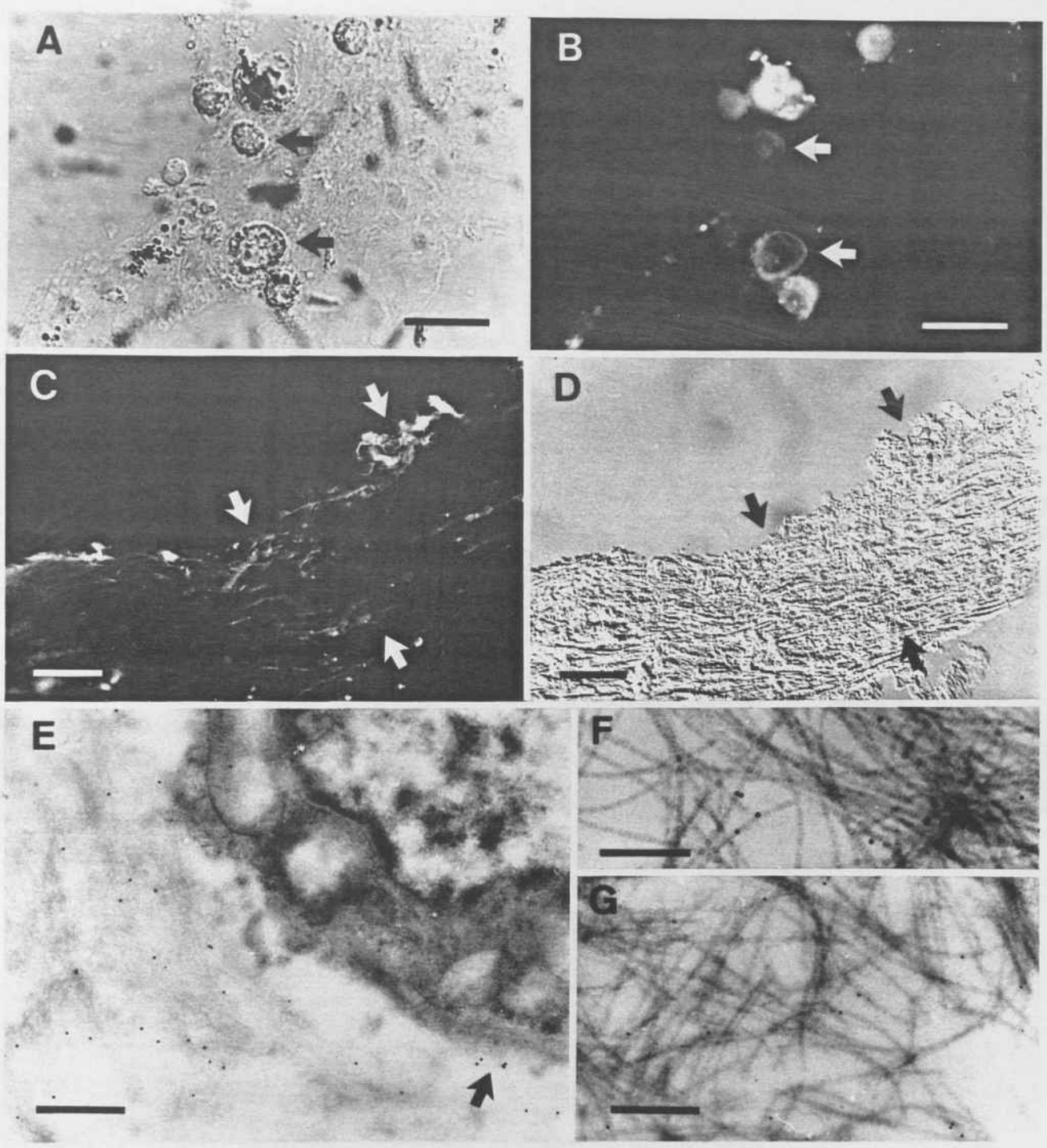
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Figure 3.

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 microscope 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 non-reactive for FN (*between arrows*). (*X 315, bar=40μm*).

A
B
C

Figure 4.

Figure 4. Expression of β_1 subunit complex and $\alpha_v\beta_3$ integrin in ERM. Semithin cryosectioned specimens were analyzed by indirect immunofluorescence after incubation with anti-ASβ_1 antisera and mAb LM609 directed against $\alpha_v\beta_3$ receptor. **(A)** Wide variety of cells presented fluorescent staining for β_1 complex receptor (*arrows*) (*X 730, bar=20μm*). **(B)** The VN receptor ($\alpha_v\beta_3$) was occasionally found in isolated cells or organized in clusters or nest (*arrows*) (*X 360, bar=40μm*). **(C)** VN immunolocalization in the same sample, shows a pericellular pattern of distribution (*arrows*) as its receptor (*X 450, bar=30μm*). **(D)** High magnification showed immunofluorescence for β_1-subunit complex closely related to the plasma membrane (*arrow*) (*X 1,130, bar=10μm*). **(E)** Beta-1 labelling for plasma membrane (pm) was corroborated by immunoelectron microscopy (*X 39,500, bar=0.5μm*).

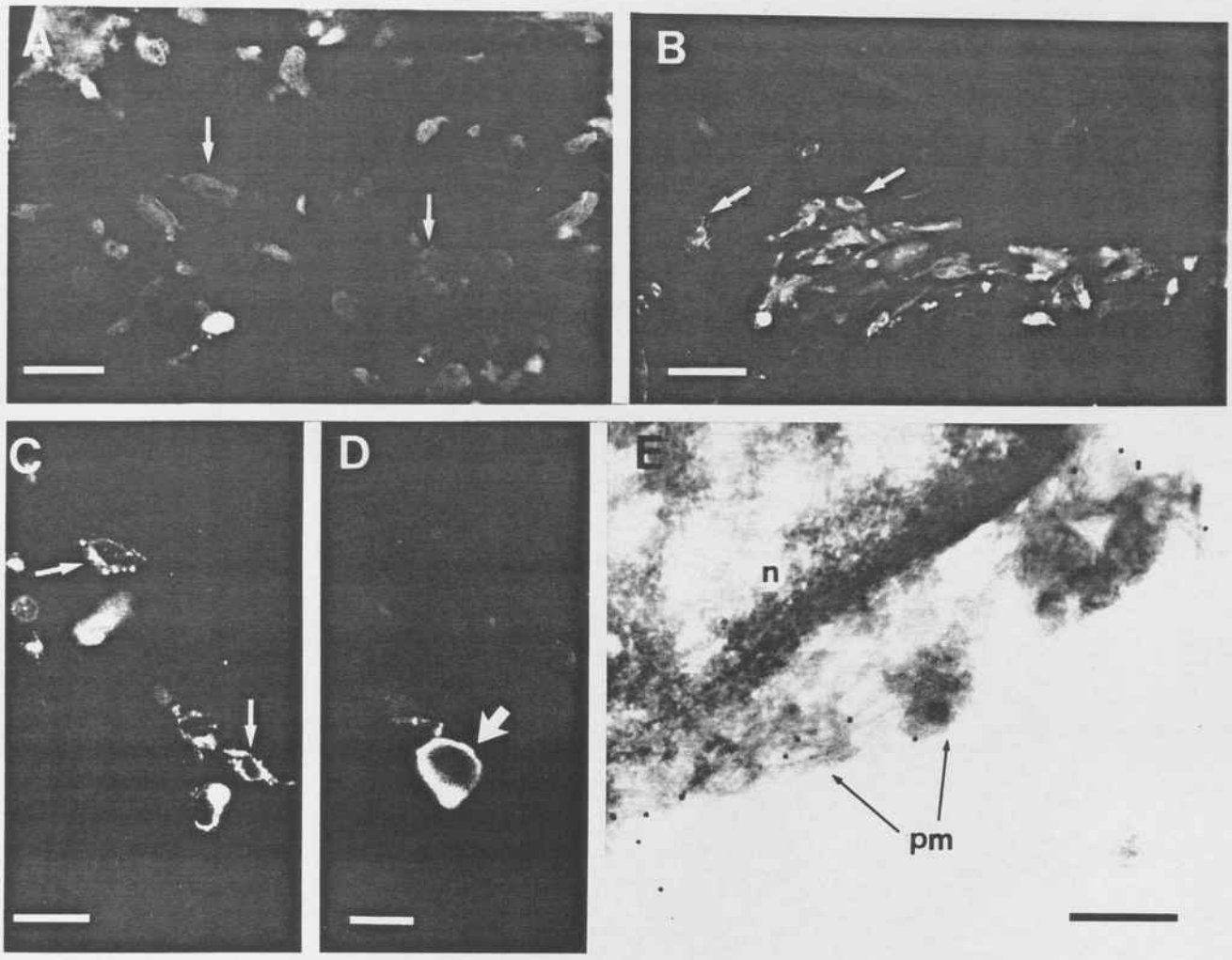
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Figure 5.

Figure 5. Colocalization of FN and VN in collagenic matrix in ERM. Ultrathin sections were incubated with mAb VN7 anti-VN and then with anti-FN antisera. **(A)** In single-labelling experiments, strong immunoreactivity for FN was observed in densely arranged collagenous material (*X 27,500*). **(B)** High magnification show labelling across collagen bundles (*X 39,500*). **(C)** Double-label experiments always showed more abundant FN immunoreactivity (pA-Au 16nm) (*arrowhead*) than VN (IgG-Au 10nm) (*arrows*) (*X 30,000*). **(D)** Colocalized label for FN and VN (*arrows*) was usually seen (*X 27,500*); (*bar=0.5μm*).

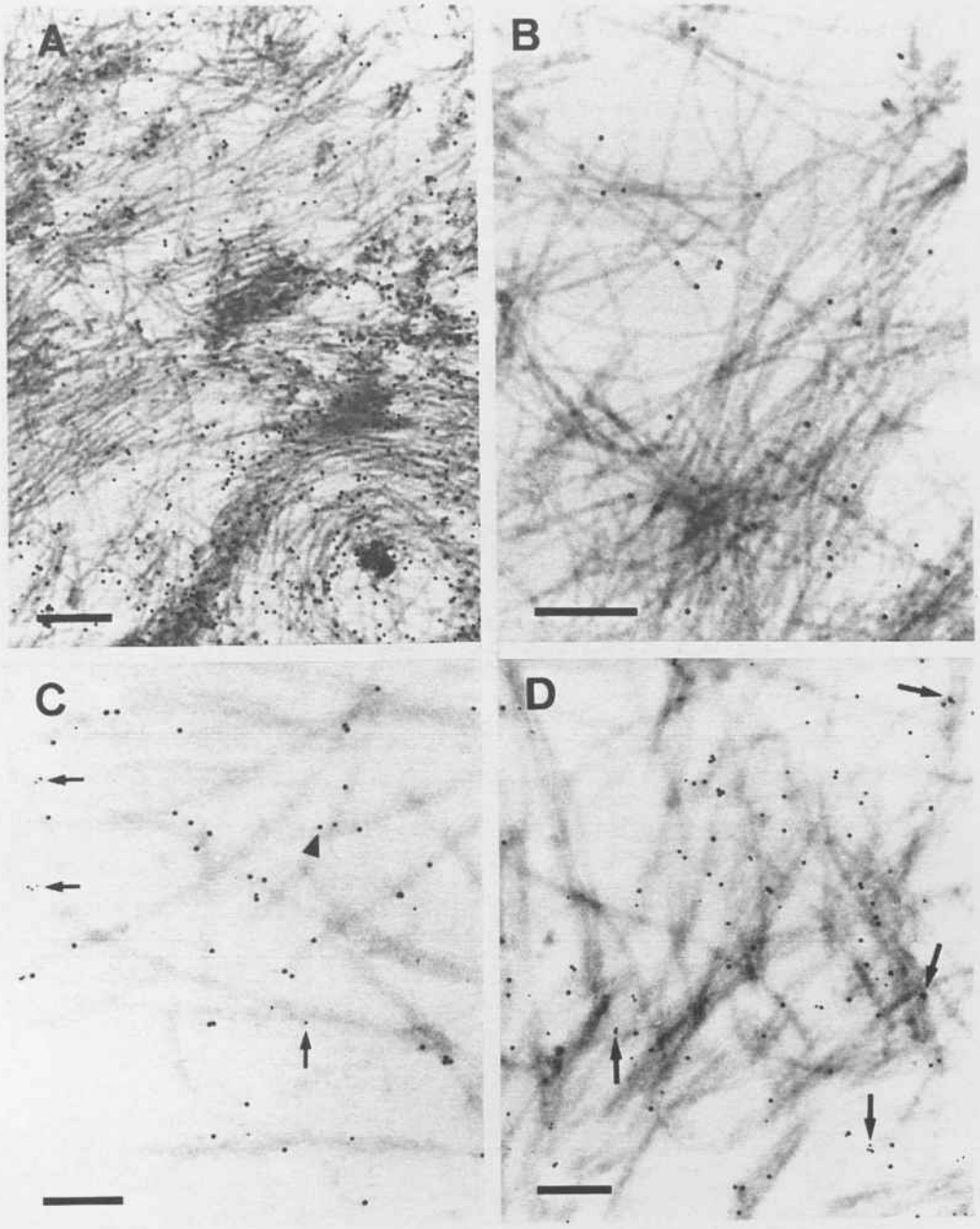
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Figure 6.

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 pathological samples in both subretinal fluid (SRF) in early stages (A and B) and vitreous aspirates in later stages (C and D) of pathology. Proteins of molecular mass higher than 66 kD observed in normal human serum (S) were less evident in pathological 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).

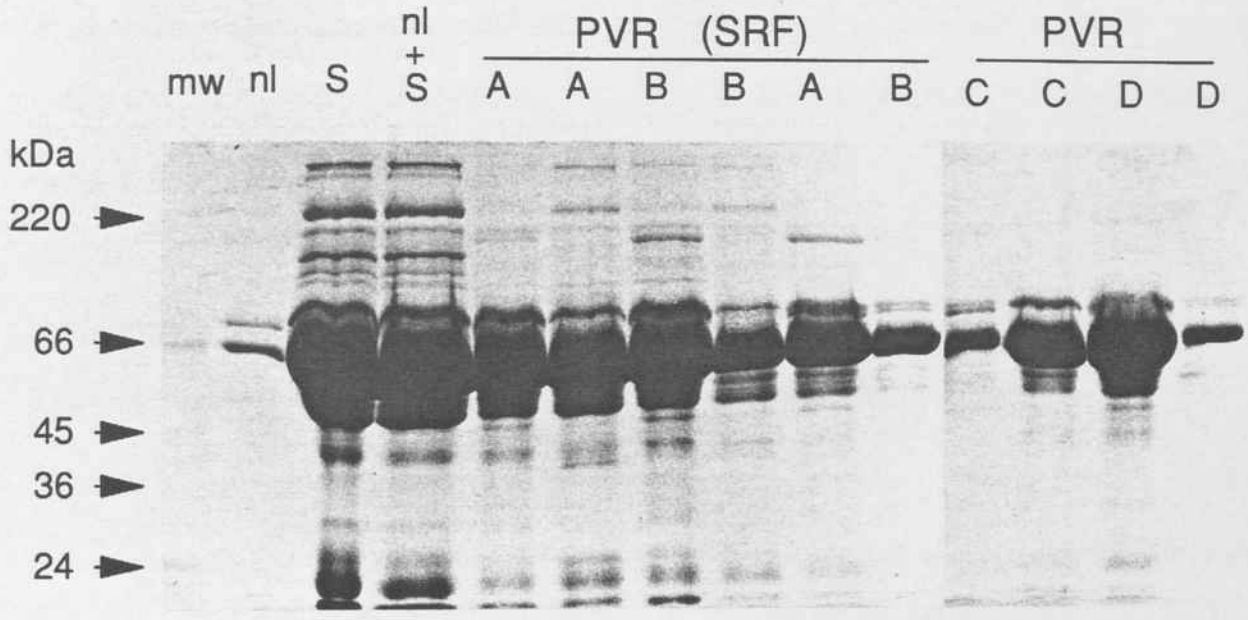
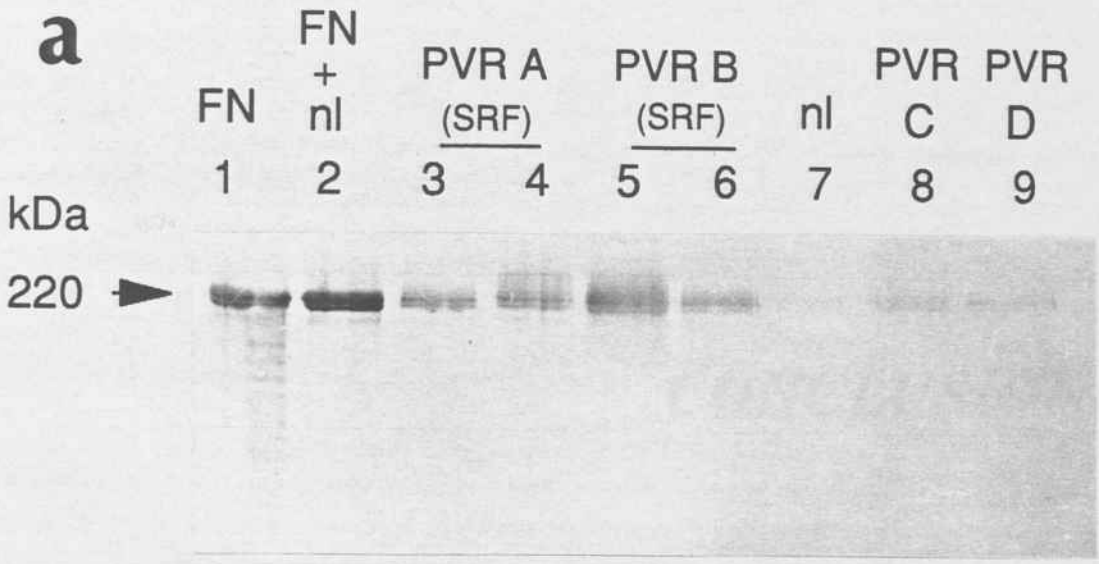
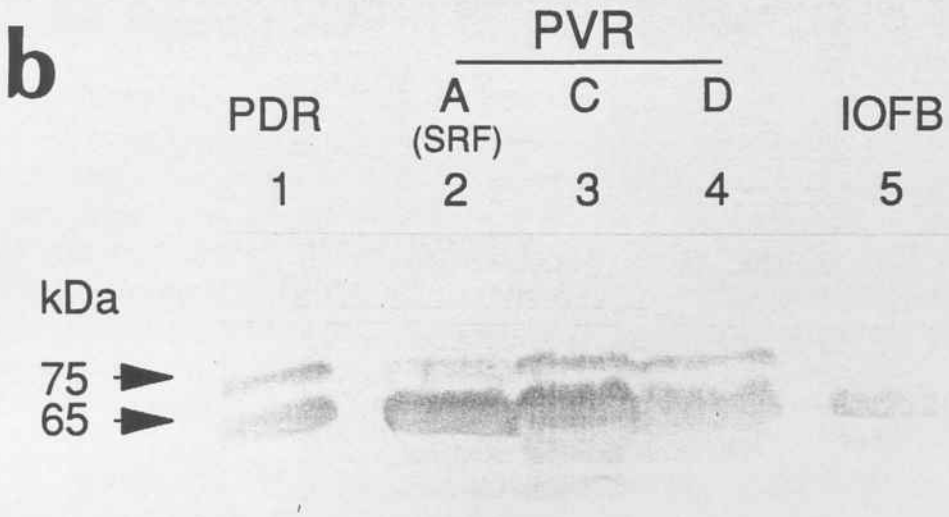


Figure 7.

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, 4*) and B (*lanes 5, 6*) of PVR, as well as later stages C (*lane 8*) and D (*lane 9*) were positive for FN. Purified FN (*lanes 1, 2*) was used as positive control. **(b)** On 10% SDS-PAGE, PVR samples in different stages (*lanes 2, 3, 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 (65-75 kD) was observed and low molecular mass fragments of degraded glycoprotein were also detected.


a
FN
FN + nl
PVR A (SRF)
PVR B (SRF)
nl
PVR C
PVR D
1 2 3 4 5 6 7 8 9
kDa
220


b
PDR
PVR
A (SRF)
C
D
IOFB
1 2 3 4 5
kDa
75
65

CONCLUSIONES

CONCLUSIONES

De los presentes resultados obtenidos se desprenden las siguientes conclusiones principales:

Primera. La vitreorretinopatía proliferativa (VRP), el síndrome de la interfase vitreorretiniana (SIV, "macular pucker") y la retinopatía diabética proliferativa (RDP) son enfermedades proliferativas intraoculares caracterizadas por la aparición de un tejido conjuntivo fibrocelular (VRP, SIV) y fibrogliovascular (RDP) de neoformación. El conjunto de los resultados ultraestructurales y estereológicos indican que la formación del tejido fibrocelular y fibrogliovascular posee un comportamiento similar a la formación de un tejido reparacional que se observa en los procesos de cicatrización, indicando que la enfermedad proliferativa intraocular puede representar un proceso reparacional modificado por circunstancias particulares del medio intraocular, con consecuencias indeseables para la arquitectura y funcionalidad retiniana.

Segunda. Los estudios inmunocitoquímicos en microscopía óptica y electrónica para la caracterización de las proteínas de los filamentos intermedios presentes en las células de las membranas epirretinianas de la VRP sugieren que: 1) determinados tipos celulares con características ultraestructurales similares son capaces de expresar, por lo menos, dos de las proteínas internas estudiadas; 2) determinados tipos celulares resultaron negativos para dichas proteínas. Los resultados sugieren que existe un proceso activo de diferenciación-rediferenciación celular en la VRP.

Tercera. La fibronectina (FN), laminina (LN), vitronectina (VN) y sus receptores, las integrinas del complejo β_1 y la $\alpha_v\beta_3$ (receptor-VN), están presentes en la estructura de los capilares de neoformación de la RDP. Las glucoproteínas y sus receptores se presentan situados en regiones que les permite mediar la adhesión célula-substrato y contribuir a la formación y mantenimiento de las láminas basales. Asimismo, la localización de los receptores en el dominio apical capilar, en contacto con la circulación, sugiere un papel en la captación de glucoproteínas a partir del plasma. Los resultados sugieren que glucoproteínas y receptores participan, estructural y funcionalmente, en el proceso angiogénico y en el mantenimiento del entorno diabético intraocular.

Cuarta. La fibronectina (FN), laminina (LN), vitronectina (VN) y sus receptores, las integrinas del complejo β_1 y la $\alpha_v\beta_3$ (receptor-VN), están relacionados con las células y la matriz extracelular de las membranas epirretinianas en la VRP: 1) glucoproteínas y receptores se encuentran localizados en una posición que les permite mediar la interacción de la membrana plasmática con el substrato; 2) la FN cambia su patrón de distribución con relación al tiempo de duración del tejido fibrocelular, obedeciendo al modelo de ensamblaje característico propuesto para la proteína.

Quinta. En la VRP las concentraciones vítreas de proteínas totales y de la VN aumentan, de forma proporcionada, con relación a los estadíos clínicos de la enfermedad, sugiriendo un aporte esencialmente plasmático. Las concentraciones intravítreas de FN aumentan de forma precoz y desproporcionada, sugiriendo un aporte suplementario al plasmático e indicando que puede mediar en los fenómenos iniciales pre-membranogénicos del proceso proliferativo.