

Expression of Extradomain-B–containing Fibronectin in Subretinal Choroidal Neovascular Membranes

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• **PURPOSE:** To investigate the presence of the fibronectin isoform containing the extradomain B (B-FN), a marker-protein of angiogenesis, in surgically excised human choroidal neovascular membranes (CNVM) to evaluate whether B-FN could be used as a therapeutic target for specific antibody-photosensitizer immunoconjugates.

• **DESIGN:** Laboratory investigation.

• **METHODS:** The setting was an institutional practice. The study population consisted of 15 eyes (15 patients) with CNVM undergoing membrane excision (four eyes with age-related macular degeneration, seven with pathologic myopia and four with multifocal choroiditis). The control group consisted of eight eye bank eyes (four subjects) without choroidal neovascularization. Light microscopic immunohistochemistry on cryostat sections of tissues was obtained. B-FN was detected by a human recombinant antibody, CGS-1, and compared with immunostaining for endothelial cells with factor VIII-related antigen. The main outcome measure was the presence of CGS-1 positively stained cells or areas of the extracellular matrix. Staining of CGS-1 was scored on a scale from 0 to 3.

• **RESULTS:** Fourteen of 15 neovascular membranes stained strongly with CGS-1 (score 2 or 3). One membrane from a patient with pathologic myopia was negatively stained (score 0). CGS-1 positive staining was detected around endothelial cells and in the extracellular matrix of CNVMs. The retina of eyes without choroidal neovascularization was negative with CGS-1 in all eight donor eyes, while the choroid contained some weakly CGS-1 positive cells (score 0 and 1, respectively).

• **CONCLUSIONS:** The extradomain B is abundantly expressed in CNVMs, but its expression is more restricted in eyes harboring no apparent choroidal neovascularization. In the future, B-FN might serve as a target for the delivery of antibody-photosensitizer immunoconjugates to newly developed vessels to enhance the selectivity of photodynamic therapy. (*Am J Ophthalmol* 2003;135:7–13. © 2003 by Elsevier Science Inc. All rights reserved.)

THE COMPLEX AND STILL POORLY UNDERSTOOD PROCESS of choroidal neovascularization (CNV) involves the migration of choroidal endothelial cells through the Bruch membrane into the subretinal pigment epithelial space and the formation of new vessels that can spread under and over retinal pigment epithelial cells.^{1–3} The choroidal neovascular membrane (CNVM) is a fibrovascular tissue that represents a nonspecific inflammatory reaction and wound healing process.^{4,5} Previous studies on surgically excised CNVMs investigated their cellular and some of their extracellular matrix (ECM) components, which include collagen types I, IV, and in smaller amounts, types III, V, and VI, fibronectin, laminin,⁵ tenascin-C,⁶ different growth factors,^{1,2,7} matrix metalloproteinases and their inhibitors,⁸ and mucopolysaccharides and lipids.⁵

Light and electron microscopic studies have shown that the formation of new vessels in different tissues is accompanied by the remodeling of the ECM.^{9–12} This phenomenon occurs, for example, during tumor growth, fetal

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This study was performed at the Ophthalmology Clinic, Ancona (surgical excision) and the Laboratory of Cellular Biology, National Institute for Cancer Research, Genoa (immunohistochemistry).

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development, and wound healing, processes in which angiogenesis plays a crucial role. During angiogenic processes a number of new matrix proteins are expressed. One of these is an isoform of fibronectin (FN) containing an extradomain of 91 aminoacids, also referred to as B-fibronectin (B-FN).

Fibronectins are high-molecular-weight glycoproteins present in the ECM and in body fluids, and they are made up of repeating homology sequences. The FN polymorphism is due to alternative splicing patterns of the single FN primary transcript in three regions, namely IIICS, extradomain A, and extradomain B (EDB), as well as to posttranslational modifications.¹³⁻¹⁶ Although B-FN is virtually undetectable in normal adult tissues (except the regenerating endometrium), it shows a much greater expression in fetal and tumor tissues¹⁷ and in healing wounds,¹⁸⁻²⁰ where it localizes around newly formed vessels.^{21,22}

The recombinant antibody CGS-1 is a single chain fragment (scFv) of the immunoglobulin molecule composed only of the hypervariable regions of the heavy and light chains connected through a short linker and containing a Myc tag. It was obtained from a human combinatorial scFv library by selection against the recombinant FN fragment containing the complete type III repeat extradomain B.²³

Previous animal studies showed that a high affinity antibody against B-FN conjugated with a photosensitizer selectively accumulates in the newly formed vessels of the rabbit cornea angiogenesis model, and after irradiation with light of the adequate wavelength, mediates the complete and selective occlusion of corneal neovascularization.²⁴ The aims of this study were to investigate whether B-FN was also present in surgically excised neovascular membranes and, utilizing donor eyes without obvious CNV for control purposes, to evaluate the possible use of B-FN as a target for antibody-photosensitizer immunoconjugates in the treatment of CNV.

METHODS

SURGICALLY EXCISED CNVMS WERE OBTAINED FROM 15 eyes of 15 patients, 12 women and 3 men, ranging in age from 27 to 84 years (51.7 ± 18.2 years, mean \pm SD). The underlying disease was age-related macular degeneration (AMD) in four patients (aged 73.5 ± 11.9 years), pathologic myopia (PM) in seven patients (aged 47.7 ± 12.3 years) and multifocal choroiditis (MFC) in four patients (aged 37.0 ± 11.8 years). Onset of symptoms had begun less than 1 month preoperatively in all patients. The best-corrected visual acuity of all patients was 20/70 or less. All CNVMS, regardless of the underlying disease, were classified as classic, well defined, and subfoveal. None of the patients had undergone previous laser photocoagulation. All subjects gave their informed consent for the

surgical procedure, and the entire study adhered to the tenets of the Declaration of Helsinki. All surgical extractions were performed using the pars plana technique described by Scheider and associates.²⁵ After excision, the membranes were placed on filter paper and transported by overnight courier to the laboratory in tissue culture medium for immunohistochemical analysis. Tissues were embedded on edge in Tissue-Tek, OCT compound (Sakura, Zoeterwoude, The Netherlands), were snap frozen in liquid nitrogen, and if not used immediately, stored at -80 C.

Additionally, eight eyes of four donor subjects, three women and one man, ranging in age from 55 to 93 years (75.5 ± 15.6), were obtained from the Veneto Eye-bank Foundation (Venice, Italy) and used for control examinations. Cerebral and cardiorespiratory events or neoplasm were the causes of death in all cases. No clinical information was available concerning any previous eye disease. Between death and inclusion in OCT, eyes were stored at 4 C in a moist chamber. Eyes were opened by removing the anterior pole. Internal examination was performed under a dissection microscope (Olympus, Nagano, Japan), and special attention was paid to diagnose atrophy and hypertrophy of the retinal pigment epithelium, exudates, hemorrhages, or fibrotic changes in the macular area. Thereafter, different pieces from the peripheral to the central regions, including the macula, were obtained and the specimens were embedded in Tissue-Tek, snap frozen in liquid nitrogen, and stored at -80 C.

For immunohistochemistry analysis, $5\text{-}\mu\text{m}$ thick serial sections were cut in a cryostat at -20 C, mounted on poly-L-lysine-coated glass microslides, and then fixed in Dealonay liquid (alcohol 99% and acetone 1:1) or in ice-cold acetone for 10 minutes for hematoxylin-eosin (HE) staining and for immunohistochemistry, respectively. Immunostaining was performed using the standard streptavidin-biotin alkaline phosphatase complex technique. Recombinant antibody CGS-1 against the ED-B domain of FN was used, as previously described.¹⁵ Briefly, after saturating the nonspecific binding sites for 30 minutes with swine serum, slides were incubated with primary and anti-Myc tag antibody for 60 minutes, then washed with tris-phosphate buffer, and finally incubated with biotinylated secondary antibody for 30 minutes. The streptavidin-biotin alkaline-phosphatase complex (BioSpa, Milan, Italy) was then added, and staining was developed with Fast Red (Sigma-Aldrich, Steinheim, Germany). Slides were mounted with either Glycergel or Ultramount (Dako, Carpinteria, California, USA). Factor VIII-related antigen immunostaining was also performed according to manufacturer (Dako, Glostrup, Denmark) instructions to detect vascular endothelial cells. Slides were stained as described above, omitting the primary antibody to detect any nonspecific staining. Some slides were incubated in the presence of EDB in excess, to check if the soluble antigen blocked the specific staining of tissues. Slides were

examined with a Leica microscope (Leica, Wetzlar, Germany) and photomicrographs were taken with a 3-CCD color camera (JVC, Kanagawa, Japan).

RESULTS

ALL SURGICAL SPECIMENS PROVED TO BE FIBROVASCULAR tissue that contained retinal pigment epithelial cells, vascular endothelial cells, fibroblasts and myofibroblasts, macrophages, and inflammatory cells. The Bruch membrane or fragments of it could be identified in six membranes (2 AMD, 1 PM, 3 MFC). Photoreceptor cell fragments were also detected in three membranes (2 AMD, 1 PM). Retinal pigment epithelium cells were often multilayered, adjacent to the remnants of Bruch membrane and tended to show migration into stroma and to envelop vascular endothelial cells.

Immunohistochemistry to detect vascular endothelial cells was positive in all cases. Immunohistochemistry to detect B-FN gave a strong positive reaction (Figure 1, B and D) in 14 of the 15 membranes (Table 1). Positivity was seen in close proximity to and around vascular endothelial cells (Figure 1, C and D), as well as diffusely throughout the stroma (Figure 1, B). In one membrane from a patient with MFC, myofibroblasts stained positively for B-FN (data not shown). Photoreceptor fragments (Figure 1, D) and Bruch membrane or remnants of it were negative for B-FN in all cases. Staining characteristics for B-FN were similar in all membranes regardless of the underlying disease (Figure 2). The only membrane that did not stain for B-FN came from a patient with pathologic myopia. Negative controls showed no staining.

Gross examination of the donor eyes under the dissection microscope revealed alterations in the structure of the retinal pigment epithelium, namely atrophy and hypertrophy in the macular region, in one of eight eyes. Serous retinal detachment involving the macula was seen in all postmortem eyes. The subretinal fluid was mixed with brownish pigments that made the detailed macroscopic evaluation of drusen difficult. Drusen, hemorrhage, and fibrotic tissue were not visible.

On light microscopic examination nodular drusen could be detected in six of eight donor eyes, whereas soft drusen were seen in three cases (Table 2). Areas with retinal pigment epithelium atrophy or hypertrophy or clumping were seen in all eye bank eyes. As expected, staining for factor VIII-related antigen revealed numerous endothelial cells throughout the whole choroidal layer as well as in retinal vessels in all specimens (Figure 1, E).

Immunohistochemistry for B-FN was negative in the retina, retinal vessels, retinal pigment epithelium cells, drusen, and Bruch membrane in all eight donor eyes. The choroid contained a few (0 to 5 cells per high-power magnification field), evenly dispersed, weakly positive cells in all eight donor eyes (Figure 1, F). These cells

were seen mainly in close proximity to the Bruch membrane (Figure 1, G), probably in the choriocapillaries and in the wall of some large choroidal vessels (Figure 1, H). We found no correlation between positively stained cells and the presence of drusen. There was no difference between the staining pattern of regions with retinal pigment epithelium alterations and those with an intact retinal pigment epithelium cell layer. Controls omitting the primary antibody were negative in all eight donor eyes. Incubation of slides in the presence of soluble ED-B blocked staining of tissues.

DISCUSSION

CURRENT THERAPEUTIC APPROACHES FOR CNV PRESENT numerous drawbacks and limitations. Laser photocoagulation is the most widely studied and accepted treatment for the disorder, but it causes irreversible damage to the chorioretinal tissues and is associated with a high recurrence rate. Laser treatment of subfoveal lesions is especially inappropriate, as it leads to immediate and permanent loss of vision.²⁶ Photodynamic therapy (PDT), although showing promise as a new therapeutic tool for subfoveal CNV, is available only for a small proportion of cases and recurrences require repeated treatments.²⁷ Surgical approaches, including membrane extraction,²⁸ retinal pigment epithelium cell transplantation,²⁹ and macular translocation,³⁰ are other treatment modalities whose advantages for visual outcome are still controversial and under investigation. As a consequence, the discovery of new, more effective, and less invasive therapies for CNV has become one of the most important goals of ophthalmologic research.

Recently, significant efforts have focused on the search for substances able to inhibit the angiogenic processes underlying CNV. Because the selective delivery of antiangiogenic drugs to the neovascular tissue is essential to this therapeutic approach, the identification of angiogenic markers would be useful. B-FN, which is highly expressed in neovascularized tissues, is a known marker of angiogenesis in tumors.^{15,21}

We studied surgically excised CNVMs using immunohistochemical techniques and found that B-FN was strongly expressed in these tissues, regardless of the underlying disease. By contrast, the choroid of eyes without CNV showed only a few cells expressing B-FN, and with one exception, all retinal vessels were negative.

The presence and the possible roles of FN in the human,^{31,32} rat,³³ simian,³⁴ and bovine eye³⁵ are discussed in the literature. These proteins are generally thought to play a role in cell-ECM interactions. The FN isoform containing the ED-B is generated by alternative splicing of the primary FN transcript. The ED-B isoform is a type-III homology repeat composed of 91 amino acids whose sequence is highly conserved in different species (and

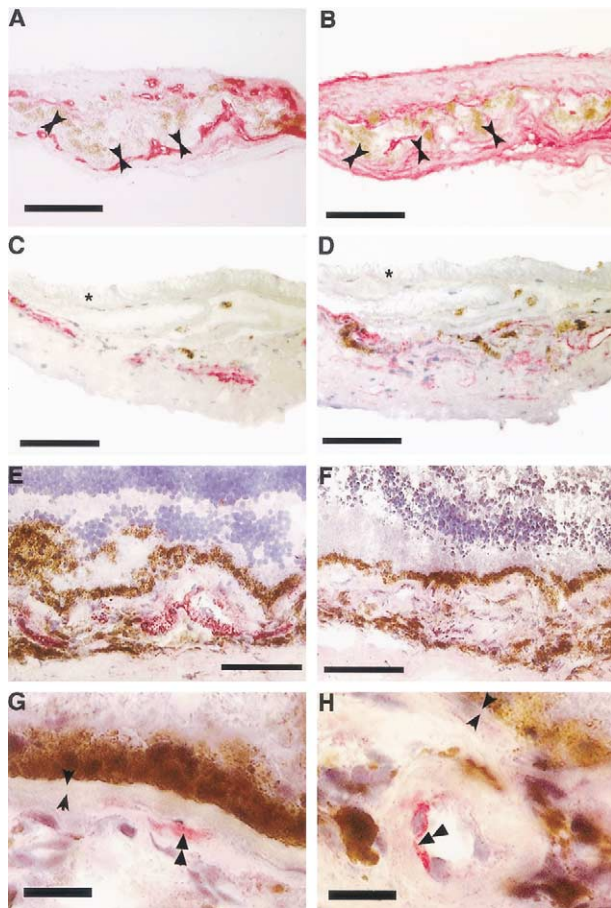


FIGURE 1. Representative photomicrographs after immunohistochemistry of serial sections of two neovascular membranes, excised from patients with age-related macular degeneration (A–D) and of the donor eye tissues (E–H) stained for factor VIII-related antigen (A, C, and E) and for ED-B-containing fibronectin (B, D, F, G, and H). Note the different staining patterns of neovascular membranes: diffuse (B) and localized around vessels (D). Reaction product is shown in red. Counterstain: Gill hematoxylin. Bars represent 100 μm (A–F) or 50 μm (G and H). The Bruch membrane is indicated between arrows (A, B, G, and H), the asterisk shows photoreceptor fragments (C and D), while double arrows indicate CGS-1 positive cells in the choroid of the donor eyes (G and H).

identical in humans and mice).²³ B-fibronectin is virtually undetectable in normal adult tissues¹⁶ (except for the regenerating endometrium) but is upregulated during fetal development, wound healing, and tumor growth.^{36,37} Previous studies demonstrated that B-FN expression is upregulated in healing corneal wounds,^{18,19} and still another study showed FN overexpression in healing areas of previous retinal laser photocoagulation.³⁸

It is known that certain FN isoforms that are expressed during wound repair subsequently disappear as the healing process evolves.³⁹ Here we show that B-FN is also present in human CNVMs, which represents a typical, nonspecific

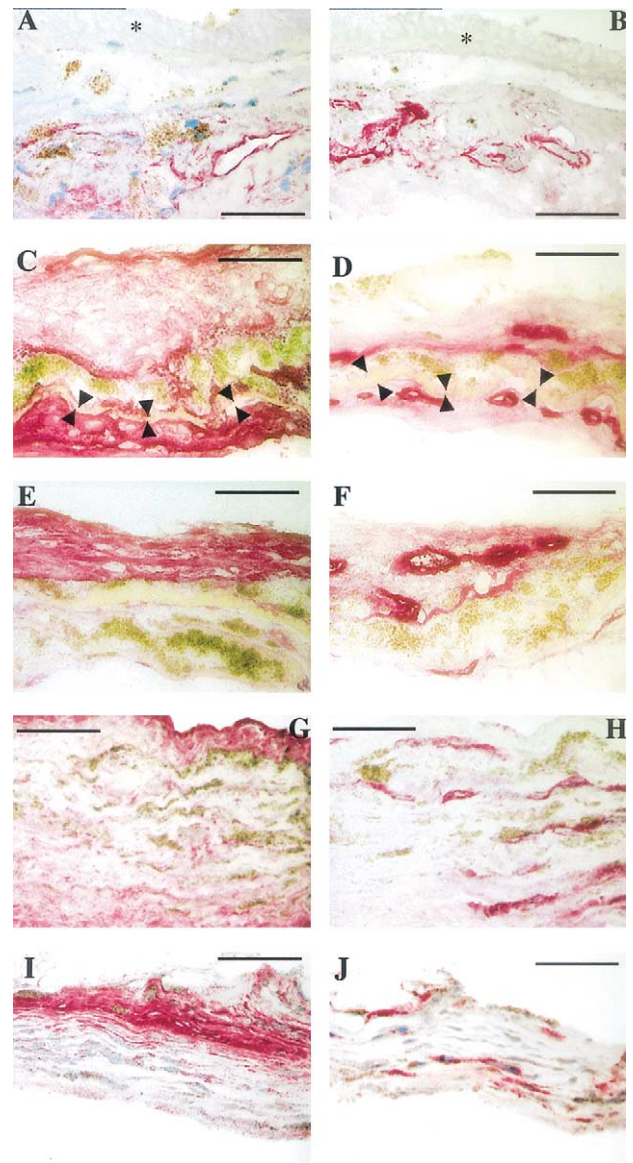


FIGURE 2. Representative photomicrographs after immunohistochemistry of neovascular membranes excised from patients with age-related macular degeneration (A–F), pathologic myopia (G and H), and multifocal choroiditis (I and J) stained for ED-B-containing fibronectin (A, C, E, G, and I) and for factor VIII-related antigen (B, D, F, H, and J). Photomicrographs A, B and C, D were taken with higher magnification from the same membrane as shown in Figure 1, C, D and A, B, respectively. Reaction product is shown in red. Counterstain: Gill hematoxylin. Bars represent 50 μm . The Bruch membrane is indicated between arrows (C and D); the asterisk shows photoreceptor fragments (A and B).

wound repair-like response to an unknown specific stimulus.⁴⁰ In tumors, ED-B is detectable only around the newly forming vessels of the tumor stroma, but not around mature, preexisting, vessels.^{15,41,42} B-fibronectin is a marker of angiogenesis, but it could also be present in

TABLE 1. Histologic Data of Surgically Excised CNVMs

CNVM	Diagnosis	Age (years)	Laterality	Gender	BM	RPE*	CGS-1 Staining†
1	AMD	57	OS	F	Absent	Dispersed	3
2	AMD	73	OS	F	Present	In line on BM/dispersed	2
3	AMD	80	OD	M	Absent	Few dispersed	2
4	AMD	84	OS	F	Present	In line on BM/dispersed	2
5	CMF	27	OD	F	Present	Dispersed	3
6	CMF	32	OS	F	Present	Dispersed/in line at edge	3
7	CMF	35	OS	F	Absent	In line at edge/dispersed	2
8	CMF	54	OD	F	Present	In line at edge	3
9	Myopia	36	OS	F	Absent	Dispersed	2
10	Myopia	38	OD	F	Absent	Dispersed	0
11	Myopia	41	OD	F	Absent	Dispersed	3
12	Myopia	44	OD	M	Absent	Dispersed	3
13	Myopia	50	OS	F	Absent	Dispersed	2
14	Myopia	53	OS	M	Absent	Dispersed	2
15	Myopia	72	OS	F	Present	Dispersed	3

AMD = age-related macular degeneration; BM = Bruch membranes; CMF = multifocal chorioretinitis; CNVM = choroidal neovascular membrane; F = female; M = male; myopia = degenerative myopia; OD = right eye; OS = left eye.

*Distribution of the retinal pigment epithelial cells (RPE).

†CGS-1 staining is expressed qualitatively (0: no staining; 1: weak staining; 2: strong, focal staining; 3: strong and diffuse staining).

TABLE 2. CGS-1 Staining of Donor Eyes

Eye	Drusen	Age (years)	Laterality†	Gender	BM	RPE*	CGS-1 Staining†	
							Retina	Choroid
1	H/S	93	OD	F	Thicker	A/H	0	1
2	H/S	93	OS	F	Thicker	A/H	0	1
3	H	78	OD	F	Thicker	A/H	0	1
4	H	78	OS	F	Thicker	A/H	0	1
5	H	76	OD	M	Thicker	A/H	0	1
6	H/S	76	OS	M	Thicker	A/H	0*	1
7	Nd	55	OD	F	Normal	A/H	0	1
8	Nd	55	OS	F	Normal	A/H	0	1

BM = Bruch membrane; F = female; M = male; H = nodular drusen present; S = soft drusen present; Nd = not detected; OD = right eye; OS = left eye.

*Distribution of the retinal pigment epithelium cells (RPE): A, atrophy of RPE; H, hypertrophy of RPE.

†CGS-1 staining is expressed qualitatively (0 = no staining; 1 = weak staining in some cells; 2 = strong, focal staining; 3 = strong and diffuse staining).

*One vessel stained positively.

tissues undergoing ECM remodeling. This could explain the diffuse staining found in some of the membranes.

We found one membrane from a patient with PM that stained negatively for B-FN, even though factor VIII-positive endothelial cells were present. It is generally recognized that B-FN is expressed in the ECM undergoing remodeling during angiogenesis and that its expression is downregulated as the matrix matures.¹⁸ In this study we did not examine other ECM components

(collagens, laminin, elastin, and so forth), whose presence could provide further insight into the maturation stage of these membranes. Although the patients enrolled in the study showed symptoms of recent disease onset and the CNVMs were small in size, we cannot rule out—without precise data on the duration of these membranes—that the negatively stained membrane from the myopic patient was actually relatively older scar tissue.

Our results demonstrating the presence of B-FN in human CNVMs further uphold the concept that the isoform is a marker of angiogenesis. Our findings that B-FN is predominantly expressed in the neovascular tissue and that it is practically absent in normal adult tissues make the protein a potential candidate for the targeted delivery of antiangiogenic drugs. In a recent experimental study, newly developed corneal vessels in rabbits were shown to express B-FN, and vessels were successfully coagulated with a photosensitizer coupled to an scFv against B-FN without damaging the neighboring tissues.²⁴ Using engineered antibodies for the targeted delivery of photosensitizers to CNVMs may further improve PDT outcomes, by enhancing the target to background ratio of the photosensitizer. Age-related macular degeneration and other choroidal neovascular disorders could greatly benefit from this therapeutic approach in the future. We are planning to conduct further studies in this direction.

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