CHAPTER 7

INSULIN-LIKE GROWTH FACTOR-BINDING PROTEINS ARE EXPRESSED IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Purpose: The insulin-like growth factor-I (IGF-I) protein is a growth promoting polypeptide that can act as an angiogenic agent in the eye. Earlier we detected IGF-I and type 1 IGF receptor in neovascular AMD. The diverse activities of IGF-I are mediated through binding and activation of the type I IGF receptor. IGF-I is usually bound to one of the six major IGF-binding proteins (IGFBPs). These complexes prolong half-life in the circulation and function as a reservoir for IGF-I. Furthermore most IGFBPs have actions that are independent of IGF-I binding. The purpose of our study is to localize the expression of IGFBP proteins and mRNA *in situ* in the normal human eye and in eyes with neovascular age-related macular degeneration (AMD).

Methods: Formalin-fixed, paraffin-embedded slides of 4 normal control eyes and 17 eyes with choroidal neovascularization (CNV) secondary to AMD were used. IGFBP mRNA was detected by *in-situ* hybridization with digoxigenin-labeled RNA probes, and IGFBP proteins were studied by immunohistochemistry.

Results: In the normal retina, we found mRNA of IGFBP-2 and IGFBP-4; mRNA of IGFBP-1 was not detected. IGFBP-1, -2, -3, -4 and -6 were immunolocalized throughout the eye in a spatially differentiated pattern. In eyes with CNV we localized mRNA of IGFBP-2 and -4 in CNV resulting from AMD, as well as most IGFBP proteins, in vascular endothelial cells, RPE cells and fibroblasts.

Conclusions: The finding of these members of the IGF family may indicate a role of the IGF family in the pathogenesis of neovascular AMD, since IGF-I is involved in angiogenesis. The functional role of the various IGF family members in AMD needs to be established.

INTRODUCTION

Neovascular age-related macular degeneration (AMD) is characterized by choroidal neovascularization (CNV), in which newly formed vessels from the underlying choroid grow beneath the retinal pigment epithelium (RPE) and the retina. CNV may cause (sub) acute blindness because of bleeding or scar formation.[1] Although the morphology of angiogenesis in CNV secondary to AMD has been described in detail, the pathogenesis is still poorly understood.

Growth factors are acknowledged to play an important role in retinal neovascularizations. Vascular endothelial growth factor (VEGF), an endothelial specific mitogen, is regarded as one of the most important ocular angiogenic factors, especially under hypoxic circumstances.[166,251] Other angiogenic factors in ocular neovascularization include basic fibroblast growth factor, transforming growth factor-beta (TGF-β), platelet derived growth factor and insulin-like growth factor-I (IGF-I).[144,251]

In an earlier study we detected production of IGF-I and the type 1 IGF receptor (IGF-IR) in the normal eye and in eyes with neovascular AMD [manuscript submitted]. IGF-I is a growth promoting polypeptide that has mitogenic and differentiating effects on many cell types, among which ocular cell types.[171,172,174,252,253] In animal models, IGF-I is associated with ocular angiogenesis.[177,178]. Furthermore, *in vitro* IGF-I can act as a direct angiogenic factor on retinal endothelial cells,[173] or indirectly through increased VEGF gene expression in cultured RPE cells.[156] The diverse activities of IGF-I are mediated through binding and activation of IGF-IR. Intravascularly and in the extracellular space, IGF-I is usually bound to one of the IGF-binding proteins (IGFBP). Six major IGFBPs are described currently. Most IGFBPs have additional actions that are independent of IGF-I binding, including inhibition or enhancement of cell growth and induction of apoptosis (reviewed by Baxter[167]). IGF-I and the IGFBPs are mainly produced by the liver, however they are also synthesized locally by most tissues, where they act in an autocrine or paracrine manner.[168] In many situations on pathological growth, multiple components of the IGF system may be dysregulated.[169]

The purpose of this study is to explore mRNA and protein expression of the six IGFBPs *in situ* in normal human eyes and in eyes with CNV.

MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

Patients

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. Four enucleated eyes without ischemic disease (enucleated for other reasons) were used to study the normal human eye. Term placental tissue was used as positive control.[277] Seventeen eyes (8 enucleated eyes, 4 donor eyes and 5 surgically removed subretinal neovascular membranes) of 16 patients with neovascular AMD were used. The clinical and histological diagnosis of the eyes and the classification of CNV are described in Table 7.1. The eyes were processed for routine diagnostic procedures by fixation in 10% buffered formaldehyde and were embedded in paraffin. Five-µm sections were prepared for *in-situ* hybridization and immunohistochemistry.

RNA probes and antibodies

The cDNA containing plasmids for human IGFBP-1 to 6 were kindly provided by Shunichi Shimasaki (Department of Reproductive Medicine, University of California, La Jolla, CA, USA). Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) using T7 RNA polymerase for antisense and T3 RNA polymerase for sense probes.

Goat polyclonal antibodies against IGFBP-1 to 6 (respectively M-19; M-18; M-19; C-20; C18; M20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies against pankeratin (monoclonal mouse antibodies; 1:100 dilution); factor VIII (monoclonal mouse antibodies; 1:50 dilution) and glial fibrillary acidic protein (GFAP; polyclonal rabbit antibodies; dilution 1:200) were obtained from DAKO (Glostrup, Denmark). Monoclonal mouse antibodies against smooth muscle actin (SMA; 1:150 dilution) were obtained from Biogenex (San Ramon, CA, USA).

In-situ hybridization

Sections were deparaffinized with xylene and rehydrated. Before hybridization, the slides were incubated in the following solutions: 0.2 N HCL, 0.3% Triton X-100 in phosphate-buffered saline (PBS), RNase-free proteinase K (5 µl/ml for 20 minutes at 37°C), 4% formaline in PBS, acetic anhydride in 0.1 M triethanolamine, and 2xSSC.

TABLE 7.1 DIAGNOSIS OF STUDIED EYES AND HISTOLOGICAL CLASSIFICATION OF CNV

No	Age/ sex	OD / OS	Clinical description	Histological classification CNV		
				subret/ sub-RPE	FV/FC	other characteristics
Co1	83/M	OS	corneal ulcer			
Co2	57/M	OS	recurrent conjunctival melanoma			
Co3	42/M	OS	choroidal melanoma			
Co4	69/F	OD	orbital metastasis, post irradiation			
CNV1	79/M	u	surgically excised CNV	mixed	FV + FC	hemorrhage
CNV2	79/F	u	surgically excised CNV	subretinal	FV + FC	hemorrhage
CNV3	76/F	OD	surgically excised CNV	subretinal	FV	BLD
CNV4	79/M	u	surgically excised CNV	mixed	FV + FC	BLD, hemorrhage
CNV5	78/M	u	surgically excised CNV	sub-RPE	FV + FC	BLD, confluent soft drusen
CNV6	72/M	OS	disciform MD	mixed	FV + FC	BLD, hemorrhage
CNV7	86/M	OS	disciform MD, acute glaucoma	sub-RPE	FV + FC	BLD, hemorrhage, retinal detachment; posterior uveitis
CNV8	91/M	OS	disciform MD, donor eye	mixed	FC	disciform MD, BLD
CNV9	87/M	OS	disciform MD, donor eye	mixed	FV + FC	disciform MD, BLD
CNV10	83/M	OD	painful eye, suspected uveal melanoma	mixed	FV + FC	ischemic retinal disease; disciform MD, BLD, hemorrhage
CNV11	73/M	OS	disciform MD	subretinal	FV + FC	
CNV12	73/M	OD	disciform MD, post irradiation	subretinal	FV	
CNV13	82/M	OD	disciform MD	mixed	FC	confluent soft drusen
CNV14	85/F	OS	post surgical endophthalmitis	subretinal	FV	endophthalmitis, uveitis
CNV15	84/F	OD	expulsive hemorrhage preoperatively	subretinal	FV	hemorrhage
CNV16	80/F	OS	disciform MD, hemorrhage, secondary glaucoma	mixed	FV + FC	BLD, hemorrhage
CNV17	84/F	OS	disciform MD	mixed	FV + FC	BLD, hemorrhage

OD = right eye; OS = left eye; FV = fibrovascular; FC = fibrocellular; PDR = proliferative diabetic retinopathy; u = unknown; MD = macular degeneration; BLD = basal laminar and linear deposits.

The slides were preincubated in 50% formamide in 2xSSC at 37ºC. For hybridization, antisense and sense probes were diluted in hybridization solution (50% deionized formamide, 10% dextran sulphate, 4xSSC (IGFBP-2 and IGFBP-4) or 2xSSC (IGFBP-1), 1xDenhardt's solution, 1 µg/ml tRNA, 250 µg/ml herring sperm RNA) to a concentration of 400 ng/ml, and incubated at 68ºC for 30 minutes. The

hybridization solution was then layered onto the sections and hybridized overnight at 55ºC in a humid chamber. Posthybridization washes were performed at 45ºC for 30 minutes in the following solutions: 50% formamide in 2xSSC, 50% formamide in 1xSSC, 0.1xSSC. The slides were incubated with RNAse T1 (2 U/ml) in 2xSSC/1mM EDTA in 37ºC for 15 minutes and washed at 45ºC with 1xSSC and at room temperature with 2xSSC. The digoxigenin-labeled hybrids were detected by antibody incubation performed according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) with following modifications. A 1:1000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase was used for a 2.5 hour incubation overnight at 4ºC. Afterwards, an extra washing step of 0.025% Tween in Tris-buffered saline (pH 7.5) was introduced. For staining, sections were layered with detection buffer (0.1 M Tris-HC, 0.1 M NaCl, 0.05 M $MgCl_2$ pH 9.5) containing NBT (4-nitroblue tetrazolium chloride), BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (both from Vector, Burlingame, CA, USA) and 6% polyvinylalcolhol (m.w. 31.000-50.000, from Aldrich Chemical Milwaukee, WI, USA). The color reaction was performed in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Sections were washed in 10 mM Tris-HCl, 1 mM EDTA pH 8.0, counterstained with nuclear red solution, dehydrated with ethanol gradients and mounted. The slides were examined by light microscopy. Staining of the antisense probe was scored positive if the sense probe scored negative in the same region. Intensity of staining between slides could not be used as reliable quantitative criterion because of possible differences in fixation time.

Immunohistochemistry

The sections were deparaffinated and rehydrated. Antigen retrieval was performed for the IGFBP-6 and pancytokeratin antibodies (microwave-heating for 10 minutes), and for the factor VIII antibodies (pronase treatment for 20 minutes at 37°C). Immunohistochemistry with the IGFBPs was performed as follows. After blocking with 1,5% normal rabbit serum (Dako) in PBS for 30 minutes, the slides were incubated with the primairy antibodies (1:150 for IGFBP-1, IGFBP-2, and IGFBP-4; 1:100 for IGFBP-3 and IGFBP-6) overnight at 4°C. The sections were further incubated with biotinylated rabbit-anti-goat antibodies (Dako) for 1 hour, followed by alkaline phosphatase-labeled antibiotin (Dako) for 30 minutes. For immunohistochemistry with Factor VIII, SMA, GFAP and pankeratin, slides were blocked with normal goat serum in PBS (Dako, 1:10) for 15 minutes. The primairy antibodies were incubated for 1 hour at room temperature, followed by biotinylated multilink antibodies for 30 minutes. The avidin-biotin complex was visualized by incubation with new fuchsin (as a red chromogen) for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. Negative controls for immunohistochemistry included 1) omission of the

primary antibody and 2) preabsorbtion of the antibodies with a tenfold of the immunizing IGFBP peptide (Santa Cruz) for 4 hours.

RESULTS

In two eyes with neovascular AMD, (Table 7.3: CNV 8 and CNV13) no mRNA could be detected with digoxigenin-labeled mRNA *in-situ* hybridization, regardless of the probe. All mRNA appeared to be lost in these eyes. These eyes were considered as 'not classifiable'.

IGFBP-1

The sense probe for IGFBP-1 mRNA showed light aspecific staining in the retina, and negative controls for IGFBP-1 immunohistochemistry showed no staining. In the normal eye (Table 7.2, Figure 7.1A,D), mRNA for IGFBP-1 could not be detected. IGFBP-1 protein was found in all nuclear and plexiform layers of the neuroretina, including photoreceptor inner segments, in the RPE at the basal side. Retinal and choroidal vessels stained positive, choriocapillary cells incidentally stained positive. Lens epithelium, non-pigmented epithelium of the ciliary body and corneal layers also stained for IGFBP-1 protein.

In eyes with CNV (Table 7.3, Figure 7.2A,D), faint staining for IGFBP-1 mRNA was only detected in one sample. All CNVs showed positive immunohistochemical staining in capillary endothelial cells, RPE and/or fibroblasts.

IGFBP-2

The sense probe for IGFBP-2 mRNA, and negative controls for IGFBP-2 immunohisto-chemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1B,E), we found IGFBP-2 mRNA in the ganglion cell layer, inner nuclear layer, photoreceptor layer and RPE. IGFBP-2 protein was found with higher intensity but in the same pattern as IGFBP-1, that is in all nuclear and plexiform layers of the neuroretina, including photoreceptor inner segments, in the RPE and in the choriocapillaris. Lens epithelium, non-pigmented epithelium of the ciliary body and corneal layers also stained for IGFBP-2 protein.

In CNV (Table 7.3, Figure 7.2B,E) IGFBP-2 mRNA was found in 9 out of 14 CNV, localized in endothelial cells, RPE or fibroblasts. Nearly all cellular components of CNV, including capillaries, RPE and fibroblasts, stained intensely positive for IGFBP-2 protein.

TABLE 7.2 EXPRESSION OF IGFBP MRNA AND PROTEIN IN CONTROL EYES

	IGFBP-1		IGFBP-2		IGFBP-3		IGFBP-4		IGFBP-6	
	RNA	**prot**	**RNA**	**prot**	**RNA**	**prot**	**RNA**	**prot**	**RNA**	**prot**
GGL	-	++	++	+		++	++	++		+
INL	-	+	+	++		+	++	+		-
ONL	-	+	-	++		-	+	-		-
PHR	-	++	++	++		++	++	++		+
RPE	-	++	+	++		++	++	++		+
ChC	-	+	-	+		-	+	+		-

Expression of mRNA (RNA) and protein (prot) expression: - = no expression; + = moderate expression; ++ = intense expression. NFL = neural fiber layer; GGL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; PHR = photoreceptor layer; RPE = retinal pigment epithelium; ChC = choriocapillaris. IGFBP-3 and –6 mRNA in-situ hybridization was not performed.

IGFBP-3

mRNA for IGFBP-3 was not determined because of technical problems with the probe. Negative controls for IGFBP-3 immunohistochemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1F), we found IGFBP-3 protein in the ganglion cell layer, the plexiform layers, the photoreceptor inner segments and in the RPE. In the RPE, staining was localized to the basal side. Retinal and choroidal vessels were positive, while the choriocapillaris stained incidentally. Often intravascular fluid stained positive. Lens epithelium was negative, and the cornea and non-pigmented epithelium of the ciliary body showed only slight staining.

In CNV (Table 7.3, Figure 7.2F), variable protein staining was found in newly formed vessels and in fibroblasts. RPE cells stained positive in all CNV.

IGFBP-4

The sense probe for IGFBP-4 mRNA, as well as negative controls for IGFBP-1 immunohistochemistry, showed no staining. In the normal retina (Table 7.2, Figure 7.1C,G), we found IGFBP-4 mRNA in the neuroretina, the RPE and in the corneal layers. IGFBP-4 protein was detected in the nerve fiber layer, ganglion cell layer, inner nucelar layer, the photoreceptor inner segments and in the RPE. In the RPE, staining was localized to the basal side. Positive staining was also found in large choroidal vessels and retinal vessels. Lens epithelium was positive, as was corneal endothelium and non-pigmented epithelium of the ciliary body. Stroma of the choroid, iris and sclera stained intensely positive.

In CNV (Table 7.3, Figure 7.2C,G), IGFBP-4 mRNA was detected in endothelial cells, RPE, fibroblasts and other cells. IGFBP-4 protein staining was found mostly in RPE and fibroblasts, and to a lesser extend in vascular endothelial cells. The stroma of most CNV stained intensely.

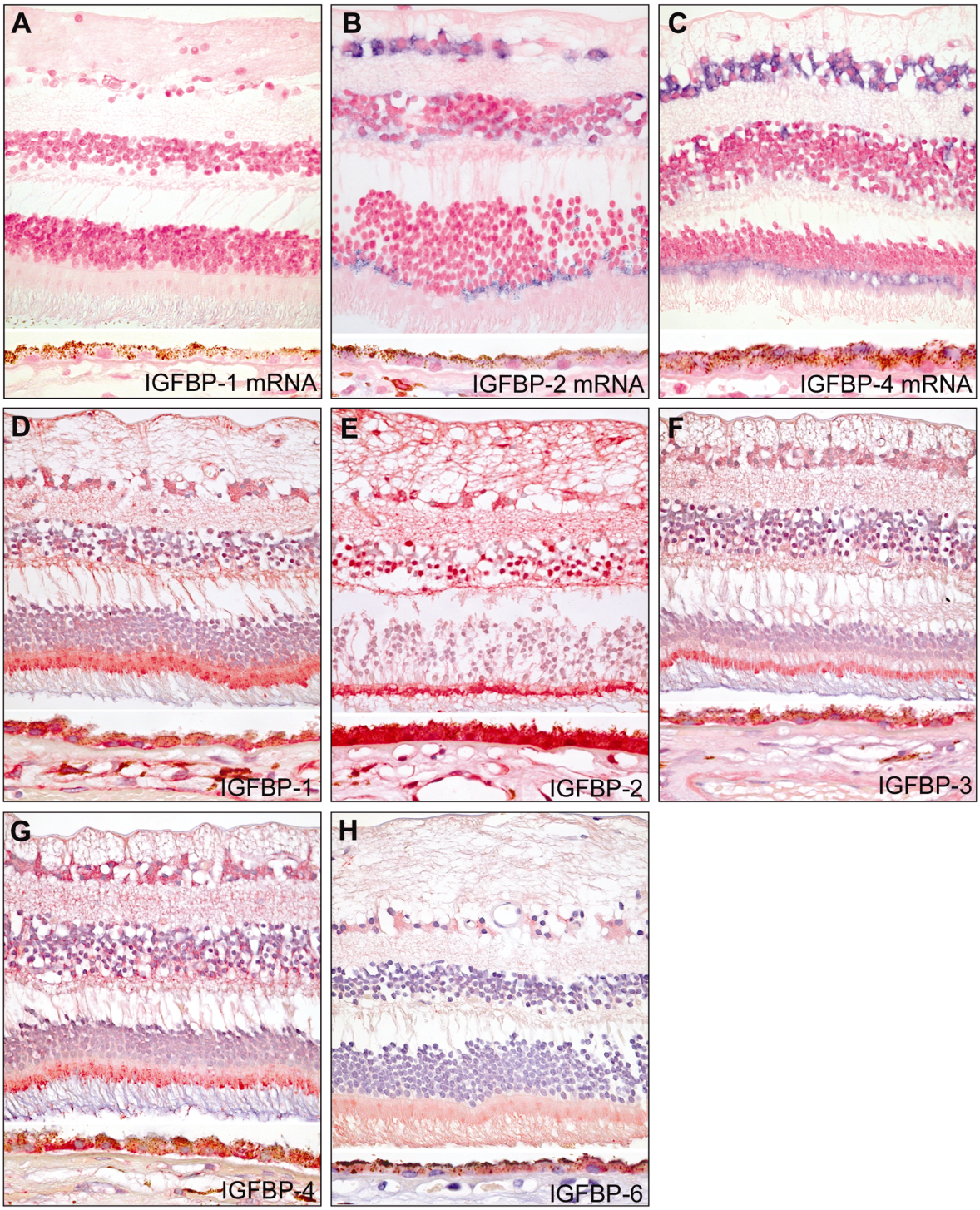


Figure 7.1 ***Localization of IGFBPs in the posterior pole of the human eye****. Expression of IGFBP mRNA was detected in paraffin-embedded tissue by in-situ hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGFBP proteins was detected in paraffin-embedded tissue with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin.* ***(A)*** *IGFBP-1 mRNA****;*** ***(B)*** *IGFBP-2 mRNA;* ***(C)*** *IGFBP-4 mRNA.* ***(D)*** *IGFBP-1 protein;* ***(E)*** *IGFBP-2 protein;* ***(F)*** *IGFBP-3 protein;* ***(G)*** *IGFBP-4 protein;* ***(H)*** *IGFBP-6 protein. mRNA for IGFBP-3, -5 and -6 were not determined because of technical problems; neither was IGFBP-5 protein. Original magnification X400.*

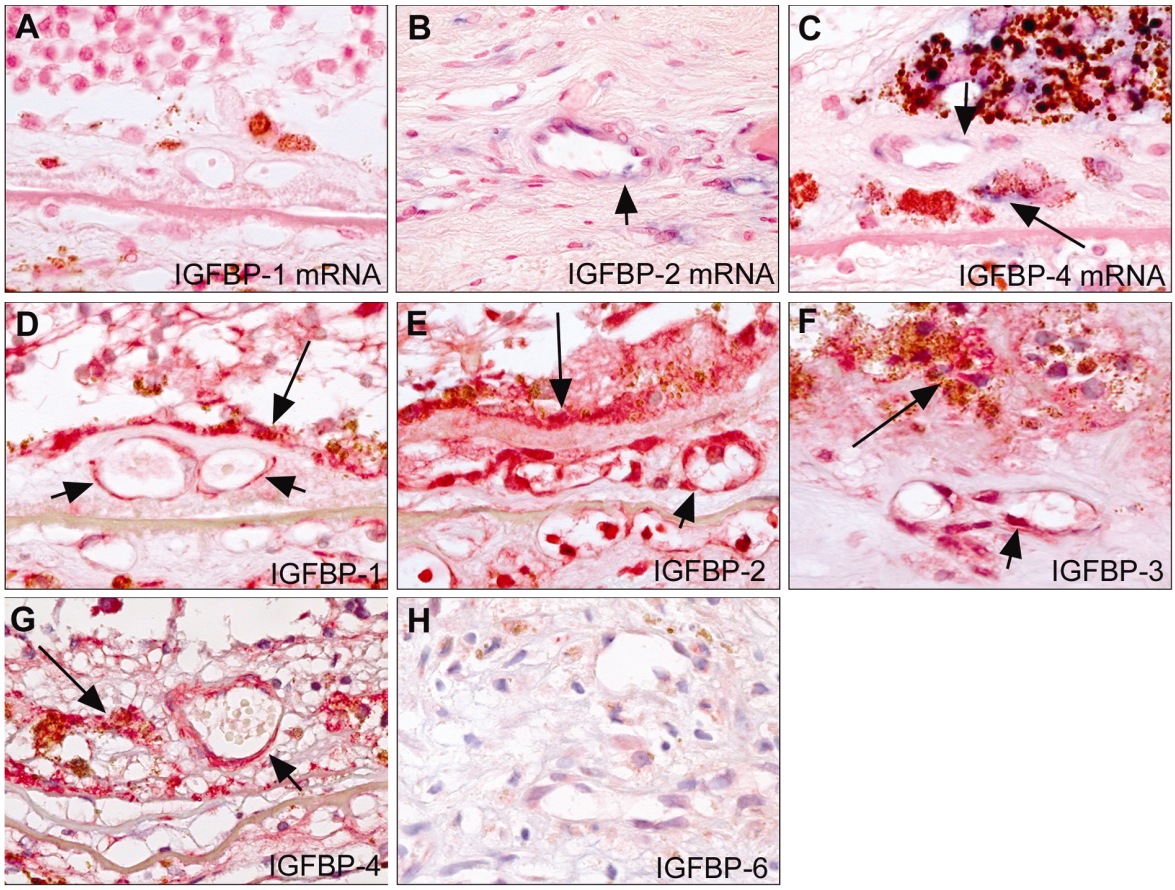


Figure 7.2. ***Localization of IGFBPs in CNV secondary to AMD****. Expression of IGFBP mRNA was detected in paraffin-embedded tissue by in-situ hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGFBP proteins was detected in paraffin-embedded tissue with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin.* ***(A)*** *IGFBP-1 mRNA in CNV11;* ***(B)*** *IGFBP-2 mRNA in CNV 11;* ***(C)*** *IGFBP-4 mRNA in CNV12;* ***(D)*** *IGFBP-1 protein in CNV11;* ***(E)*** *IGFBP-2 protein in CNV11;* ***(F)*** *IGFBP-3 protein in CNV1;* ***(G)*** *IGFBP-4 protein in CNV9;* ***(H)*** *IGFBP-6 protein in CNV2. mRNA for IGFBP-3, -5 and -6 were not determined because of technical problems; neither was IGFBP-5 protein. Original magnification X400.*

IGFBP-5

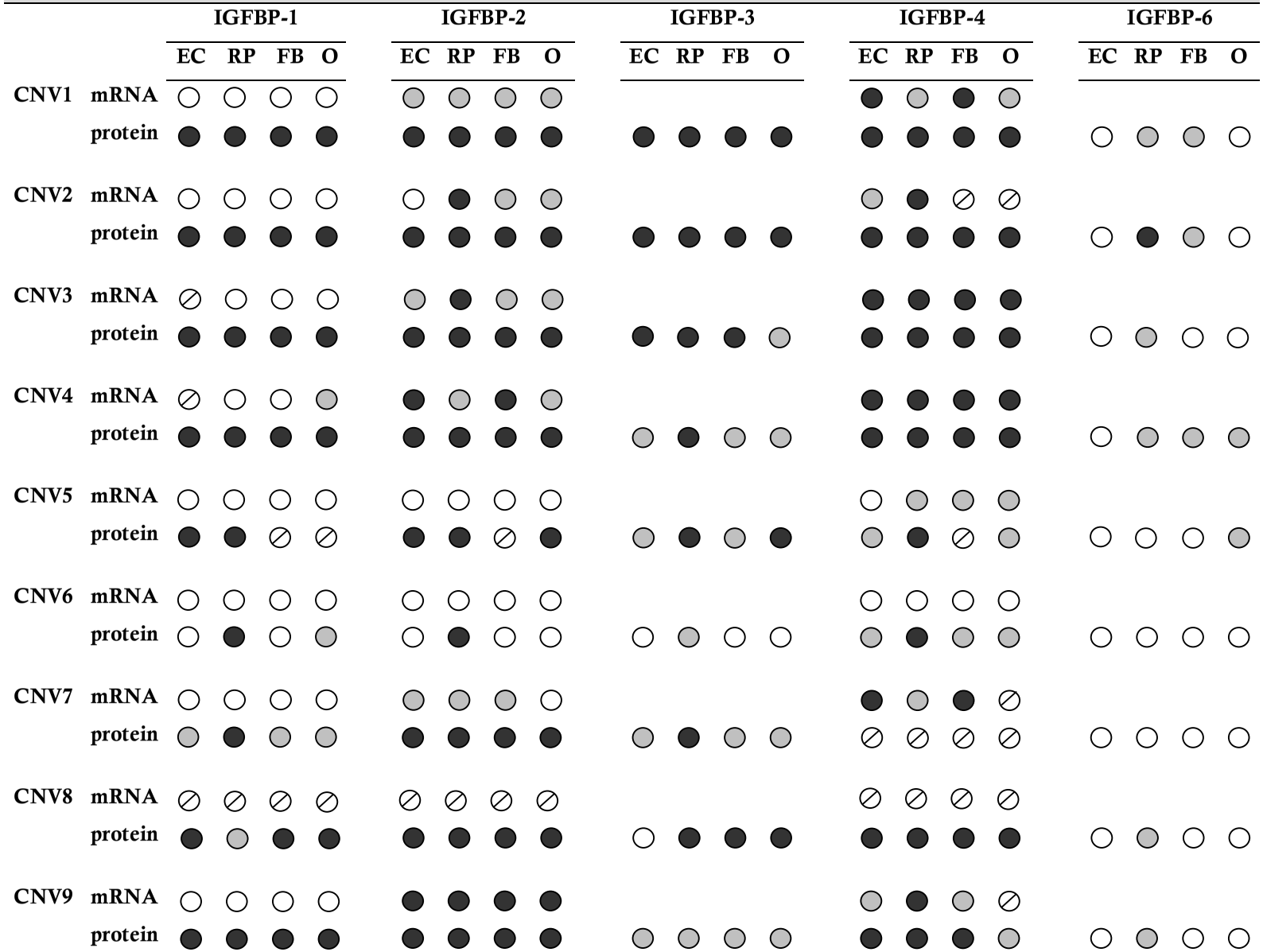
mRNA and protein for IGFBP-5 were not determined because of technical problems with the probe and the antibodies.

IGFBP-6

mRNA for IGFBP-6 was not determined because of technical problems with the probe. Negative controls for IGFBP-6 immunohistochemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1H), we found little IGFBP-6 protein in the ganglion cell layer and the photoreceptor inner segments, and some staining in the RPE. Non-pigmented epithelium of the ciliary body showed slight staining.

In CNV (Table 7.3, Figure 7.2H) only some RPE and fibroblasts showed positive staining for IGFBP-6 protein.

TABLE 7.3 MRNA ANP PROTEIN EXPRESSION OF IGFBPS IN NEOVASCULAR AMD



		IGFBP-1				IGFBP-2				IGFBP-3				IGFBP-4				IGFBP-6			
		EC	RP	FB	O	EC	RP	FB	O	EC	RP	FB	O	EC	RP	FB	O	EC	RP	FB	O
CNV10	mRNA	○	○	○	○	◔	◔	○	○					◔	◔	◔	◔				
	protein	◔	●	◔	◔	●	●	●	●	○	○	◔	◔	○	◔	◔	◔	○	○	○	○
CNV11	mRNA	○	○	○	○	◔	●	●	○					⊘	⊘	⊘	⊘				
	protein	●	●	●	●	●	●	●	●	●	●	●	◔	●	●	●	◔	○	○	○	○
CNV12	mRNA	○	○	○	○	◔	◔	○	○					◔	●	◔	◔				
	protein	●	●	●	●	●	●	●	●	●	●	●	●	◔	●	◔	◔	○	○	○	○
CNV13	mRNA	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘					⊘	⊘	⊘	⊘				
	protein	●	●	●	●	●	●	●	●	◔	◔	◔	◔	○	●	◔	○	○	○	○	○
CNV14	mRNA																				
	protein	◔	◔	◔	○	◔	◔	◔	○	○	◔	○	○	○	◔	⊘	⊘	○	◔	◔	○
CNV15	mRNA																				
	protein	◔	◔	◔	○	◔	◔	◔	○	○	◔	○	○	○	◔	◔	⊘	⊘	⊘	⊘	⊘
CNV16	mRNA	○	○	○	○	○	○	○	○					◔	○	○	◔				
	protein	●	●	●	○	●	●	●	◔	◔	◔	◔	◔	◔	●	◔	◔	○	○	○	○
CNV17	mRNA	⊘	○	⊘	○																
	protein	◔	●	●	◔	●	◔	●	●	○	●	◔	○	○	◔	◔	○	○	◔	○	○

Categories of expression: ○ *= no expression;* ◔ *= 1 to 50% of cells;* ● *= 51 – 100% of cells. EC = endothelial cells; RP = retinal pigment epithelium; FB = fibroblast-like cells; O = other cell types.* ⊘ *= not classifiable; open spaces indicate not performed*

DISCUSSION

In the normal retina, including RPE, we localized mRNA expression of IGFBP-2 and 4. The localization is consistent with earlier findings of *in-situ* studies.[278,279] In contrast, in adult rat retina, mRNA was not detected for IGFBP-2 and IGFBP-4.[261] This difference can be explained by difference in species, or in different sensitivity of used techniques.

We did not detect IGFBP-1 mRNA in the normal eye, consistent with earlier findings.[261,280] However, the presence of IGFBP-1 mRNA can not be excluded in the eye, since in general only a part of total mRNA is detected with digoxigenin-labeled mRNA *in-situ* hybridization in paraffin-embedded material.[281]

IGFBP proteins were found in a spatially differentiated pattern in the normal human eye. IGFBP-2 was detected in a more extensive pattern than described by Miyamura and coworkers.[279] This can partly be explained by the shorter intervals between enucleation and fixation of our material, because most specimens we studied were surgically enucleated eyes or excised CNV, while Miyamura used donor eyes with postmortem times ranging from 4 to 21 hours. Furthermore different techniques and different antibodies used could account for the difference in expression. IGFBP-3 was also found in intravascular fluid, consistent with the fact that IGFBP-3 is the most abundant IGFBP in serum, and binds more than 95% of the IGF.[282] The circulating IGF-I/IGF-BP complexes limit access of IGF-I to specific tissues and to the IGF receptor and also function as a reservoir for IGF-I. Intravascularly, the IGF-I/IGFBP3 dimer forms a complex with the acid-labile subunit, resulting in a prolonged half-life of several hours. When released from this complex, IGF-I can enter target tissues with help of other binding proteins.[168]

Little IGFBP-6 was detected in the normal eye. This could be explained by the higher affinity of IGFBP-6 to IGF-II than to IGF-I.[283] IGF-II expression in the eye is low,[278] and IGF-II is important in fetal growth but has no effect on postnatal somatic growth.[284]

The function of IGFBPs in the normal eye is still unclear. The presence in the outer retina suggests a role in the maintenance of normal physiology of the retina.[266]

In CNV secondary to ARM we found mRNA of IGFBP-2 and IGFBP-4, and IGFBP-1, -2, -3, -4 proteins, and rarely IGFBP-6 protein localized in endothelial cells, RPE cells and fibroblasts. Earlier we detected IGF-I and IGF-IR in neovascular AMD. Since IGF-I has the capability of stimulating angiogenesis in the eye,[170] its presence could indicate a function in the pathogenesis of neovascular AMD. The presence of IGFBPs strengthen this conclusion, since IGFBPs are major modulators of IGF-I function. The interaction of IGF-I with one of the six major IGFBPs generally blocks receptor activation.[167] Besides inhibition of IGF-I action, some of the IGFBPs

(IGFBP-1, IGFBP-5 and mostly IGFBP-3) have paradoxical stimulatory effect on IGF activity.[167] Furthermore, some IGFBPs have actions that are independent of IGF-RI binding. IGFBP-1 stimulates cell migration through interaction with the $\alpha_5\beta_1$-integrin (fibronectin receptor).[285] IGFBP-3 can inhibit the growth of cells independent of IGF-IR,[286] and induces apoptosis.[287] IGFBP-3 has a number of ligands in addition to IGF-I, such as plasminogen and certain cell-surface and matrix components.[167] Finally, at least some IGF-R1 independent actions of IGFBP-3 and IGFBP-5 are assumed to be mediated through signaling receptors located on the plasma membrane of target cells, of which the type V TGF-β receptor may be one.[288]

The influence of the various members of the IGF-I family on CNV needs to be established. Several authors suggest hypoxia or relative hypoxia as a stimulus in the pathogenesis of neovascular AMD.[74,144] Averbukh and coworkers showed that both hypoxia and relative hypoxia may cause IGF system stimulation in the retina of a neonatal rat model, through upregulation of IGF-IR and IGFBPs.[289] This stimulation may result in neovascularization, suggesting that the IGF system may play an important role in angiogenesis induced by relative tissue hypoxia.[289]

In conclusion, we localized mRNA of IGFBP-2 and -4 in CNV resulting from AMD, as well as most IGFBP proteins. The mRNA and proteins were detected in newly formed vessels, in RPE and in fibroblasts. Together with the finding of IGF-I and its receptor in CNV membranes, the presence of these members of the IGF family may indicate a role of the IGF family in the pathogenesis of neovascular AMD. The functional role of the various IGF family members in the pathogenesis of AMD needs to be established.