Part III

Molecular Aspects of Neovascular Age-related Macular Degeneration

CHAPTER 6

INSULIN-LIKE GROWTH FACTOR-I AND ITS RECEPTOR 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. The purpose of our study is to localize the expression of IGF-I and its receptor (IGF-IR) mRNA and IGF-IR protein *in situ* in the normal human eye and to examine the presence of expression in eyes with neovascular age-related macular degeneration (AMD).

Methods: Formalin-fixed, paraffin-embedded slides of 4 normal control eyes, 14 eyes with choroidal neovascularization (CNV) secondary to AMD were used. Three eyes with proliferative diabetic retinopathy were studied as positive control. IGF-I and IGF-IR mRNA was detected by *in-situ* hybridization with digoxigenin-labeled RNA probes. IGF-IR protein was studied by immunohistochemistry.

Results: In the normal retina, IGF-I and IGF-IR mRNA expression was found throughout the neuroretinal layers, in the retinal pigment epithelium (RPE) and in some choriocapillary and retinal capillary endothelial cells. In eyes with CNV we found IGF and IGF-IR mRNA in capillary endothelial cells, some transdifferentiated RPE, and fibroblast-like cells.

IGF-IR protein was found in normal eyes in all neuroretinal layers, in the RPE, and in the choroidal vessels. In eyes with CNV, we found IGF-IR protein in the RPE monolayer, in transdifferentiated RPE and in newly formed vessels.

Conclusions: The co-localization of protein and receptor indicates an autocrine function of IGF-I in the normal human retina. Since IGF-I participates in ocular neovascularization, synthesis of IGF-IR and IGF-I in endothelial cells, RPE cells and fibroblast-like cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD.

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 neuroretina. 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, platelet derived growth factor and insulin-like growth factor-I (IGF-I).[144,251]

In an earlier study we demonstrated that most CNV in AMD express somatostatin receptor type 2A that bind potential anti-angiogenic somatostatin analogues.[248] Somatostatin receptors are present on cultured human retinal endothelial cells. Proliferation of both retinal endothelial cells[173] as well as choroidal endothelial cells[174] can be inhibited by somatostatin analogues. In mice, inhibition of IGF-I by somatostatin analogues or by downregulation of growth hormone (GH), can decrease ischemia-induced retinal neovascularization.[177] IGF-I is a growth promoting polypeptide that has mitogenic and differentiating effects on many cell types, among which are ocular vascular endothelial cells[252] as well as RPE[172,253] and neuronal cells.[171] IGF-I can act as a direct angiogenic factor on retinal endothelial cells,[173,174] or indirectly through increased VEGF gene expression of cultured RPE cells.[156]

Most studies describe the role of IGF-I in *in vitro* models[173,174,254-257] or *in situ* in diabetic retinopathy.[258,259] So far IGF-I has not been studied in ARM.

The purpose of our study is to localize the expression of IGF-I and its receptor (IGF-IR) mRNA and IGF-IR protein *in situ* in normal human eyes and to examine the presence of expression in eyes with CNV, in order to elucidate its possible role in angiogenesis in AMD.

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) and 3 enucleated eyes with proliferative diabetic retinopathy (PDR) were used as controls. Fourteen eyes (5 enucleated eyes, 4 donor eyes and 5 surgically removed subretinal neovascular membranes) of 13 patients with neovascular AMD were studied. The clinical and histological diagnosis of the eyes and the classification of CNV are described in Table 6.1. 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

The human IGF-I probe was a 258 bp fragment containing exon 2 and 3 of the IGF-I gene.[260] The human IGF-IR probe was generated using a cDNA clone with a unique insert of human IGF-IR (I.M.A.G.E. cDNA clone 150361, Research Genetics, Huntsville, AL, USA). The insert was reduced to 270 bp by restriction with BamHI and AvaI, and ligated in vectors pBluescript SK (antisense) and KS (sense) (Stratagene Europe, Amsterdam, The Netherlands). E. Coli X12Blue were transformed with these vectors and proper colonies were isolated and grown. Sequence analysis was performed to verify the inserts. Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) using T7 RNA polymerase.

In-situ hybridization

Sections were deparaffinated with xylene and rehydrated. 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 37ºC) and 4% formaline in PBS. Subsequently, acetylation was performed with acetic anhydride in 0.1 M triethanolamine. The slides were rinsed in 2x SSC (1xSSC = 150 mM NaCl and 15 mM sodium citrate) and 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 (IGF-I) or 2xSSC (IGF-IR), 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. Post hybridization washes were performed at 45ºC for 30 minutes in the following solutions: 50% formamide in 2xSSC, 50% formamide in 1xSSC, 0.1xSSC (IGF-IR) or 0.5xSSC (IGF-I). The slides were incubated with RNAse T1 (2 U/ml) in 2xSSC/1mM EDTA in 37ºC for 15 minutes and washed at 45ºC with

TABLE 6.1 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			
DM1	34/M	OS	PDR			
DM2	63/F	OD	PDR			
DM3	75/F	OD	PDR			
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
CNV16	80/F	OS	disciform MD, hemorrhage, secondary glaucoma	mixed	FV + FC	BLD, hemorrhage

CNV = choroidal neovascularization; 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.

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 the following modifications. A 1:1000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase was used for a 2.5 hour incubation at room temperature or 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.

Immunohistochemistry

Polyclonal rabbit antibodies against IGF-IR (1:750 dilution) were obtained from Research Diagnostics (Flanders, NJ, USA). Immunohistochemistry against IGF-I was not performed because of lack of adequate antibodies for paraffin-embedded material. Antibodies against pankeratine (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). Sections were deparaffinated and rehydrated. Antigen retrieval was performed for the IGF-IR and pankeratin antibodies (microwave-heating for 10 minutes), and for the factor VIII antibodies (pronase treatment for 20 minutes at 37°C). After blocking with normal goat serum (Dako, 1:10) for 15 minutes, the slides were incubated with the primary antibodies for 1 hour at room temperature or overnight at 4°C (IGF-IR). The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both Biogenex) for 30 minutes. The 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, 2) incubation with an irrelevant polyclonal rabbit antibody and 3) preabsorbtion of the IGF-IR antibodies with a tenfold of the immunizing IGF-IR peptide (Research Diagnostics) for 4 hours.

Grading of expression and statistics

The slides were examined by light microscopy. Grading of mRNA and IGF-IR protein expression was performed in a masked fashion by two authors (AL and CM) twice or once, respectively. Slides were blinded and randomly graded per cell type in 3 categories (Table 6.2): 1: no staining; 2: staining in less than 50% of cells; 3: staining in more than 50% of cells.

Positive cell types in CNV were identified using pankeratine staining (RPE cells), factor VIII staining (vascular endothelial cells), GFAP staining (neuronal cells) and SMA (myofibroblasts) in consecutive slides, combined with cellular morphology at examination by light microscopy. Cells not meeting these criteria were classified as "other".
For inter- and intra-observer variability a kappa-value was calculated.

RESULTS

In two eyes with neovascular AMD (Table 6.2: CNV 8 and CNV13) the normal retina, regarded as positive internal control, was negative with *in-situ* hybridization. All mRNA appeared to be lost in these eyes.

TABLE 6.2 EXPRESSION OF IGF-I AND IGF RECEPTOR TYPE 1 IN CNV

	IGF-I mRNA				IGF-IR mRNA				IGF-IR protein			
	EC	**RP**	**FB**	**O**	**EC**	**RP**	**FB**	**O**	**EC**	**RP**	**FB**	**O**
1. CNV1	0	0	0	1	2	2	2	2	1	1	1	1
2. CNV2	0	0	0	0	2	2	2	2	1	2	2	1
3. CNV3	1	2	1	1	2	1	1	2	1	1	0	0
4. CNV4	1	2	1	1	nc	nc	nc	nc	1	2	0	0
5. CNV5	1	1	0	1	1	2	1	1	1	2	nc	nc
6. CNV6	0	0	0	0	1	1	1	0	1	1	0	1
7. CNV7	nc	0	nc	1	1	2	1	1	0	1	0	0
8. CNV8	0	0	0	0	0	0	0	0	0	1	1	0
9. CNV9	2	2	1	1	2	2	2	2	nc	nc	nc	nc
10. CNV10	1	1	1	1	1	1	1	0	1	1	1	0
11. CNV11	0	1	1	nc	2	2	2	2	1	1	0	1
12. CNV12	0	1	1	1	1	1	1	1	2	2	1	1
13. CNV13	0	0	0	0	0	0	0	0	0	1	0	0
14. CNV16	0	1	0	1	1	1	1	1	0	1	0	0

0 = no expression; 1 = 1 to 50% of cells; 2 = 51 – 100% of cells. EC = endothelial cells; RPE = retinal pigment epithelium; FB = fibroblasts and fibrocytes; O = other cell types.

In-situ hybridization

IGF-I

In the normal retina, IGF-I mRNA expression (Figure 6.1A,D) was found in the ganglion cell layer, inner nuclear layer, outer limiting membrane, RPE monolayer and in some cells in the choroid. Choriocapillary endothelial cells and retinal vessels were positive infrequently. Further expression was found in the lens epithelium, and in all

corneal layers (not shown). Hybridization with the sense probe was negative. In eyes with CNV we found IGF mRNA in the retina in the same pattern as in normal retina. Staining in preexistent RPE monolayer was similar to normal eyes. In 8 out of 14 eyes with CNV, expression was found in vascular endothelial cells, some RPE cells, and fibroblast-like cells (Table 6.2; Figure 6.2A,D,G,J). Eyes with PDR showed identical expression in retinal layers. In the preretinal membranes endothelial cells from newly formed capillaries and fibroblast-like cells stained positive (Figure 6.1G). Hybridization with the sense probe was negative.

IGF-I Receptor

In the normal eye mRNA of the IGF-IR (Figure 6.1B,F) was seen in the ganglion cell layer, inner and outer nuclear layer and outer limiting membrane. The RPE was strongly positive. Endothelial cells of the choriocapillaris, choroidal and intraretinal vessels were frequently positive. Further expression was found in the non-pigmented epithelium and to a lesser extent in the pigment epithelium of the ciliary body, the iris dilator muscle, the iris pigment epithelium and iris endothelial cells. The lens epithelium and all corneal layers were also positive. Hybridization with the sense probe was negative. In all eyes with CNV we found IGF-IR mRNA in endothelial cells of newly formed vessels, in RPE-cells and in the RPE monolayer, and in fibroblasts (Table 6.2; Figure 6.2B,E,H,K). Eyes with PDR showed expression in retinal layers and RPE similar to normal eyes. In the diabetic preretinal membranes (Figure 6.1H) endothelial cells from newly formed capillaries and fibroblast-like cells were positive. Hybridization with the sense probe was negative.

Immunohistochemistry

IGF-I Receptor

In the normal eyes, we found IGF-IR protein in the choroidal vessels, in the RPE, and in all layers of the neuroretina (Figure 6.1C,F). Choriocapillaris was negative. Negative controls showed no staining, except for the peptide control, in which staining of the RPE monolayer was not totally blocked compared to retinal staining, indicating an aspecific component in the RPE staining. In all eyes with CNV, we found the IGF-IR protein in RPE cells, and in 9 out of 13 classifiable eyes, staining was seen in newly formed vessels (Table 6.2; Figure 6.2C,F,I). Eyes with PDR showed expression in the retina similar to normal eyes, and in the diabetic preretinal membranes endothelial cells from newly formed capillaries were positive (Figure 6.1I).

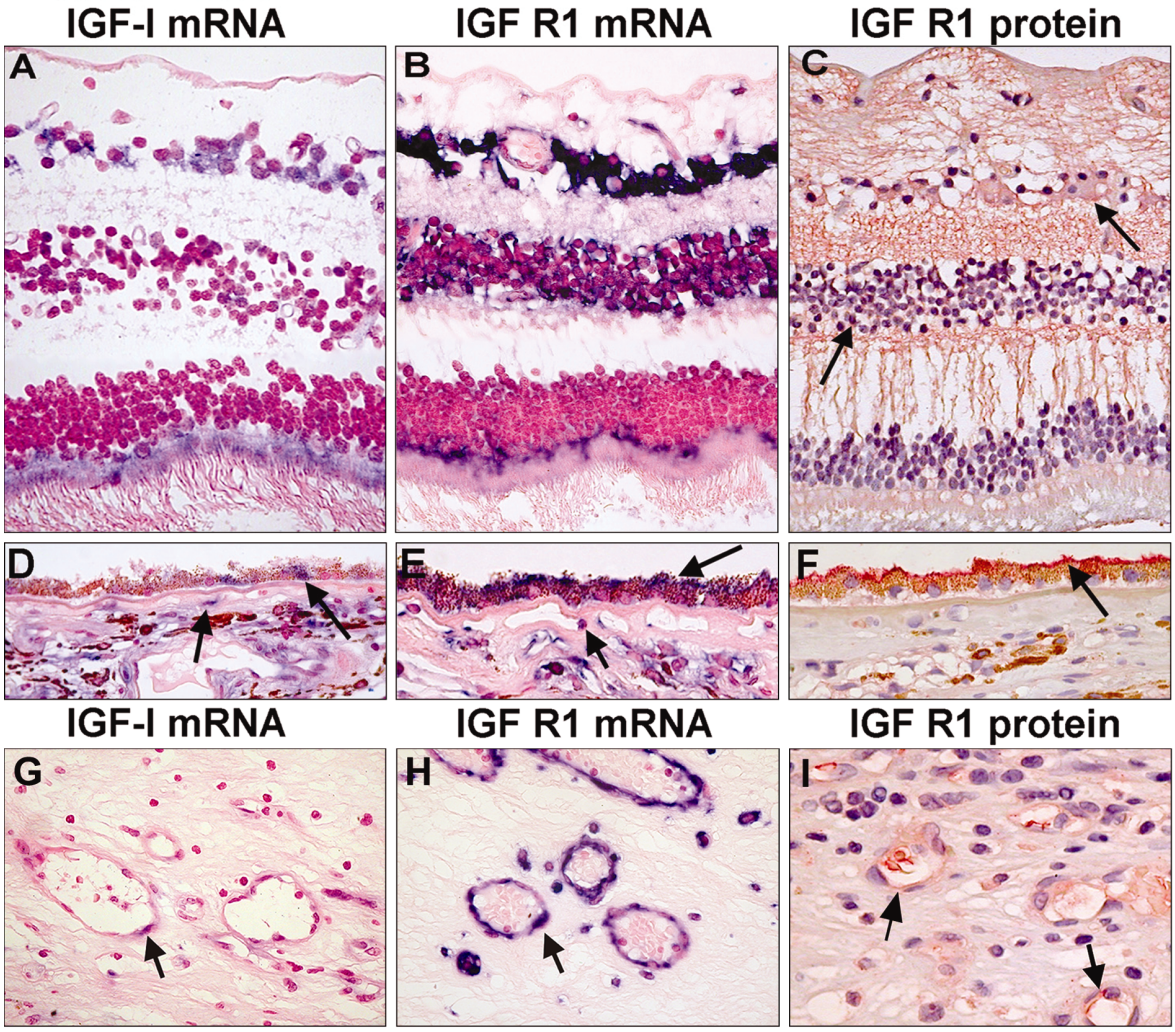

Figure 6.1 ***Localization of IGF-I and IGF-IR in the human eye and in eyes with diabetic proliferative retinopathy.*** *Expression of IGF-I mRNA (left column), IGF-IR mRNA (middle column) in paraffin-embedded tissue, detected by* in-situ *hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGF-IR protein (right column) in paraffin-embedded tissue, detected with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin. Short arrows indicate capillary endothelial cells, long arrows indicate RPE cells.* ***(A to F)*** *Posterior pole of normal eye (Co1) with IGF-I mRNA expression* ***(A, D)****; with IGF-IR mRNA expression* ***(B, E)****; and with IGF-IR protein expression* ***(C, F)****.* ***(G, H, I)*** *Diabetic preretinal membrane (DM2) with IGF-I mRNA expression* ***(G)****; with IGF-IR mRNA expression* ***(H)****; and with IGF-IR protein expression* ***(I)****. Original magnification X400*

Statistics

Grading of mRNA and IGF-IR protein expression was performed in a masked fashion by two authors (AL and CM), twice with a time interval of 7 weeks and once, respectively. For inter-observer variability, kappa was 0.75; for intra-observer variability kappa was 0.83.

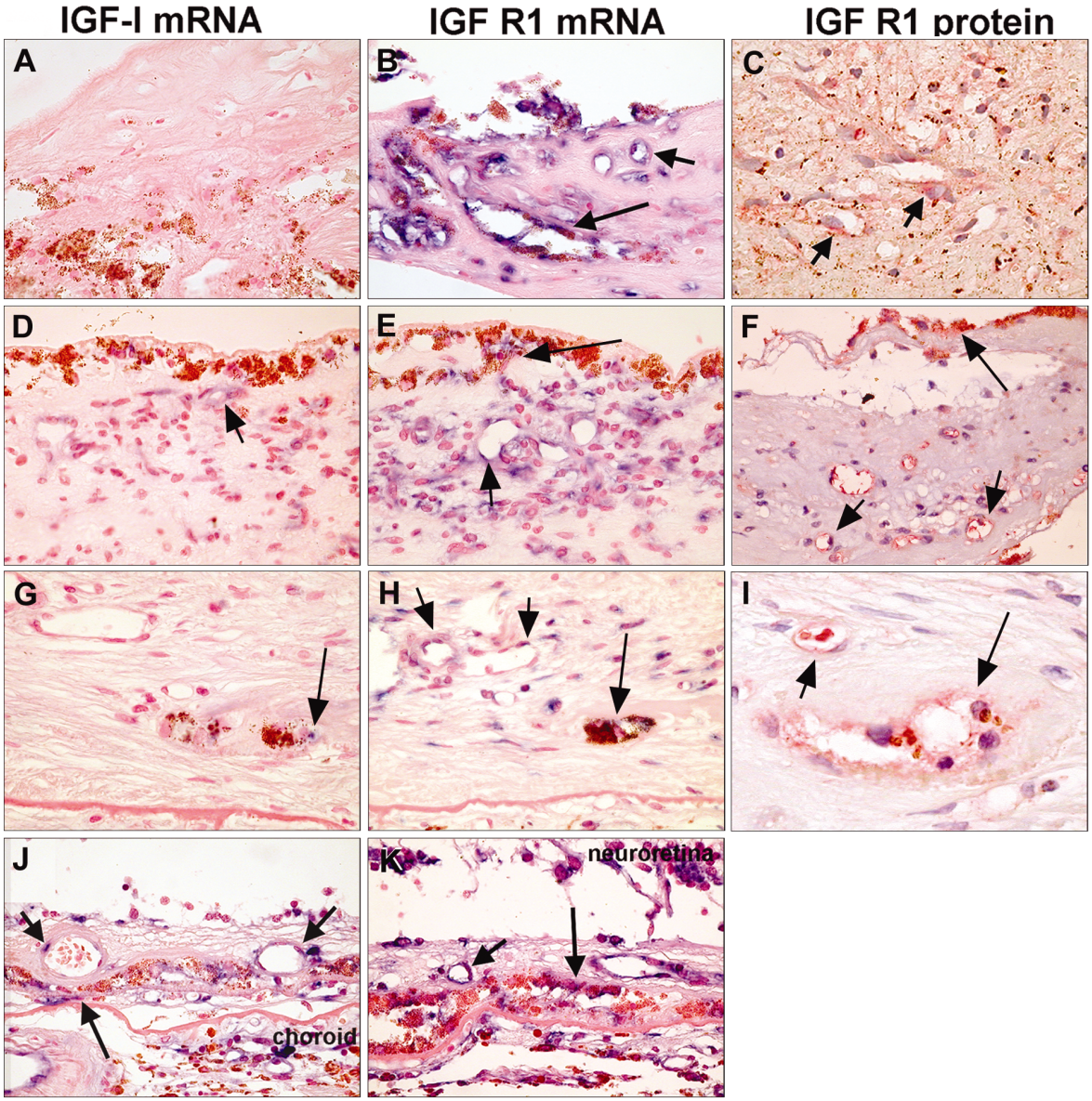

Figure 6.2 ***Localization of IGF-I and IGF-IR in eyes with neovascular AMD****. Expression of IGF-I mRNA (left column), IGF-IR mRNA (middle column) in paraffin-embedded tissue, detected by* in-situ *hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGF-IR protein (right column) in paraffin-embedded tissue, detected with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin. Short arrows indicate capillary endothelial cells, long arrows indicate RPE.* ***(A, B, C)*** *Surgically excised CNV (CNV2) with IGF-I mRNA expression* ***(A)****; with IGF-IR mRNA expression* ***(B)****; and with IGF-IR protein expression* ***(C)****.* ***(D, E, F)*** *Surgically excised CNV (CNV3) with IGF-I mRNA expression pression* ***(D)****; with IGF-IR mRNA expression* ***(E)****; and with IGF-IR protein expression* ***(F)****.* ***(G, H, I)*** *Sub-RPE CNV (CNV11) with with IGF-I mRNA expression* ***(G)****; with IGF-IR mRNA expression* ***(H)****; and with IGF-IR protein expression* ***(I)****.* ***(J, K)*** *Subretinal CNV (CNV9) with IGF-I mRNA expression* ***(J)****; and with IGF-IR mRNA expression* ***(K)****. Protein detection of IGF-IR with immunohistochemistry gave aspecific staining; therefore not shown. Original magnification X400.*

DISCUSSION

In the normal human retina, we found IGF-I and IGF-IR mRNA expression throughout the neuroretina in the same pattern as described for other species.[261,262] Expression in human RPE was not described before *in-situ*, but was detected in cultured human RPE cells.[254-256] IGF-I stimulates differentiation and proliferation of vascular endothelial cells,[170] RPE cells[256] and neural retinal cells,[171,263] and is also neuroprotective.[263-265] Our finding of protein and receptor co-localization confirms a paracrine/autocrine function of IGF-I in the normal human retina.[261,266]
The observed hybridization signal of IGF-IR was much stronger than that of IGF-I. This might represent a real difference in expression, confirming the findings of higher IGF-IR expression in cultured human RPE.[254,255] On the other hand, intensity of staining is not a reliable quantitative criterion, and the IGF-IR probe might be more sensitive than the IGF-I probe.

In CNV secondary to ARM, we demonstrated mRNA expression of IGF-I and IGF-IR in vascular endothelial cells, in RPE cells and in fibroblast-like cells. Similarly, we found expression of IGF-I mRNA and its receptor in epiretinal membranes in eyes with PDR, including endothelial cells from newly formed capillaries and in fibroblast-like cells. This is in accordance with a previous diabetic retinopathy study, in which binding sites for IGF-I (receptors or binding proteins) were demonstrated in vessel walls as well as in cells in fibrous tissue in human diabetic epiretinal membranes.[258] In human and rat diabetic retinas, IGF-I mRNA levels are decreased,[259,267] but vitreal levels of IGF-I are elevated.[268-272] Our findings confirm that intraocular synthesis may contribute to these elevated concentrations.[271]
The pathogenesis of neovascular AMD involves the choroidal vasculature rather than the retinal vasculature as in diabetic retinopathy. *In vitro*, IGF-I stimulates the proliferation of choroidal endothelial cells.[174] Therefore, synthesis of IGF-IR and IGF-I in endothelial and RPE cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD, since both cell types appear to have an important role in this process.[1]
IGF-I participates in each step of ocular neovascularization.[170] It is involved in the degradation of basement membranes and extracellular matrix proteolysis, and in vascular endothelial cell migration and proliferation.[173] Intravitreous injection of IGF-I in animals produces preretinal neovascularization in rabbits[175] or microangiopathy resembling diabetic microangiopathy in pigs.[176] IGF-I also increases RPE cell migration and proliferation *in vitro*.[172] Furthermore, IGF-I induces upregulation of VEGF mRNA expression in RPE cells[156] and fibroblasts,[178] which in turn also stimulates endothelial cells.[156] Antagonism of IGF-IR suppresses retinal

neovascularization and reduces the retinal endothelial cell response to VEGF,[273] which allows for the hypothesis that IGF-I has a permissive role in VEGF-induced neovascularization.
Inhibition of IGF-I, achieved with somatostatin analogues,[177] may occur at different levels. Firstly, somatostatin inhibits the GH-IGF axis. Furthermore, somatostatin can inactivate the mitogenic potential of IGF-I directly by inactivating the phosphorylated, active form of IGF-IR.[170,274] Finally, somatostatin can act directly as an anti-angiogenic agent through binding to somatostatin receptors.[173] Therefore, somatostatin analogues may be an effective therapy for neovascular AMD.
IGF-I is recruited in normal wound repair.[275,276] This may partly explain the presence of IGF-I in CNV, because formation of the disciform lesion is regarded as normal wound repair.[1]

In conclusion, in this descriptive study we localized the expression of IGF-I mRNA and IGF-IR protein and mRNA in the normal eye. The co-localization of protein and receptor indicates an autocrine function of IGF-I in the human retina. Furthermore, we detected synthesis of both IGF-I and its receptor, and IGF-IR protein in ocular neovascular membranes of patients with AMD and diabetic patients. Since IGF-I participates in ocular neovascularization, synthesis of IGF-IR and IGF-I in vascular endothelial, RPE and fibroblast-like cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD. The exact role of the IGF family in CNV formation and its possible therapeutic possibilities need to be established.