Insulin-like Growth Factor-I and its Receptor in Neovascular Age-Related Macular Degeneration

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PURPOSE. The insulin-like growth factor (IGF)-I protein is a growth-promoting polypeptide that can act as an angiogenic agent in the eye. The purpose of the current study was 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 and 14 eyes with choroidal neovascularization (CNV) secondary to AMD were examined. Three eyes with proliferative diabetic retinopathy were studied as the 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 choriocapillaris 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 choriocapillaris. In eyes with CNV, IGF-IR protein was present in the RPE monolayer, in transdifferentiated RPE, and in newly formed vessels.

CONCLUSIONS. The colocalization of protein and receptor indicates an autocrine function of IGF-I in the normal human retina. Because 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 toward a role for this growth factor in the pathogenesis of neovascular AMD. (Invest Ophthalmol Vis Sci. 2003;44:2192–2198) DOI:10.1167/iovs.02-0410

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 morphogenesis 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 neovascularization. Vascular endothelial growth factor (VEGF), an endothelium-specific mitogen, is regarded as one of the most important ocular angiogenic factors, especially under hypoxic circumstances.2,3 Other angiogenic factors in ocular neovascularization include basic fibroblast growth factor, transforming growth factor-β, platelet-derived growth factor, and insulin-like growth factor (IGF)-I.2,4

In an earlier study, we demonstrated that most CNVs in AMD express somatostatin receptor type 2A, which binds potential antiangiogenic somatostatin analogues.5 Somatostatin receptors are present on cultured humanretinal endothelial cells. Proliferation of retinal endothelial cells6 and choroidal endothelial cells7 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.8 IGF-I is a growth-promoting polypeptide that has mitogenic and differentiating effects on many cell types, among which are ocular vascular endothelial cells,9 RPE cells,10,11 and neuronal cells.12 IGF-I can act as a direct angiogenic factor on retinal endothelial cells,6,7 or indirectly through increased VEGF gene expression of cultured RPE cells.13

Most studies describe the role of IGF-I in vitro models5,7,14–17 or in situ in diabetic retinopathy.18,19 So far, IGF-I has not been studied in ARM.

The purpose of our study was 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, 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 CNV was performed after obtaining the informed consent of the patient.

PATIENTS

All eyes were retrieved from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. Four enucleated eyes without ischemic disease (enucleated for other reasons) and three enucleated eyes with proliferative diabetic retinopathy (PDR) were used as the control. Fourteen eyes (five enucleated eyes, four donor eyes and five surgically removed subretinal neovascular membranes) of 13 patients with neovascular AMD were studied. The clinical and histologic diagnosis of the eyes and the classification of CNV are described in Table 1. The eyes were processed for routine diagnostic procedures by fixation in 10% buffered formaldehyde and were embedded in paraffin. Five-micrometer 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.20 The human IGF-IR probe was generated using a
In Situ Hybridization

Sections were deparaffinized 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 μg/mL for 20 minutes at 37°C) and 4% formalin in PBS. Subsequently, acetylation was performed with acetic anhydride in 0.1 M Denhardt solution, dehydrated with ethanol gradients, and mounted.

Immunohistochemistry

Polyclonal rabbit antibodies against IGF-IR (1:750 dilution) were obtained from Research Diagnostics (Flanders, NJ). Immunohistochemistry against IGF-I was not performed, because adequate antibodies for paraffin-embedded material are not available. Antibodies against pan-keratin (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). Sections were deparaffinized and rehydrated. Antigen retrieval was performed for the IGF-IR and pan-keratin antibodies (microwave heating for 10 minutes) and for the factor VIII antibodies (prolonged treatment for 20 minutes at 37°C). After the slides were blocked with normal goat serum (Dako, 1:10) for 15 minutes, detected by antibody incubation performed according to the manufacturer’s instructions (Roche Diagnostics) 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 4°C. Afterward, an extra washing step of 0.025% Tween in Tris-buffered saline (pH 7.5) was performed. For staining, sections were layered with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂ (pH 9.5) containing 4-nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; both from Vector, Burlingame, CA) and 6% polyvinylalcohol (molecular weight 31,000 to 50,000, from Aldrich Chemical, Milwaukee, WI). The color reaction was performed in the dark and was stopped when the desired intensity of the resultant blue precipitate was reached. Sections were washed in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0), counterstained with nuclear red solution, dehydrated with ethanol gradients, and mounted.

cDNA clone with a unique insert of human IGF-IR (IMAGE cDNA clone 150561, Research Genetics, Huntsville, AL). The insert was reduced to 270 bp by restriction with BamHI and AvaI and ligated in antisense and sense vectors (pBluescript SK and KS, respectively; Stratagene Europe, Amsterdam, The Netherlands). *Escherichia coli* X12Blue was 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 instructions (Roche Diagnostics, Mannheim, Germany), using T7 RNA polymerase.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age/</th>
<th>Eye</th>
<th>Clinical Description</th>
<th>Histologic Classification CNV</th>
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<tbody>
<tr>
<td>Co1</td>
<td>83/M</td>
<td>L</td>
<td>Corneal ulcer</td>
<td>Subretinal/Sub-RPE FC</td>
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<td>L</td>
<td>Recurrent conjunctival melanoma</td>
<td>FV/FC Hemorrhage</td>
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<td>Co3</td>
<td>42/M</td>
<td>L</td>
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<td>Mixed BLD, hemorrhage</td>
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<td>Co4</td>
<td>69/F</td>
<td>R</td>
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<td>L</td>
<td>PDR</td>
<td>Sub-RPE BLD, confluent soft drusen</td>
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<tr>
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<td>Mixed BLD, hemorrhage</td>
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<tr>
<td>DM3</td>
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<td>CNV1</td>
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<td>Mixed FC BLD, hemorrhage</td>
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FV, fibrovascular; FC, fibrocellular; PDR, proliferative diabetic retinopathy; U, unknown; MD, macular degeneration; BLD, basal laminar and linear deposits.

**TABLE 1.** Diagnosis of Studied Eyes and Histologic Classification of CNV
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 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) preabsorption of the IGF-IR antibodies with ten times the amount of the immunizing IGF-IR peptide as of the antibody (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 (ACL, CMM) twice, and randomly graded per cell type in three categories (Table 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 with pankeratin 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 that did not meet these criteria were classified as other.

For inter- and intraobserver variability, a $\kappa$ statistic was calculated.

RESULTS
In two eyes with neovascular AMD (Table 2; eyes CNV8 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.

In Situ Hybridization
**IGF-I.** In the normal retina, IGF-I mRNA expression (Figs. 1A, 1D) was found in the ganglion cell layer, inner nuclear layer, outer limiting membrane, RPE monolayer, and some cells in the chorioid. 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 the preexistent RPE monolayer was similar to normal eyes. In 8 of 14 eyes with CNV, expression was found in vascular endothelial cells, some RPE cells, and fibroblast-like cells (Table 2; Figs. 2A, 2D, 2G, 2J). 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 (Fig. 1G). Hybridization with the sense probe was negative (Fig. 2L).

**IGF-I Receptor.** In the normal eye mRNA of the IGF-IR (Figs. 1B, 1F) 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 and the choroidal and intraretinal vessels were frequently positive. Further expression was found in the nonpigmented 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 2; Figs. 2B, 2E, 2H, 2K). Eyes with PDR showed expression in retinal layers and RPE similar to normal eyes. In the diabetic preretinal membranes (Fig. 1H) endothelial cells from newly formed capillaries and fibroblast-like cells were positive. Hybridization with the sense probe was negative (Fig. 2M).

**Immunohistochemistry**

In the normal eyes, we found IGF-IR protein in the choroidal vessels, in the RPE, and in all layers of the neuroretina (Figs. 1C, 1F); the choriocapillaris was negative. The negative controls showed no staining, except for the peptide control, in which staining of the RPE monolayer was not totally blocked compared with 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 of 13 classifiable eyes, staining was present in newly formed vessels (Table 2; Figs. 2C, 2F, 2I). Eyes with PDR showed expression in the retina similar to that in normal eyes, and, in the diabetic preretinal membranes, endothelial cells from newly formed capillaries were positive (Fig. 1I).

**Statistics**

Grading of mRNA and IGF-IR protein expression was performed in a masked fashion by two authors (ACL, CMM), twice,
with an interval of 7 weeks, and once, respectively. For inter-observer and intraobserver variabilities, $\kappa$ was 0.75 and 0.83, respectively.

**DISCUSSION**

In the normal human retina, we found IGF-I and IGF-IR mRNA expression throughout the neuroretina in the same pattern, as described in other species. Expression in human RPE has not been described before in situ, but has been detected in cultured human RPE cells. IGF-I stimulates differentiation and proliferation of vascular endothelial cells, RPE cells, and neural retinal cells, and is also neuroprotective. Our finding of protein and receptor colocalization confirms a paracrine-autocrine function of IGF-I in the normal human retina.

The observed hybridization signal of IGF-IR was much stronger than that of IGF-I. This may represent a real difference in expression, confirming the findings of higher IGF-IR expression in cultured human RPE. However, intensity of staining is not a reliable quantitative criterion, and the IGF-IR probe may 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 epi-retinal 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 epi-retinal membranes. In human and rat diabetic retinas, IGF-I mRNA levels are decreased, but vitreous levels of IGF-I are elevated. Our findings confirm that intraocular synthesis may contribute to these elevated concentrations.

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. Therefore, synthesis of IGF-IR and IGF-I in endothelial and RPE cells in CNV may play a role for this growth factor in the pathogenesis of neovascular AMD, because both cell types appear to have an important role in this process.

IGF-I participates in ocular neovascularization. It is involved in the degradation of basement membranes, and proteolysis of extracellular matrix, and in migration and proliferation of vascular endothelial cells. Intravitreal injection of IGF-I produces preretinal neovascularization in rabbits or microangiopathy resembling diabetic microangiopathy in pigs. IGF-I also increases RPE cell migration and proliferation in vitro. Furthermore, IGF-I induces upregulation of VEGF mRNA expression in RPE cells and fibroblasts, which in turn also stimulates endothelial cells. Antagonism of IGF-IR suppresses retinal neovascularization and reduces the retinal endothelial cell response to VEGF, which allows for the hypothesis that IGF-I has a permissive role in VEGF-induced neovascularization.

Inhibition of IGF-I, achieved with somatostatin analogues, may occur at different levels. First, 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. Finally, somatostatin can act directly as an antiangiogenic agent through binding to somatostatin receptors. Therefore, somatostatin analogues may be an effective therapy for neovascular AMD.
FIGURE 2. Localization of IGF-I and IGF-IR in eyes with neovascular AMD. Expression of IGF-I mRNA (A, D, G, J, L), IGF-IR mRNA (B, E, H, K, M) in paraffin-embedded tissue, detected by in situ hybridization with digoxigenin-labeled probes, colored with NBT/BCIP (blue), and counterstained with nuclear red. Expression of IGF-IR protein (C, F, I) in paraffin-embedded tissue detected with polyclonal antibodies and visualized with an alkaline phosphatase system, using a red chromogen and hematoxylin counterstaining. Short arrows: capillary endothelial cells, long arrows: RPE. Surgically excised specimens of CNV: CNV2 with expression of IGF-I (A) and IGF-IR (B) mRNA and IGF-IR protein (C); CNV3 with expression of IGF-I (D) and IGF-IR (E) mRNA and IGF-IR protein (F); sub-RPE CNV11 with expression of IGF-I (G) and IGF-IR (H) mRNA and IGF-IR protein (I); subretinal CNVg with expression of IGF-I (J) and IGF-IR (K) mRNA. Protein detection of IGF-IR with immunohistochemistry produced aspecific staining and is therefore not shown. Expression of sense mRNA probes of IGF-I (L) and IGF-IR (M) in subretinal CNV (CNV9). Original magnification, ×400.
IGF-I is recruited in normal wound repair. This may partly explain the presence of IGF-I in CNV, because formation of the disciform lesion is regarded as normal wound repair.

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 colocalization 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 patients with diabetes. Because IGF-I participates in ocular neovascularization, synthesis of IGF-IR and IGF-I in vascular endothelial, RPE, and fibroblast-like cells in CNV may indicate a role for this growth factor in the pathogenesis of neovascular AMD. The exact role of the IGF family in CNV and its possible therapeutic possibilities are yet to be established.

References


