Aqueous Humor Levels of Hepatocyte Growth Factor in Retinitis Pigmentosa

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Purpose. To determine the level of hepatocyte growth factor (HGF) in the aqueous humor of patients with retinitis pigmentosa (RP).

Methods. This was a prospective, comparative control study. Aqueous humor samples were collected from the eyes of 15 RP patients. The level of HGF was determined with a commercially available enzyme-linked immunoabsorbent assay kit. The control group was composed of aqueous samples from 15 patients about to undergo cataract surgery with no other ocular or systemic diseases.

Results. The concentration of HGF was markedly higher in the aqueous humor of the patients with RP than in that of the control subjects (Mann–Whitney U test, $P < 0.001$). The level of HGF was 958.75 ± 271.52 (mean ± SD) pg/mL in the eyes of the RP patients and 403.52 ± 116.27 pg/mL in eyes of the control group.

Conclusions. The concentration of HGF in aqueous humor is higher among patients with RP than in non-RP subjects. This finding provides new evidence that should be taken in account when considering HGF as a neuroprotective treatment for patients with RP. (*Invest Ophthalmol Vis Sci.* 2010;51:3157–3161) DOI:10.1167/iovs.09-4390

Retinitis pigmentosa (RP) is the most common cause of inherited blindness, with a prevalence of approximately 1 in 4000.[1] Visual impairment in RP is due primarily to loss of photoreceptors, which leads to subsequent damage of the retinal pigment epithelium (RPE) and other layers of the retina.[2] Apoptosis is reported to be the final and most common cause of photoreceptor degeneration in all the RP animal and patient models analyzed to date, and the apoptotic pathways engaged in the process have recently been defined.[3] RP is a highly variable disorder, with some patients developing only sectorial visual field loss and others suffering a profound loss of peripheral visual field, which is in turn associated with varying degrees of central macular function loss. Changes in retinal vasculature are prominent clinical features, with attenuation of retinal vessels in early stages and fibrotic degeneration in later stages of the disease.

Human hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for hepatocytes.[4,5] The reported biological effects of HGF include the regulation of proliferation, development, motility, morphogenesis, migration, adhesion, and survival of many types of cells from various organs.[5–10] HGF and its receptor c-met have been identified in the central nervous system (CNS),[11] and evidence generally suggests that HGF regulates neuronal survival.[12–14]

In the eye, HGF plays an important role in ocular angiogenesis and retinal neuron neuroprotection. It has been demonstrated that retinal ischemia increases the expression of HGF and its receptor c-met in retinal cells in the middle and inner layers of the retina. In addition, intravitreal injection of HGF has been shown to promote the survival of these cells in retinal ischemia–reperfusion experiments in rats, which suggests that it protects retinal cells under ischemic stress,[15] and it has been reported to promote the morphologic and physiological preservation of photoreceptors in rats with photoreceptor degeneration.[16] The antiapoptotic effect may be the mechanism of the neuroprotective action of HGF, which is known to be the mechanism of photoreceptor death in animal models of RP.[17,18] On the other hand, HGF has been shown to be involved with the angiogenesis associated with tumor growth and wound healing.[19,20] In recent studies, however, increased levels of HGF have been reported in the vitreous and serum of patients with proliferative diabetic retinopathy (PDR) and in the subretinal fluid of patients with stage 5 retinopathy of prematurity (ROP),[21] suggesting that HGF plays an important role in the initial stages of retinal angiogenesis.[22]

The objective of this study was to quantitatively measure the levels of HGF in the aqueous humor of patients with RP and to compare them with those in healthy control subjects, in an effort to identify a possible role of HGF in the pathogenesis of the disease and to aid research into neuroprotective treatments for RP.

Methods

In this prospective, comparative, control study, we investigated the levels of HGF in the aqueous humor of patients with RP. Aqueous samples from cataract patients without any other ocular or systemic diseases were collected as control samples. The study protocol complied with the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of a tertiary referral hospital. Informed consent was obtained from all subjects.

Patients were enrolled in the Retinitis Pigmentosa Reference Unit of the Valencian Community. Undiluted aqueous humor samples were collected from 15 eyes of 15 individuals with typical forms of RP and with no other confounding ocular or systemic disease. Patients were at

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least 18 years of age and displayed the typical forms of RP, which are characterized by an elevated final dark-adaptation threshold, retinal arteriolar narrowing, and a reduced and delayed electroretinogram. Patients with syndromic forms of RP, such as Laurence-Moon-Bardet-Biedl-syndrome and Usher-syndrome, were excluded from the study. The ages of patients at diagnosis are shown in Table 2. All patients underwent a full ophthalmic examination that included best corrected visual acuity (BCVA), automated visual field, and multifocal electroretinogram (mfERG).

The BCVA was measured according to the ETDRS protocol adapted for use in the Age-Related Eye Disease Study.[23]

We used the measurement of static perimetric sensitivities (i.e., total point score) (30-2 program with size V target; Humphrey Field Analyzer (HFA); Carl Zeiss Ophthalmic Systems, Inc., Dublin, CA). The size V target was used to minimize the number of locations with floor effects (sensitivity, ≤0 dB). The FASTPAC test strategy was used to test the central 30-2 visual field in as short a time as possible.[24,25]

Before the mfERG was recorded, the appropriate refraction was measured and corrected for the testing distance. The pupils were dilated with 1% tropicamide (Alcon Cusí, El Masnou, Spain) and 10% phenylephrine (Alcon Cusí). A commercial multifocal system (RETIscan; Roland Consult, Wiesbaden, Germany) was used to determine electrical function of the macular area. The room light was on during stimulation. The stimulus, consisting of 103 hexagons covering a visual field of 30°, was presented on a monitor (20-in. VGA; ELSA, Beijing, China) with a frame rate of 75 Hz at a distance of 28 cm from the patient's eye. The patient was instructed to maintain fixation on a red central-fixation cross 2 mm in diameter. DTL fiber electrodes and one ground electrode, were placed in the center of the forehead and one reference electrode in the temporal region of the studied eye. Each element alternated between black and white (93% contrast, mean luminance 51.8 cd/m^2). For a 50,000 amplification, the filters were set between 5 and 100 Hz. Each recording session was subdivided into eight recording segments of approximately 47 seconds. The signals were registered with sampling intervals of 83 ms. The results obtained with the retinal scanning equipment were analyzed according to the ISCEV (International Society for Clinical Electrophysiology of Vision) guidelines for multifocal ERG recordings.[26] The results were distributed in five concentric rings. The response density was obtained for each ring (in nanovolts/square degrees) and was calculated by dividing the response amplitude (nanovolts) by the retinal area (square degrees). The total response density of the central retinal 30° was calculated by adding the response densities of the five concentric rings in each patient.

Aqueous humor samples from 15 eyes of 15 patients about to undergo surgery for cataracts were obtained as the control. The control group was age-matched with the RP group.

Aqueous samples of RP patients were collected under sterile conditions using a 30-gauge needle under the slit lamp, with one drop of povidone iodine applied before and after the anterior chamber was punctured. Antibiotic prophylaxis was subsequently administered for several days. The aqueous samples of control subjects were collected with a 30-gauge needle before cataract surgery began. Undiluted aqueous samples of at least 0.05 mL were collected from each patient, placed in sterile tubes, and stored immediately at −80°C until use. All specimens were assayed for HGF in a double-blind arrangement with respect to their group.

An enzyme-linked immunosorbent assay (ELISA) of aqueous humor samples was performed to quantify the levels of HGF (Searchlight Human Angiogenesis Array; Pierce Biotechnology, Inc., Woburn, MA). The sensitivity of the HGF assay is 1.6 pg/mL. All procedures were performed according to the manufacturer's instructions (http://www.piercenet.com/files/1601463%20ArrayBrochure.pdf).

Demographic characteristics of the patients were summarized with descriptive statistics (SPSS for Windows; SPSS Inc., Chicago, IL). The Mann–Whitney U test for independent samples was used to compare the HGF levels of the groups. $P < 0.05$ was considered significant. Spearman's ρ was used to calculate the correlation between the aqueous humor level of HGF with the visual field sensitivity and with the total response density of the mfERG in the RP patients.

Results

Thirty aqueous humor samples were collected from 15 RP patients and 15 control patients. All participants were Caucasian. No statistically significant differences were found between the mean ages of the RP (mean ± SD; 50.4 ± 11.6 years [range, 36–75]) and the control (49.6 ± 11.8 years [range, 35–72]) groups (independent-samples *t*-test, $P = 0.84$; Table 1). Table 2 shows detailed information for each patient.

The aqueous humor level of HGF was 958.75 ± 271.52 (mean ± SD) pg/mL in the eyes of RP patients and 403.52 ± 116.27 pg/mL in the eyes of the control group. HGF levels in the two groups differed significantly (Mann–Whitney U test, $P < 0.001$), with those of the RP patients significantly higher than those of the control subjects (Fig. 1).

Visual acuity in the RP patients was 0.86 ± 0.21 (mean ± SD) logMAR. The lower end of the range of normal visual acuity is 0.5 logMar (60 EDTRS letters). Table 3 shows detailed clinical information on all the RP patients.

The visual field sensitivity of the RP patients (HFA 30-2 program, size V target) was 423.7 ± 294.6 dB (mean ± SD). The lower end of the range of normal for the 30-2 program field (size V target) is 2500 dB. The correlation between the aqueous humor level of HGF and the visual field sensitivity was −0.05 (Spearman's ρ), indicating no correlation.

The mfERG of the RP patients showed that amplitudes were decreasing with increasing eccentricity from the fixation point, with significantly higher values in rings 1 and 2; from rings 3 to 5 the amplitudes were near 0 nV/deg^2 (range, 8–0.4). The total mean ± SD response density of the central retinal 30° was 98.9 ± 29.8 nV/deg^2. The lower end of the range of normal total response density in our laboratory is 208.4 nV/deg^2. The correlation between the aqueous humor level of HGF and the mfERG response density was −0.48 (Spearman's ρ) indicating a weak inverse correlation. The correlation between the mfERG response density and visual field sensitivity was 0.7 (Spearman's ρ), indicating a strong direct correlation.

We did not find any relationship between our RP patients on the basis of the hereditary pattern and the aqueous humor level of HGF, and no relationship was shown between the hereditary pattern and the mfERG recordings.

Table 1. Patient Baseline Characteristics and Level of HGF in Aqueous Humor

Disease Group	Age (y)	Sex (M:F)	Level of HGF in Aqueous Humor (pg/mL)
RP ($n = 15$)	50.4 ± 11.6 (36–75)	(8:7)	958.75 ± 271.52
	$P = 0.84$*		$P < 0.001$*
Control ($n = 15$)	49.6 ± 11.8 (35–72)	(8:7)	403.52 ± 116.27

Data are expressed as the mean ± SD (range).
* Compared with Control group.

TABLE 2. Data of All Patients

	RP Patients			Control Patients		
Patients	Age (y)	Sex	Level of HGF in Aqueous Humor (pg/mL)	Age (y)	Sex	Level of HGF in Aqueous Humor (pg/mL)
1	44	F	1046.99	44	F	195.46
2	40	F	854.16	42	M	380.04
3	36	M	1140.08	35	F	375.91
4	49	M	1037.37	49	F	544.93
5	39	F	1726.96	37	M	464.95
6	65	F	882.59	66	M	444.18
7	75	M	708.88	72	F	362.38
8	45	F	771.30	48	M	183.45
9	52	M	776.56	54	F	485.22
10	46	F	739.71	43	M	341.83
11	45	M	728.65	40	F	607.68
12	48	M	130.88	49	M	431.30
13	43	M	965.91	40	M	400.77
14	68	M	812.60	71	M	328.3
15	62	F	884.70	54	F	506.25

DISCUSSION

The confirmation that HGF levels are higher in patients with RP than in healthy control subjects raises important questions. Recently, we have shown that the concentration of vascular endothelial growth factor A (VEGF-A) is lower in the aqueous humor of RP patients than in that of non-RP subjects.[27] With these results we postulated that the lack of angiogenic action attributable to VEGF-A may explain some of the clinical manifestations of this disease, such as the narrowing and fibrotic degeneration of retinal blood vessels. Recent reports have demonstrated a direct neuroprotective effect of VEGF-A on retinal neurons.[28] Like VEGF-A, HGF plays an important role in ocular angiogenesis and retinal neuroprotection. These dichotomous results, with the low intraocular concentration of VEGF and the high concentration of HGF, lead us to believe that HGF plays role in these patients that goes beyond a strict relation to angiogenesis. Other reports have also shown a poor correlation between VEGF and HGF in angiogenic and nonangiogenic fibroproliferation.[21,29] Among PDR samples, VEGF concentration was shown to be significantly higher in a subgroup with higher angiogenic activity, which was represented by iris neovascularization, although there were no significant differences in HGF concentration between the subgroups.[29] In eyes with ROP, VEGF concentrations in the subretinal fluid were significantly higher in stage 4, during which there is active angiogenesis, than in stage 5, which is associated with more fibroproliferation. Significantly higher concentrations of HGF were observed during stage 5.[21] These findings indicate that intraocular production of HGF is more important in mediating inflammatory and fibroproliferative processes than in angiogenesis itself.[30]

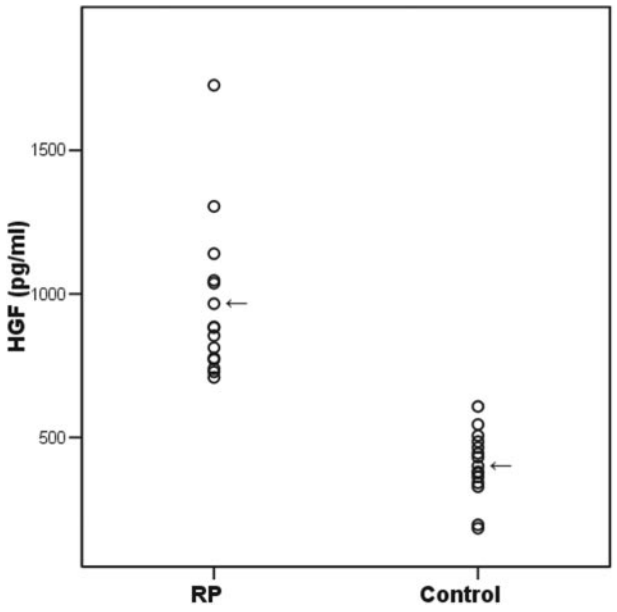

FIGURE 1. Hepatocyte growth factor (HGF) levels in the aqueous humor of 15 eyes with retinitis pigmentosa (RP) and 15 control eyes. HGF levels in the two groups were significantly different (Mann–Whitney U test, $P < 0.001$), with those in RP eyes being significantly higher than those in control eyes. *Arrows*: the mean aqueous humor level of HGF in each group.

It has been demonstrated that the stressed retina expresses HGF and its receptor in the retinal pigment epithelium (RPE), glial cells, and the inner and middle layers of the retina.[15,16] It has also been shown that the mRNA of HGF is highly expressed in the retina of RCS rats (Shuler RK et al. *IOVS* 2004;45:ARVO E-Abstract 705). The neurons of the inner and middle layers of the retina are the ones that survive longest during the degenerative process of RP, and the longer survival may be one of the reasons for the high concentration of HGF in these patients. In our study, we were able to find only a weak inverse correlation between the mfERG and the intraocular levels of HGF. These findings could make us believe that the more advanced the disease, the higher the levels of HGF, but still we must exercise caution in drawing conclusions, because correlation is not sufficient to demonstrate the presence of a relationship; and, again, the correlation proved to be weak.

With respect to the possible consequences of the high intraocular levels of HGF in RP, it should be pointed out that the cone function has been shown to be significantly preserved by intravitreal injection of HGF in animal models of RP.[16] It has also been demonstrated in vitro that blue light irradiation inhibits the production of HGF by RPE cells, and this may enhance the phototoxic effect of visible light on the RPE and retinal neurons in a lifetime.[31] The high levels of HGF could act as a countersystem for retinal degeneration in RP patients and may explain, at least in part, the preservation of central macular function until advanced stages of the disease. Moreover, the increase in fibroproliferative actions attributable to HGF

Table 3. Detailed Clinical Data of RP Patients

Patient	Age at Diagnosis (y)	Genotype	EDTRS Visual Acuity (logMar)	HFA 30-2 Field (Total Point Scores, dB)	mfERG (Total Response Density, nV/deg^2)	Level of HGF in Aqueous Humor (pg/mL)
1	35	AR	0.84	164	81.3	1046.99
2	27	AD	0.96	211	80.2	854.16
3	32	S	0.70	255	92.6	1140.08
4	34	S	0.70	180	79.3	1037.37
5	29	S	0.84	156	59.1	1726.96
6	48	S	0.98	481	87.4	882.59
7	51	AD	0.96	137	69.8	708.88
8	14	AR	1.50	229	119.4	771.30
9	33	AD	0.60	316	101.5	776.56
10	32	S	0.84	239	105.0	739.71
11	40	AD	0.60	894	187.4	728.65
12	22	AR	0.82	957	85.9	1304.88
13	9	AR	0.90	672	82.5	965.91
14	50	S	0.84	790	104.3	812.60
15	35	AR	0.96	160	65.5	884.70

The lower normal level for visual acuity was +0.5 logMar (60 EDTRS letters); +0.68 logMar (51 EDTRS letters) indicated a Snellen visual acuity of 20/30. Lower normal level for HFA 30-2 program field (size V target) was 2500 dB. The lower normal level of the total response density in our laboratory is 202.4 nV/deg^2. AD, autosomal dominant; AR, autosomal recessive; S, simplex.

could explain some of the clinical manifestations of this disease, such as visually significant vitreous opacities.

We assume that there are many more factors that contribute to the pathogenesis of RP. Yet, by demonstrating the presence of high levels of HGF in RP, we believe that we can help to comprehend further this devastating disease, and at the same time open the way to new lines of research in animal models of RP.

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