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A Reassessment of Insulin-Like Growth Factor Binding Protein Gene Expression in the Human Retinal Pigment Epithelium

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Abstract The role of insulin-like growth factors (IGF) in regulating cell differentiation and proliferation is in part modulated by the IGF binding protein (IGFBP) family of genes. Previous studies of the human retinal pigment epithelium (RPE) have detected expression of IGFBP-2, -3, and -6. However, recent experiments in our lab have suggested a broader pattern of *IGFBP* gene family expression in the RPE cell than has previously been recognized. We have examined the gene expression profile of IGFBP-1 to -6 and the related protein, IGFBP-rP1, in RPE cell lines derived from ten donors eyes using RT-PCR, ELISA, and Western methods. Transcripts of IGFBP-1 to -6 and -rP1 were consistently detected in human RPE cells. IGFBP-3, -5, -6, and -rP-1, appear to be constitutively expressed in the RPE, whereas IGFBP-1, -2, and -4, were expressed at variable levels in the cell lines examined. IGFBP secretion by the RPE in vitro was confirmed by ELISA (IGFBP-1, -2, -3, -4, and -6) and Western blot analysis (IGFBP-5 and -rP1). There was, in general, a strong correlation between gene-specific transcription levels and protein secretion by the RPE. Our studies demonstrate that the major IGFBP family genes are ubiquitously expressed in explanted human RPE cells in vitro. This broad expression profile and the recent evidence that IGFBPs have IGF-independent biological activity suggest that the IGFBP family genes may constitute a previously unrecognized and complex regulatory system in the human retina and RPE. J. Cell. Biochem. 89: 933–943, 2003.

Key words: gene expression; gene regulation; growth factors; insulin-like growth factor binding protein; insulin-like growth factor; pigment epithelium of eye; retina; tissue culture

The insulin-like growth factors (IGF-I, IGF-II) play an important role in regulating the differentiation, growth, and proliferation of many types of cells, including human retinal pigment epithelial (RPE) cells, through a receptor-mediated paracrine and autocrine signaling cascade [Waldbillig et al., 1991; Blakesley et al., 1999]. There are six major IGF binding protein genes (*IGFBP-1* to *-6*) that have been identified and cloned, and four IGFBP-related proteins [Clemmons, 1991; Drop et al., 1992; Oh et al., 1996]. The physiologic activity of the IGF proteins is regulated by activation of IGF-specific receptors, and is modulated through interactions with the IGFBPs [Ooi and Boisclair, 1999]. The IGFBPs have been shown to play a role in regulating the biological activity of IGFs by serum transport, inhibiting or enhancing IGF availability at the tissue level, and modulating IGF-1 receptor binding [Binoux et al., 1991; Clemmons, 1991, 1998; McCusker et al., 1991; Drop et al., 1992]. Recent evidence also suggests that the IGFBPs have biological activities independent of their ability to bind to and regulate the IGFs [Oh et al., 1993; Rajah et al., 1997; Schneider et al., 2000, 2002].

Previous studies of *IGFBP* gene expression in isolated human RPE cell lines have identified

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proteins consistent with IGFBP-2, -3, and -6 [Randolph et al., 1993; Feldman and Randolph, 1994; Moriarty et al., 1994; Takagi et al., 1994; Matsunaga et al., 1999; Miyamura et al., 2001] However, recent experiments in our laboratory have suggested that there is a broader *IGFBP* gene expression profile in the human RPE than has previously been recognized. In order to characterize *IGFBP* gene expression in the RPE, we quantified the transcription and synthesis of IGFBPs-1 to -6 and the major IGFBP-related protein IGFBP-rP1 in ten recently explanted human RPE cell lines and three of these RPE cell lines at later passages in vitro using semi–quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), Western, and quantitative ELISA techniques. Our studies demonstrate that the major IGFBP-family genes are ubiquitously expressed in explanted RPE cells and RPE cell lines in vitro.

MATERIALS AND METHODS

RPE Cell Culture

Human RPE cells were isolated from donor eyes provided by the Mid-South Eye Bank (Memphis, TN) using procedures previously described [Chaum and Yang, 2002]. The ages of the donors ranged from 29 to 94 years. The causes of death included myocardial infarction (4), respiratory failure (3), trauma (1), cerebral aneurysm (1), and unknown (1). Two patients had diabetes mellitus one was insulin-dependent. One patient used topical medication for glaucoma. All studies and procedures were approved by the University of Tennessee Institutional Review Board. RPE cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, Atlantic Biologicals, Norcross, GA) plus L-glutamine, penicillin, and streptomycin in an atmosphere of humidified 95% air and 5% CO_2 at 37°C. The RPE cells were seeded into T25 flasks and cultured until confluent. They were then split 1:2 into DMEM plus 5% FBS and cultured for 3 days at 37°C. The tissue culture media was collected and stored at −80°C for ELISA analysis.

RT-PCR Studies

Sub-confluent RPE cells were washed twice with ice cold phosphate buffered saline (PBS) and lysed in the flask using TRI reagent (Sigma, Saint Louis, MO). The total RNA was isolated using the protocol recommended by the manufacturer. The concentration was measured by spectrophotometry and the RNA was stored at −80°C. RNA samples were cleaned of possible trace genomic DNA contaminants by treatment with DNAse I from the DNA-Free kit using the protocol recommended by the manufacturer (Ambion, Austin, TX). The purified RNA was dissolved in RNAse free water at the concentration of 0.2 μg/μl. One microgram of total cellular RNA was reversed transcribed (RT) in 20 μl of reaction volume using the Reverse Transcription System (Promega, Madison, WI). The RT product was diluted 1:5 with RNAse-free water and PCR amplification was performed in 25 μl of 1 × PCR buffer containing 1 × PCR Master Mix (Promega), 1.25 μl of forward and reverse primers (10 pmol/μl), and 5 μl of the diluted RT product. The PCR reaction was started at 95°C for 5 min followed by 30 cycles of 95°C for 45 s, the annealing temperature for 45 s, and then 72°C for 45 s, plus a final extension at 72°C for 10 min. Over-amplification of specific samples was performed as described above for an additional five cycles. Forward and reverse PCR primers of IGFBP-1 to -6 and -rP1 were designed using PrimerExpress 1.5 (ABI Applied Biosystems, Foster City, CA) and are listed in the 5′ to 3′ orientation (IGFBP-1: forward, TAT GAT GGC TCG AAG GCT CTC; reverse, TAT GAT GGC TCG AAG GCT CTC; Tm = 58°C; 131 bp. IGFBP-2: forward, CGT GGA CAG CAC CAT GAA CAT; reverse; TTG AGG TTG TAC AGG CCA TGC; Tm = 60°C, 324 bp. IGFBP-3: forward, TCC ACC CCC TCC ATT CAA A; reverse, ATT CTG TCT CCC GCT TGG ACT; Tm = 59°C, 135 bp. IGFBP-4: forward, TCC ACC CCC TCC ATT CAA A; reverse, CCC ATT GAC CTT CAT CTT GCC; Tm = 58°C, 107 bp. IGFBP-5: forward, TGT ACC TGC CCA ATT GTG ACC; reverse, TTC ATC CCG TAC TTG TCC ACG; Tm = 59°C, 109 bp. IGFBP-6: forward, AGA GTA AAC CCC AAG CAG GCA; reverse, GTC TTG GAC ACC CGC AGA ATT; Tm = 60°C, 116 bp. IGFBP-rP1: forward, TGG CCC AGA AAA GCA TGA AG; reverse, TGA TGC TGA AGC CTG TCC TTG; Tm = 57°C, 115 bp. β-actin: forward, TCA TGA AGT GTG ACG TTG ACA TCC GT; reverse, CCT AGA AGC ATT TGC GGT GCA CGA TG; Tm = 58°C, 285 bp). The PCR products were separated in 1.5 or 2.5% agarose gels and stained with 0.2 μg/ml ethidium bromide for 30 min. The stained gel was scanned using Typhoon 8600 imaging system

and analyzed using ImageQuant (Amersham Pharmacia, Piscataway, NJ). The band densities were normalized to β-actin-specific amplicons and quantified.

Enzyme-Linked Immunosorbant Assays (ELISA)

Secreted IGFBPs -1, -2, -3, -4, and -6, were measured in the culture media in triplicate using DuoSet ELISA kits according to the protocol recommended by the manufacturer (R&D Systems, Minneapolis, MN). ELISA assays have not been developed for IGFBP -5 or -rP1. Secretion of these two proteins into the media was detected and quantified using standard Western methods described below. The ELISA assays were performed using 96-well plates coated with 100 μl of capture antibody diluted in 10 mM PBS, pH 7.2–7.4, and incubated at room temperature (RT) overnight. The final concentrations of the mouse anti-human IGFBP capture antibodies were optimized for each assay and were between 2 and 4 μg/ml. After washing three times with wash buffer (0.05% Tween 20 in 10 mM PBS, pH 7.2–7.4), the 96-well plates were blocked with 300 μl of block buffer (5% Tween-20, 5% sucrose in 10 mM PBS) for 1.5 h at RT. After washing with buffer three times, the plates were incubated with 100 μl/well of diluted (1:4–1:8), or in some cases undiluted RPE cell-conditioned media or serial diluted IGFBP standards in reagent diluent (5% Tween 20 in PBS, pH 7.2–7.4). Goat serum (2%) was also used as a blocking agent for the IGFBP-3 ELISA. After incubation for 2 h at RT, the plates were washed three times and 100 μl/well of diluted detection antibody (biotinylated goat anti-human IGFBP in reagent diluent plus 2% normal goat serum) was added to each well. The final concentration of the detection antibody was optimized for each assay and was between 0.1 and 0.4 μg/ml. After incubating for 2 h at RT, the plates were washed with buffer three times and incubated with 100 μl/well of peroxidase-conjugated streptavidin (1:200) for 20 min at RT. After washing three-times, the plates were color developed with 100 μl per well of substrate solution from the ImmunoPure TMB substrate kit (Pierce, Rockford, IL) for 15 min and the reaction was then stopped with 50 μl of 2 M H_2SO_4. The optical density (OD) of each reaction well was determined immediately at 450 nm on an EL_X800 UV universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The protein level in the culture media was calculated using KCjunior software (Bio-Tek Instruments) by comparison to an IGFBP-specific standard curve.

Western Blotting

The secreted IGFBP-5, and -rP1 proteins were detected by Western blot analysis. Briefly, the culture media was concentrated (for IGFBP-5 only) using Amicon Ultra-4 columns (Millipore, Billerica, MA). Tris-glycine gels (12%) (Invitrogen, Carlsbad, CA) were used for PAGE separation of proteins in the media under reducing conditions. The gels were semidry transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked with 5% non-fat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline with 0.1% Tween20 (pH 7.6, TBS-T) for 2 h at RT. Membranes were incubated with primary mouse anti-human IGFBP-5 antibody (United States Biological, Swampscott, MA) or mouse anti-hIGF-rP1 antibody (Diagnostic Systems, Webster, TX) in TBS-T overnight at 4°C. After washing three-times with TBS-T, membranes were incubated with a secondary antibody of biotin-SP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min at RT. After washing, the membranes were incubated with peroxidase-conjugated streptavidin (Jackson) for 45 min at RT. The membranes were incubated with the ECL + Plus chemiluminescent Western blotting detection system (Amersham) or Supersignal West Pico chemiluminescent substrate (Pierce) for 5 min at RT. The signals were scanned and analyzed using the Typhoon 8600 Imager.

RESULTS

IGFBP Gene Expression in Human RPE Cells

IGFBP-1 expression. IGFBP-1 transcript-specific amplicons were detected at low levels in 3 of 13 cell lines after 30 cycles of RT-PCR amplification (Table I). However, the ELISA assay detected IGFBP-1 in the media from RPE cell lines that did not appear to be expressing the transcript based upon our initial RT-PCR studies. Therefore, we over-amplified the PCR product for an additional five cycles (total of 35 cycles) and were able to detect a single amplicon band that corresponded to the IGFBP-1 transcript seen in the cell lines with high levels of transcription (Fig. 1). This IGFBP-1 amplicon was seen in every cell line (except late passage

TABLE I. Relative Level of IGFBP-Specific Transcripts in the RPE

RPE[a]	Passage	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGFBP-rP1
8	3			++		+	+	+++
8	12			+++		++	++	+++
12	3			++		+	+	+++
12	20			++		++	++	+++
13	2			++		+	+	+++
13	21		+	+++		++	++	+++
14	2			++		+	+	+++
15	4	+	+++	++	+++	+++	+++	+++
16	4	+	+++	++	+++	+++	+++	+++
17	4	+	+++	++	+++	++	+++	+++
18	3		+	++		++	+	+++
20	4			++		+	+	+++
21	2		+	+++	++	+++	+	+++

RT-PCR performed for 30 cycles. +, low; ++, intermediate; +++, high.
[a]RPE cell line.

HPE-12, discussed below), although most of the cell lines transcribed the gene at very low levels (Table II).

The ELISA studies showed moderate levels of secreted IGFBP-1 protein in the diluted media of RPE cells lines 15, 16, and 17 (range 3.9–13.5 ng/ml). Initially, IGFBP-1 was not detected in similarly diluted media from the cell lines expressing low levels of the IGFBP-1 transcript (data not shown). The ELISA studies were replicated using undiluted samples of the conditioned media. Small amounts of IGFBP-1 protein were found in the undiluted conditioned media from most of the cell lines in which the transcripts were weakly expressed (Table III). The level of protein secreted into the media ranged from 0.14 to 0.42 ng/ml. Control wells containing DMEM media only and DMEM with 5% FCS did not have detectable levels of the IGFBP-1 protein in the ELISA assay. IGFBP-1 protein was not detected in early or later passage RPE-8 cells or RPE-18 cells, although IGFBP-1 transcripts were seen in these cell lines. This suggested that the protein secretion was below the level of detection in the ELISA assay in these cell lines. Late passage RPE-12 failed to demonstrate both the transcript and the protein; however, both were detected in the RPE-12 cell line at an earlier passage in vitro.

IGFBP-2 expression. IGFBP-2 transcript-specific amplicons were detected in high levels in cell lines RPE-15, -16, and -17, similar to IGFBP-1. Low levels of transcripts were also seen in cell lines RPE-13, -18, and -21, following standard RT-PCR amplification (Table II). However, IGFBP-2 transcripts were detected in all of the RPE cell lines after over-amplification (Fig. 1).

IGFBP-2 protein was detected in the media of all of the RPE cell lines by ELISA. In cell lines RPE-15 to -17, which expressed high levels of the IGFBP-2 transcript, IGFBP-2 protein concentration in the media ranged from 11.6 to 54.5 ng/ml. In contrast, low concentrations of the IGFBP-2 protein (0.1–7.0 ng/ml) were seen in the cell lines in which the transcript was only

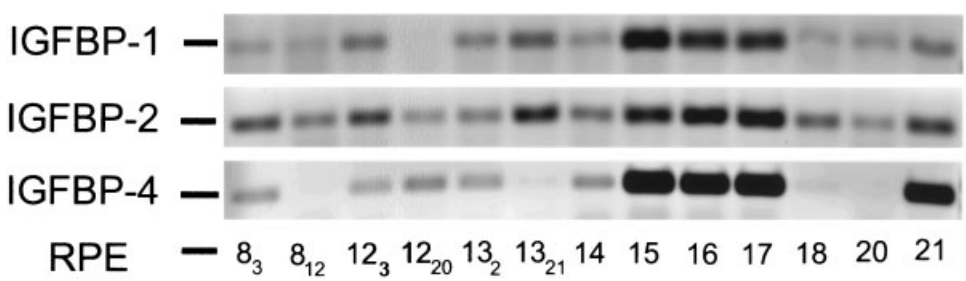

Fig. 1. Expression of IGFBP-1, -2, and -4 transcripts in the RPE. Transcripts were amplified using *IGFBP* gene-specific primers for 35-cycles. Variable levels of transcript expression were identified for IGFBP-1 (**upper panel**), IGFBP-2 (**middle panel**), and IGFBP-4 (**lower panel**). The relative levels of expression were quantified and are compiled in Table II. Passage number is indicated in the cells lines examined at two time points.

TABLE II. Relative Level of IGFBP-1, -2, and -4 Transcripts in the RPE

RPE[a]	Passage	IGFBP-1	IGFBP-2	IGFBP-4
8	3	+	++	+
8	12	+	+	−
12	3	+	++	+
12	20	−	+	+
13	2	+	+	+
13	21	++	++	+
14	2	+	+	+
15	4	+++	+++	+++
16	4	+++	+++	+++
17	4	+++	+++	+++
18	3	+	+	+
20	4	+	+	−
21	2	++	++	++

RT-PCR performed for 35 cycles. +, low; ++, intermediate; +++, high.
[a]RPE cell line.

weakly expressed (Table III). Control wells containing unconditioned media with 5% FCS did not have detectable levels of the IGFBP-2 protein in the ELISA assay.

IGFBP-3 expression. Moderate to high levels of IGFBP-3 transcript-specific amplicons were found in all of the RPE cell lines as expected (Table I). ELISA studies confirmed the expression of IGFBP-3 protein in every RPE cell line (range, 29.5–58.1 ng/ml). The control wells containing unconditioned media with 5% FCS did not have detectable levels of the IGFBP-3 protein in the ELISA assay.

The level of IGFBP-3 protein secreted into the media was remarkably similar in all of the RPE cell lines (mean 48.9 ± 8.96 ng/ml). The consistent and narrow range of IGFBP-3 secretion into the media in vitro suggests that the IGFBP-3 protein is constitutively expressed in the RPE.

IGFBP-4 expression. High levels of IGFBP-4 transcript-specific amplicons were found in cell lines RPE-15, -16, and -17, similar to the results seen for IGFBP-2. A moderate level was also seen in RPE-21, but IGFBP-4 transcripts were not detected in any of the other RPE cell lines after standard amplification (Table I). Over-amplified transcripts from these cell lines again demonstrated that, with the exception of RPE-20 and later passage RPE-8, all of the RPE cell lines express low levels of the IGFBP-4 transcript (Fig. 1).

In cell lines RPE-15 and -17, which expressed high levels of the IGFBP-4 transcript, the IGFBP-4 protein concentration in the media was 51.9 and 54.9 ng/ml, respectively. Although high levels of transcription were detected in RPE-16, only low levels of the protein were detected in the media (5.24 ng/ml). Low concentrations of the IGFBP-4 protein were also seen in the cell lines in which the transcript was only weakly expressed (2.1–7.6 ng/ml, Table III). IGFBP-4 protein was not detected in the media from cell lines RPE-18 or -20, however, transcript-specific amplicons were detected in RPE-18. RPE-20 was the only cell line in which an IGFBP could not be detected by either RT-PCR or ELISA methods. Control wells containing unconditioned media with 5% FCS had a barely detectable signal in the ELISA assay (0.09 ± 0.2 ng/ml).

IGFBP-5 expression. IGFBP-5 transcript-specific amplicons were detected in every RPE

TABLE III. Secreted IGF Binding Proteins in RPE-Conditioned Media

		IGFBP-1		IGFBP-2		IGFBP-3		IGFBP-4		IGFBP-6	
RPE[a]	Passage	Ave[b]	SD[c]	Ave	SD	Ave	SD	Ave	SD	Ave	SD
8	3	ND[d]	—	1.98	0.59	52.7	3.54	7.59	1.29	30.3	0.94
8	12	ND	—	2.38	0.53	51.6	6.17	3.78	1.29	53.0	2.65
12	3	0.19	0.01	1.14	0.62	58.1	11.0	4.49	0.36	29.7	2.21
12	20	ND	—	0.08	0.01	42.9	0.65	2.84	1.45	17.5	0.79
13	2	0.17	0.03	0.60	0.07	56.1	6.42	4.24	2.71	27.3	0.88
13	21	0.42	0.00	4.78	0.91	55.3	2.79	2.83	1.59	28.7	1.41
14	2	0.14	0.02	0.16	0.08	44.4	1.17	5.26	2.22	40.2	2.19
15	4	13.5	0.69	11.6	4.62	29.5	5.36	54.9	6.19	153.6	64.6
16	4	3.9	0.26	54.5	21.1	42.3	11.5	5.24	4.04	136.3	5.4
17	4	5.1	0.58	28.1	6.94	46.9	3.39	51.9	6.45	520.7[e]	92.4
18	3	ND	—	5.30	0.10	49.2	4.51	ND	—	13.6	0.47
20	4	0.34	0.01	0.92	0.10	54.1	0.40	ND	—	13.1	0.26
21	2	0.32	0.03	6.78	0.57	52.8	5.04	2.06	2.55	31.8	1.24

[a]RPE cell line.
[b]The average values (Ave) and [c]standard deviation (SD) from studies done in triplicate. All values are in ng/ml.
[d]Not detected by the ELISA assay.
[e]Calculated value (see text).

cell line. The level of transcription varied between cell lines but was easily detectable after standard amplification (Table I).

In the Western blot analysis, the purified human IGFBP-5 protein is a 28 kDa doublet protein band [Martin and Baxter, 1990]. This 28 kDa doublet band was identified in the cell culture media of every RPE cell line (Fig. 2). The IGFBP-5 protein was detected in media that had been concentrated by spin columns and, therefore, it was not quantified. However, the density of the protein bands varied only slightly in intensity among the cell lines and suggested that the level of protein secretion was similar in all of the RPE cell lines. Control lanes containing unconditioned media with 5% FCS did not have detectable levels of the IGFBP-5 protein in the Western assay (data not shown).

IGFBP-6 expression. The expression pattern of IGFBP-6 was very similar to that seen for IGFBP-5. The IGFBP-6 transcript was expressed at the highest levels in cell lines RPE-15, -16, and -17, but transcript-specific amplicons were easily detectable in every cell lines after standard amplification (Table I).

The ELISA assay demonstrated moderate levels of protein secretion into the tissue culture media as expected (range, 13.1–153.6 ng/ml) (Table III). The protein levels were highest in the cell lines with the highest levels of transcription, RPE-15, -16, and -17. The level of protein detected in the diluted media from RPE-17 was calculated from the control IGFBP-6 serial dilution curve (KCjunior software) and is likely artificially elevated because the OD reading occurred in the asymptotic portion of the curve. The control wells containing unconditioned media with 5% FCS did not have detectable levels of the IGFBP-6 protein.

IGFBP-rP1 expression. All of the RPE cell lines demonstrated high levels of the IGFBP-rP1 transcript-specific amplicon (Fig. 3). This high level of transcription correlated well with secretion of the IGFBP-rP1 protein into the culture media, as demonstrated by Western analysis.

The IGFBP-rP1 protein appears as a 37 kDa band. The Western blot demonstrated that this 37 kDa IGFBP-rP1 band is present in every RPE cell line (Fig. 2). High levels of the IGFBP-rP1 protein were seen in RPE-14, -18, -20, and -21 and in late passage RPE-8 and RPE-13. The protein was easily detectable in the undiluted media from every cell line using Western methods. Control lanes containing unconditioned media with 5% FCS did not have detectable levels of the IGFBP-rP1 protein (data not shown).

The Effect of Tissue Culture on *IGFBP* Gene Expression

The expression of IGFBPs by the RPE was examined in three cell lines at early and late passages in vitro. Cell lines RPE-8, -12, and -13 were examined shortly after explantation (passages 2–4) and again after passage 12 (RPE-8) or passage 20–21 (RPE-12 and -13). There was a modest but clear increase in the transcription of IGFBP-3, -5, and -6 with increasing time in culture. The IGFBP-1 transcript was present at low levels in early passage cells. Apparent transcription levels varied over time in culture, decreasing to undetectable levels in late passage RPE-12, but increasing in late passage RPE-13 cells (Table I). IGFBP-2 transcription showed similar changes in its pattern of expression in the three cell lines. IGFBP-4 expression appeared to be at a constant low level in all three cell lines, but was not detected in late passage RPE-8 cells (Table II).

IGFBP-2 transcription was, in general, correlated with changes in the level of protein

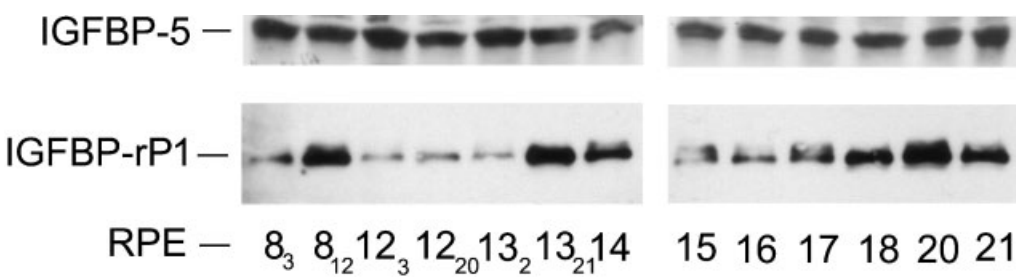

Fig. 2. Secretion of IGFBP-5 and IGFBP-rP1 proteins in vitro. Western blot analysis. of IGFBP-5 and IGFBP-rP1 secretion into conditioned media. The intensity of the IGFBP-5 signal is very consistent between cell lines (**upper panel**), whereas IGFBP-rP1 secretion is quite variable (**lower panel**) despite similar levels of transcription. There was an increase in IGFBP-rP1 secretion with increasing time and passages in vitro. Passage number is indicated in the cells lines examined at two time points.

Fig. 3. Expression of IGFBP-rP1 transcripts. IGFBP-rP1-specific amplicons of were detected at high levels in every RPE cell line. Similar results were seen for IGFBP-3, -5, and -6 transcripts and are compiled in Table I. Passage number is indicated in the cells lines examined at two time points.

secretion. Although there was no significant difference in the level of protein between early and later passage RPE-8 cells (2.0 vs. 2.4 ng/ml, respectively), the changes in transcription correlated well with the observed decrease in protein secretion by RPE-12 and the increase in protein secretion by RPE-13 (1.1→0.1 and 0.6→4.8 ng/ml, respectively). There was no substantial change in the secretion of IGFBP-3 over time in culture (Table III). There was a modest decline in IGFBP-4 secretion in cell lines RPE-15, -16, and -17 over time, however, the decreases were statistically significant only for RPE-15 ($P < 0.02$, $P < 0.23$, $P < 0.41$ respectively; paired samples t-test). IGFBP-6 transcription appeared to increase in all three cell lines over time, however, an increase in protein secretion was only confirmed in line RPE-8 (Table III). IGFBP-5 and -rP1 were present in all cell lines at early and later passages. Although the proteins could not be reliably quantified by the Western detection method, there was an obvious increase in the IGFBP-rP1 protein secreted by the later passage cells, in particular by RPE-8 and -13 (Fig. 2).

Correlation of IGFBP Transcription and Protein Secretion by the RPE

There was, in general, a direct correlation between the level of protein secreted and the level of transcription by the RPE cell lines in vitro. The RPE cells that expressed higher levels of IGFBP transcripts secreted greater amounts of that IGFBP. IGFBP-1 transcription was highest in RPE-15, -16, and -17 and the IGFBP-1 protein was also secreted at high levels in these three cell lines. A similar correlation was seen for IGFBP-2, -4, and -6 (Table III).

The correlation between the level of IGFBP-5 transcription and protein synthesis was less direct, although the levels could not be accurately quantified due to the Western method. The level of protein secretion appeared to be quite consistent between cell lines even though the level of transcript varied from low to high (Table I). Similar results were seen with IGFBP-3 where the correlation between transcription and protein levels was not as direct. However, transcription of IGFBP-3 was moderate to high in all of the cell lines examined. Conversely, IGFBP-rP1 demonstrated quantitatively similar, high levels of transcription in all of the cell lines (Fig. 3), but the protein level in the media varied widely and may reflect post-transcriptional regulation of this gene (Fig. 2). Thus, while there was a correlation between transcription and translation for most of the IGFBPs, it was not a strict one in all cell lines for every IGFBP.

DISCUSSION

The results of our RT-PCR and ELISA studies demonstrate that newly explanted human RPE cells transcribe each of the six major IGFBPs and IGFBP-rP1, and secrete all seven IGFBPs into the culture media in vitro. The level of IGFBP message detected by RT-PCR was stratified into three groups; low, intermediate, and high levels of transcription, based upon the amplicon band density normalized to β-actin transcripts from the same RNA sample. IGFBP-3, -5, -6, and -rP1 are the major IGFBPs in the human RPE and are expressed at intermediate or high levels in all of the explanted cell lines. There was also a trend toward increasing transcription of IGFBP-3, -5, -6, and rP1, with increasing passages in vitro. IGFBP-3, -4, -5, -6, and -rP1 transcription has also recently been reported in expressed sequence tag (EST) analyses of RPE/choroid gene expression in aged human eyes [Wistow et al., 2002].

IGFBP-1, -2, and -4 were expressed at low levels in most of the RPE cell lines and were detected only when over-amplified by RT-PCR. Our findings were consistent with gene-specific amplification of low copy number transcripts of IGFBP-1, -2, and -4, in the RPE. Three cell lines (RPE-15, -16, and -17) showed high levels of expression and secretion of all of the IGFBPs and IGFBP-rP1. One of these cell lines was isolated from a patient taking insulin for

diabetes mellitus, but the others were not. Thus, *IGFBP* gene expression did not appear to be induced by insulin use. Our results clearly show that the *IGFBP* gene expression in the RPE is much broader than has previously been recognized [Randolph et al., 1993; Feldman and randolph, 1994; Moriarty et al., 1994; Takagi et al., 1994; Matsunaga et al., 1999; Miyamura et al., 2001] and demonstrate for the first time that the major proteins of the *IGFBP* gene family are ubiquitously expressed in the RPE.

The inability of previous studies to identify this broad expression profile in the RPE can be explained by several factors including the sensitivity of the original techniques. Cloned cDNAs were not available for all of the IGFBP family genes when gene expression studies were first reported. The Northern blotting techniques used are also not as sensitive as RT-PCR for detecting very low copy-number transcripts. One previous report using RT-PCR techniques [Takagi et al., 1994] identified IGFBP-2 transcripts, but did not detect transcripts from IGFBP-3, which has previously been detected in high copy number using Northern methods Transcripts thought to be IGFBP-6 were detected but were not confirmed when sequenced. It is possible that this previous study failed to detect low copy-number transcripts due to the number of amplification cycles used; however, the reason for the absence of IGFBP-3 and -6 transcripts is not clear and may be due to the primers that were used. In our studies, IGFBP-2 transcription and secretion was variable in the RPE cell lines examined at later passages in vitro. Our results did not confirm the known association between IGFBP-2 expression and in vitro aging, but this may be explained by the fact that our cells had not reached replicative senescence [Matsunaga et al., 1999; Miyamura et al., 2001].

Recent studies in vascular smooth muscle cell cultures have also shown that *IGFBP* gene expression may be context-specific in vitro [Duan, 2002]. The results of previous studies looking at IGFBP expression in the RPE may reflect an unrecognized context-dependence. Our studies were performed on sub-confluent cultures and showed some variability in IGFBP expression between the cell lines tested. The smooth muscle cell cultures also demonstrated extracellular matrix (ECM)-dependent changes in IGFBP-5 transcription, suggesting that other local factors or signaling molecules such as integrins may play a role in regulating *IGFBP* gene expression.

We confirmed the specificity of the RT-PCR results by demonstrating secretion of all of the major IGFBPs by our cell lines, using sensitive ELISA-based quantitative assays or Western blotting. The assays showed significant levels of gene-specific IGFBP secretion in each culture (compared to background), and demonstrated a direct and generally dose-dependent relationship between the level of protein secretion and the quantitative level of normalized *IGFBP* gene transcription. This quantitative correlation between IGFBP-specific transcription and secretion supports our conclusion that the amplicons in the RT-PCR assays are *IGFBP* gene-specific. Our studies assayed only the IGFBPs that were secreted into the tissue culture media. Some of the apparent differences in the levels of the IGFBPs may have been due to localization of the proteins in other tissue culture compartments, such as the cell (not yet secreted) or ECM where specific IGFBPs are known to be concentrated at the local tissue level (see below). IGFBPs in these compartments were not analyzed in the studies presented here.

The IGFs play a key role in many cellular processes including cell cycling and proliferation, cell differentiation, and inhibition of apoptosis through activation of the PI3-K and MEK/ERK signal transduction cascades [Chaum, 2003]. The RPE has been previously been shown to express both IGF-I and IGF-II and their respective receptors [Waldbillig et al., 1991; Martin et al., 1992; Takagi et al., 1994; Chaum and Yang, 2002]. The IGFBPs play an important role in regulating the physiological functions of these growth factors. The ubiquitous expression of IGFBPs in human RPE cells suggests that IGFBPs are important proteins in the maintenance the IGF-mediated functions in RPE and raises new questions about the role of this protein family in the RPE and retina. It is known that IGFBP-3 and -5 are the major IGFBPs that regulate circulating IGF-1 bioavailability, but all IGFBPs function at some level to cross the endothelium to deliver IGF molecules to local tissues. IGFBP-6 may play a larger role in regulating IGF-II bioavailability at the local tissue level in the retina due to its greater binding specificity [Ooi and Boisclair, 1999]. IGFBP-rP1 may also play a supporting role in regulating insulin activity in the retina [Oh et al., 1996]. The apparent constitutive level

of expression of these proteins by the RPE suggests a role, at least in part, in regulating exogenous IGF-I and -II activity in the RPE. Conversely, the low levels of expression of IGFBP-1, -2, and -4, suggest that they are acting specifically at the local tissue level in the RPE, retina, or both.

It is now clear that the function of IGFBPs is complex and extends beyond their role as IGF-binding molecules [Baxter, 2000; Clemmons, 2001; Firth and Baxter, 2002; Mohan and Baylink, 2002]. IGFBPs and the proteolytic fragments of their degradation have numerous biologic activities independent of IGFs, which are only now being elucidated. Both the primate RPE and human vitreous have previously been shown to degrade IGFBPs in vitro [Schoen et al., 1992] and may contribute to IGF-independent IGFBP activity in the retina and RPE in vivo. IGFBPs may also be involved in cell migration, cell growth, and cell apoptosis [Mohan and Baylink, 2002] IGFBP-1 and -2 contain an RGD (Arg–Gly–Asp) integrin binding motif at the carboxy-terminus [Jones et al., 1993]. These sequences have been shown to specifically bind the $\alpha_5\beta_1$ fibronectin receptor on the RPE cell surface [Anderson et al., 1990]. IGFBP-1 binding to the $\alpha_5\beta_1$ receptor has been shown to play a role in cell migration [Jones et al., 1993], and may regulate wound healing in the RPE through modulation of the IGF-I paracrine/autocrine system [Waldbillig et al., 1991]. IGFBP-1 has also been shown to stimulate cell migration independent of IGF-I activity [Jones et al., 1993; Gleeson et al., 2001]. Whether IGFBP-1 (and perhaps IGFBP-2) plays a similar role in the migration of the RPE during wound healing has not yet been investigated.

IGFBP-3, -5, and -6 contain heparin-binding motifs and bind to proteoglycans of the ECM. IGFBP-3 has been shown to have both growth-stimulating and growth-inhibiting activity independent of IGF-I and has been shown to modulate insulin receptor activation, induce apoptosis, and modulate the antiproliferative and apoptotic effects of TGF-β and retinoic acid [Oh et al., 1993; Gucev et al., 1996; Rajah et al., 1997; Yamanaka et al., 1997]. Of particular interest is the finding that IGFBP-3 contains a nuclear localizing sequence and may regulate the transcription of growth inhibiting genes or act as a cell cycle regulatory protein [Radulescu, 1994; Jaques et al., 1997]. Although IGFBP-1 and -2 are transcribed at low levels, they may demonstrate a significant physiologic effect in modulating the activity of IGF-I in the RPE if they are concentrated in the ECM and at the cell surface by virtue of their integrin-binding motifs. Proteoglycan-bound IGFBP-5 has a decreased affinity for IGF-I, which can potentiate the effect of IGF-I in the local cellular environment [Firth and Baxter, 2002] thus, IGFBP-5 may also have a role in modulating the biological activity of IGF-I at the tissue level in the RPE. IGF-I-mediated retinal neurogenesis in teleost fish [Boucher and Hitchcock, 1998; Otteson et al., 2002] may also be regulated by local expression of *IGFBP* genes in the retina.

The physiologic relevance of each of the IGFBP family proteins in the retina and RPE remains to be elucidated and we have speculated about possible roles for these proteins. Active expression of all of the major proteins of the *IGFBP* gene family in the RPE opens a new chapter in the molecular and cellular regulation of the RPE in ocular health and disease that warrants further in depth investigation.

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