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# Gene expression of the insulin-like growth factors and their receptors in cultured human retinal pigment epithelial cells

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Insulin-like growth factors I and II (IGF I and II) are polypeptides with both growth-promoting and insulin-like metabolic effects[16,27]. Immunoreactive IGF I is present in the retina[14] and both IGF I and II are present in vitreal fluid[12]. The type I and type II IGF receptors are also localized within the neural retina[3,15,20,33]. The presence of IGFs and IGF receptors within the eye suggests a possible growth-promoting effect of IGFs on ocular tissues. IGF may enter the eye from the blood or, alternatively, arise from an ocular cell type which synthesizes and secretes IGF. IGF I and II mRNA synthesis in scleral cells[13] and IGF I synthesis in rat retina[14] suggests endogenous IGF production in the eye. We hypothesized that IGFs and IGF receptors are synthesized by one ocular cell type, the retinal pigment-epithelium (RPE). As a first step in studying IGF production by the RPE, we analyzed expression of the IGF and IGF receptor genes by cultured human RPE cells. Using Northern analysis, RNase protection and reverse-transcriptase polymerase chain reaction (RT-PCR), we found that cultured RPE cells synthesize mRNA for IGF I and the type I and type II IGF receptors.

## INTRODUCTION

Insulin-like growth factors I and II (IGF I and IGF II) are polypeptides which share structural homology with proinsulin and exert mitogenic and growth-promoting effects in many tissues[16,27]. IGF I and IGF II bind to specific membrane receptors, the type I and type II IGF receptors, respectively[7,22,23]. In the eye, autoradiography[3,20], immunocytochemistry[20] and affinity labelling[15,20,33] have localized both the type I and II IGF receptors to the neural retina and retinal pigment epithelium (RPE). IGF I and II immunoreactivity are present in the vitreous[12], whereas only IGF I immunoreactivity is present in the retina and RPE[14]. Because the RPE, located adjacent to the choriocapillaris, is responsible for the active transport of ions, metabolites and fluid to the inner retina[37], IGF I immunostaining may reflect RPE internalization of circulating IGFs. Alternatively, IGF I immunoreactivity could reflect endogenous growth factor production. In vitro conditioned medium from cultured human RPE contains IGF I immunoreactivity[34], implying either release of internalized IGF I, as seen in endothelial cell cultures[2], or endogenous IGF I production by RPE.

As an initial approach to understanding whether ocular IGF I and II represent internalized or newly synthesized growth factors, we determined whether cultured human RPE cells express the genes for IGF I and II and their receptors. Because there are differences in the sensitivity of various assays and we were interested in the relative gene expression levels, we used three independent methods to assay levels of cellular mRNAs for the IGF and IGF receptor genes: Northern analysis, ribonuclease (RNase) protection and reverse-transcriptase polymerase chain reaction (RT-PCR), listed in order of increasing sensitivity [17,26]. Using these methods, we have detected IGF I, type I and type II IGF receptor mRNA in cultured human RPE cells. Our data provide a basis for analyzing the regulation and expression of these genes in human RPE cells, a key cell type in ocular health and disease.

## MATERIALS AND METHODS

### *Chemicals*

Minimum essential medium (MEM) and RPMI 1640 medium were purchased from Grand Island Biological Co. (Grand Island, NY). Improved minimum essential medium (IMEM) was obtained from Biofluids (Rockville, MD) and bovine calf serum (CS) from Hyclone Labs (Logan, UT). Tissue culture supplies were purchased from Costar (Cambridge, MA) and Corning Glass Works (Corning, NY). Molecular biology grade reagents were purchased from

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Sigma. All other chemicals were of reagent grade and obtained from Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Fairhaven, NJ).

*Cell culture*

Retinal pigment epithelia were isolated from human post-mortem eyes provided by the Michigan Eye Bank. Primary RPE cell cultures were established as previously described[8]. Cells were periodically subcultured at a density of 3–5 × $10^6$ cells per 75 $cm^2$ tissue culture flask in MEM containing 20% CS. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% $O_2$. Medium was changed 3 times weekly.

Breast cancer cell line MDA-MB231 was obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in IMEM with 10% CS. The human neuroblastoma cell line SK-N-SH was obtained from J. Biedler, Memorial Sloan-Kettering Institute (New York, NY) and maintained in RPMI 1640 medium with 10% fetal CS.

*Northern analysis*

Total cellular RNA was isolated from cells or tissues by guanidinium thiocyanate-phenol extraction[5]. Poly(A)$^+$ and poly(A)$^-$ RNA were selected by oligo(dT) cellulose chromatography following the manufacturer's protocol (Pharmacia, Gaithersburg, MD)[1]. The human IGF IA cDNA was kindly provided by Dr. Peter S. Rotwein (Washington University School of Medicine, St. Louis, MO)[24]. An 818-base pair (bp) region of IGF IA cDNA encoding exons 1, 2, 3 and 5 was excised from pBlueScript KS (Stratagene, La Jolla, CA) by digestion with *Eco*RI (Stratagene, La Jolla, CA). The cDNA for human IGF II was kindly provided by Dr. Graeme I. Bell (Howard Hughes Institute, Chicago, IL)[4]. An 854-bp region of IGF II cDNA encoding exons 7, 8 and part of 9 was excised from pGEM4 (Promega, Madison, WI) by digestion with *Pst*I. Type I IGF receptor cDNA was obtained from Dr. Axel Ullrich (Max Planck Institute, Munich, F.R.G.)[32]. A 1247-bp insert encoding part of the type I IGF receptor $\alpha$ and $\beta$ chains was excised by digestion of pGem4 with *Eco*RI and *Hind*III (Stratagene, La Jolla, CA). All templates were $^{32}$P-labelled by random priming (Amersham, U.K.) for use in hybridization reactions.

RNA samples (1–10 $\mu$g) were dissolved in 37.5% formamide, 1.6 M formaldehyde and 1.5 mM sodium phosphate buffer. RNA samples were electrophoresed in denaturing gels (1% agarose, 2.2 M formaldehyde, 0.02 M sodium phosphate buffer) and transferred to Nytran nylon membranes (Schleicher & Schuell, Keene, NH) by capillary blotting. After transfer, 40–100 ng of denatured IGF I and IGF II cDNA were applied to the membrane for positive and negative hybridization efficiency controls. Nucleic acids were baked onto membranes for 30 min at 65–80 °C. Membranes were hybridized in 40% formamide, 1.1 M NaCl, 60 mM $NaH_2PO_4$, 6 mM EDTA, 1.0% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100–200 $\mu$g/ml denatured salmon DNA and $^{32}$P-labelled cDNA probe (1–5 × $10^5$ cpm/ml) for 12–16 h at 42 °C. Membranes were washed twice for 15 min in 0.36 M NaCl, 20 mM $NaH_2PO_4$, 2 mM EDTA and 0.1% SDS at room temperature and twice for 15 min at 65 °C in 90 mM NaCl, 5 mM $NaH_2PO_4$, 0.5 mM EDTA and 0.1% SDS. For high stringency, membranes were washed twice for 15 min at 65 °C in 18 mM NaCl, 1 mM $NaH_2PO_4$, 0.1 mM EDTA and 1.0% SDS. Membranes were exposed to X-ray film (Kodak) in the presence of an intensifying screen at –70 °C for 1–7 days.

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*

One $\mu$g of total cellular RNA was transcribed into complimentary DNA. Briefly, in a 20 $\mu$l volume of 1× PCR buffer (Perkin Elmer Cetus, Norwalk, CT), 1 mM each dNTP (Sigma), 1 U/$\mu$l RNAsin (Boehringer-Mannheim, Indianapolis, IN), 100 pM random hexamer (Sigma) and 200 U of Moloney MuLV reverse transcriptase (Bethesda Research Labs, Bethesda, MD) were added to 1 $\mu$g of total cellular RNA (or water for control samples) and incubated for 60 min at 42 °C. The reaction was heat-inactivated at 95 °C for 5 min, then placed on ice. To each 20 $\mu$l reaction, 8 $\mu$l 10× PCR buffer, 69 $\mu$l sterile $H_2O$, 1 $\mu$l each of an upstream and downstream primer and 1 $\mu$l of 1 U/$\mu$l Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) were added and placed in a ther-

TABLE I

*Primers used in the RT-PCR assay*

| *Gene* | *Primers used* | *Location* | *Product size* | *Ref.* |
|---|---|---|---|---|
| IGF-I | TCACATCTCTTCTACCTGGC | Exon 2 | | |
| | GTAGGTCTTGTTTCCTGCAC | Exon 5 | 366 bp | 28 |
| IGF-II | GAGTGCTGCTTCCGCAGCTG | Exon 3 | | |
| | TCTCTGAACGCTTCGAGCTC | Exon 4 | 249 bp | 9 |

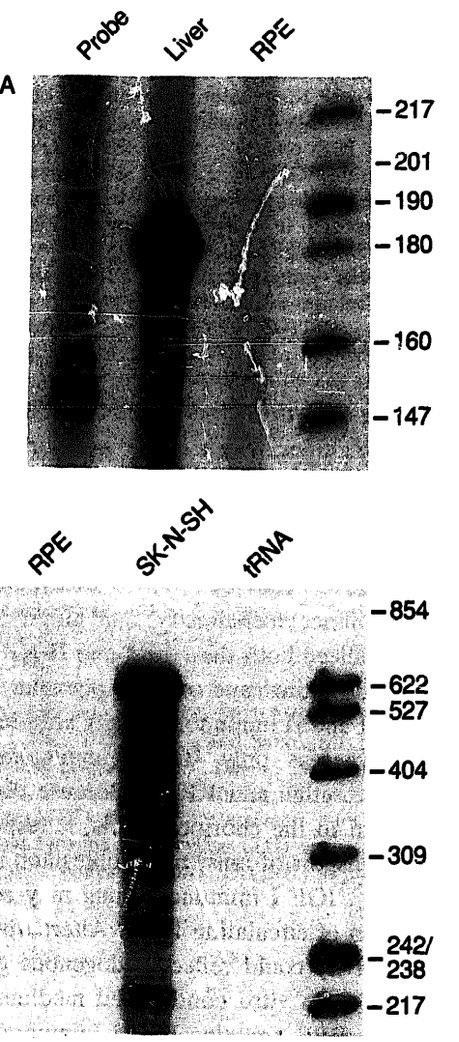


Fig. 1. A: IGF I exon 3 RNase protection assay in human cultured RPE cells. An antisense RNA probe was transcribed from a 1-kb segment of the IGF I gene that contained 182-bp of exon 3. Liver RNA protected the appropriate sized fragment, while RPE RNA did not. Radiograph exposed 36 h. B: IGF II RNase protection assay in cultured human RPE cells. An 854-bp IGF II cDNA obtained from human liver was used as a probe template. RPE did not protect this probe, while the neuroblastoma cell line SK-N-SH protected an expected 580 bp. Radiograph exposed 48 h.

mal cycler for 40 cycles, using the PCR cycle profile recommended by the Taq DNA polymerase manufacturer. The sequences of oligonucleotide primers used are outlined in Table I. Primers were constructed to conserve regions between rat and human sequences[9,25,28]. To eliminate the possibility of DNA contamination, primers spanned intronic sequences[9,28]. After PCR, 20 μl aliquots were electrophoresed in a 1% agarose gel containing 0.4 μg/ml ethidium bromide in 1× TBE buffer as described[29].

*RNase protection assay*

All riboprobes were synthesized from cDNA templates according to the manufacturer's protocol (Promega, Madison, WI). An exon 3 genomic IGF I fragment was provided by Dr. Peter Rotwein[24]. An 854-bp *Eco*RI–*Eco*RI IGF II cDNA fragment was provided by Dr. Graeme I. Bell (Howard Hughes Institute, Chicago, IL)[4]. A 293-bp *Aval*–*Aval* fragment of type I IGF receptor cDNA (bases 2737–3030), provided by Dr. Axel Ullrich (Max Planck Institute, Munich, F.R.G.)[32], was prepared as previously described[36]. Dr. William S. Sly (Washington University School of Medicine, St. Louis, MO) provided the type II IGF receptor cDNA[21]. A 377-bp *Bam*HI–*Bam*HI fragment (bases 2958–3335) of type II IGF receptor cDNA template was prepared as previously described[6].

Each RNA sample (10–50 μg) was hybridized overnight at 50 °C with $5 \times 10^4$ cpm probe in 30 μl buffer containing 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 0.4 M NaCl and 1 mM EDTA. Samples were subsequently digested with 40 μg/ml RNase A (Sigma) for 30 min at 25 °C. Digestion was terminated with proteinase K and SDS. The samples were extracted with phenol and then precipitated at –70 °C with 10 μg tRNA and two volumes of absolute ethanol. Pellets were resuspended in 5 μl of 80% formamide loading buffer and run on a 6% polyacrylamide, 8 M urea sequencing gel. Size markers were prepared by end-labeling *Msp*I-digested fragments of pBR322 (New England Biolabs, Beverly, MA). The gels were dried and exposed to X-ray film in the presence of an intensifying screen at –70 °C for 1–3 days.

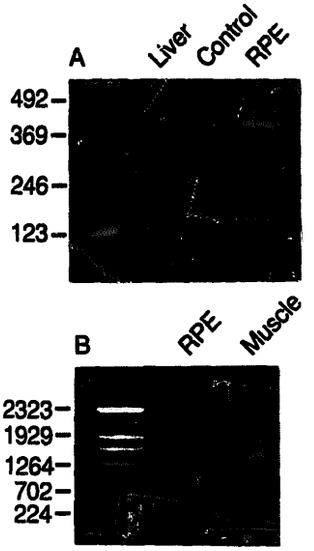


Fig. 2. RT-PCR analysis of RNA in cultured human RPE cells. One μg of total cellular RNA was reverse transcribed followed by PCR for 40 cycles with the primers for either IGF I or IGF II. Sterile $H_2O$ was added instead of RNA in control reactions; all other conditions remained constant. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. A: the IGF I primers produced the expected 366-bp product in RPE cells and rat liver but not in control reaction. B: the IGF II primers produced the expected 249-bp fragment in rat muscle but not in RPE cells.

## RESULTS

### *IGF gene expression*

We were unable to detect IGF I or IGF II mRNA in cultured human RPE cells using Northern analysis of 5 μg poly(A)$^+$ RNA (results not shown). By RNase protection analysis, 30 μg of total cellular liver RNA protected a 182-bp fragment of the IGF I exon 3 probe, whereas RPE RNA protected no visible fragments (Fig. 1A). The neuroblastoma cell line SK-N-SH protected a 580-bp fragment of the 854-bp IGF II probe in RNase protection assay (Fig. 1B). This cell line has been described to express large amounts of IGF II mRNA[35]. The 580-bp protected fragment was smaller than the probe because the IGF II transcipt produced by neuroblastoma cell lines does not contain exons 1, 2 and 3 seen in adult liver[31]. In contrast, RPE RNA did not

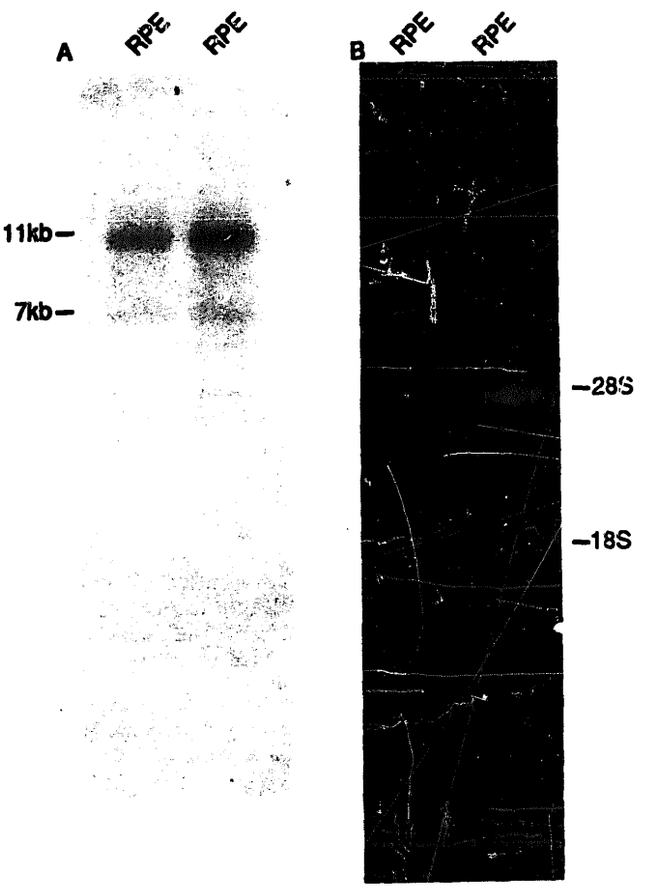


Fig. 3. Northern analysis of type I IGF receptor in cultured human RPE cells. A: Northern blot of 20 μg total RPE RNA. Two transcipts were detected, one 7-kb and one 11-kb. The 11-kb mRNA appears to be the major transcript produced by RPE. Radiograph exposed 7 days. B: photograph of ethidium bromide-stained gel from the Northern blot used in (A), showing 28S (5.0-kb) and 18S (1.8-kb) rRNA bands.

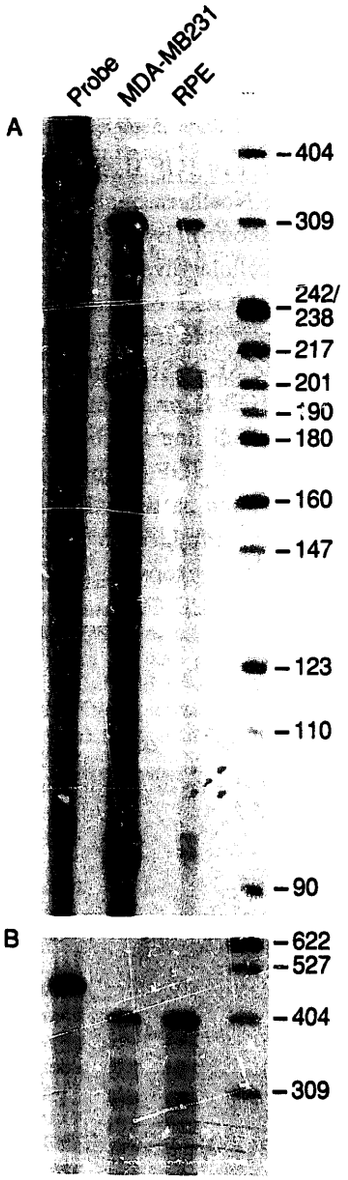


Fig. 4. A: type I IGF receptor RNase protection assay in cultured human RPE cells. RPE and MDA-MB231 RNA protected similar fragments after hybridization with the 293-bp type I IGF receptor probe: a 293-bp fragment, a 200-bp fragment and a 90-bp fragment. Radiograph exposed 5 days. B: type II IGF receptor RNase protection assay in cultured human RPE cells. The complete 377-bp type II IGF receptor probe was protected by MDA-MB231 and RPE RNA. Radiograph exposed 36 h.

protect the 854-bp IGF II probe.

RT-PCR amplified the predicted IGF I 366-bp product from rat liver and RPE RNA but not from the control water sample (Fig. 2A). The expected IGF II 249-bp product was amplified in rat muscle but not in RPE (Fig. 2B) or a control water sample (results not shown).

*IGF receptor gene expression*

Northern hybridization of total RNA (20 $\mu$g) with a $^{32}$P-labelled 1247-bp cDNA probe for type I IGF receptor detected 7-kb and 11-kb transcripts in RPE (Fig. 3A, B). The 11-kb hybridization band was of higher intensity than the 7-kb band, suggesting that the 11-kb mRNA is the major transcript produced by RPE. In RNase protection analysis experiments, RPE RNA protected three fragments of the 293-bp type I IGF receptor cDNA (Fig. 4A). MDA-MB231 RNA protected these same three fragments of the 293-bp type I IGF receptor cDNA: the complete 293-bp fragment, a 200-bp fragment and an 90-bp fragment. This pattern of expression results from alternative splicing of the type I IGF receptor gene[36]. RPE RNA also protected the complete 377-bp *Bam*HI–*Bam*HI fragment of type II IGF receptor probe (Fig. 4B). The less intense, smaller bands are due to the hybridization between sample RNA and fragments of riboprobe synthesized at less than full template length. These bands represent, therefore, an artifact of incomplete probe synthesis and not unique cellular RNAs.

## DISCUSSION

The purpose of this study was to determine if cultured human RPE cells express the genes for IGF I and II and their corresponding receptors. We found low levels of IGF I gene expression detectable only by RT-PCR and not by RNase protection or Northern blotting. Further investigation will be required to prove that these transcripts are translated into IGF I peptide, but the presence of IGF I mRNA transcripts argues for endogenous IGF I production. The presence of RPE IGF I immunoreactivity both in vitro and in vivo support this tenet[34].

In contrast to IGF I, type I IGF receptor mRNA was detected both by Northern blotting and RNase protection. Because Northern analysis is 100-fold less sensitive than RNase protection[26], which in turn is less sensitive than RT-PCR[17], our data suggest that type I IGF receptor mRNA is more abundant in cultured human RPE cells than IGF I mRNA (detectable only by RT-PCR). Northern analysis for the type I IGF receptor revealed 7-kb and 11-kb mRNA transcripts in RPE. This transcript profile is identical to the original hybridization pattern reported by Ullrich and coworkers in 3T3-LI fibroblasts and adipocytes[32]. RNase protection of RPE RNA for the type I IGF receptor protected three fragments: 293 bp, 200 bp and 90 bp. This protection pattern has been reported in the breast cancer cell line MDA-MB 231 where neither increasing amounts of RNase nor changing the time or temperature of the RNase digestion had an effect on the three fragments and is due to alternative splicing of the type I IGF receptor gene[36].

Co-expression of IGF I and IGF I receptor genes by cultured RPE cells suggests an autocrine role for IGF I on RPE. IGF I, acting through its receptor, may serve

as a survival factor for human RPE in vitro, similar to the role of basic fibroblast growth factor in maintaining photoreceptors[10]. IGF I may represent one of the active factors present in RPE-conditioned medium, which stimulates photoreceptor differentiation and Müller cell proliferation[30]. Alternatively, the low level of gene expression of IGF I compared to the type I IGF receptor may imply a paracrine role for circulating IGF I. In retinal endothelial cells, binding of circulating IGF I increases DNA synthesis and regulates endothelial cell chemotaxis[18,19]. Additional experiments examining IGF I mRNA translation into protein may clarify the primary mode of action of IGF I in RPE.

We found no IGF II gene expression in cultured human RPE cells. While present in fetal sclera and cornea[13], no adult ocular source has been reported[27], suggesting that adult vitreal IGF II immunoreactivity arises from circulating IGF II[12]. Human cultured RPE cells synthesized mRNA for the type II IGF receptor, supporting the report of Ocrant and coworkers of substantial levels of type II IGF receptor immunoreactivity in the RPE[20]. The IGF II receptor, identical to the mannose-6-phosphate receptor[16,23,27], is instrumental in RPE phagocytosis of mannose-6-phosphate containing proteins[37]. It is not yet known if the RPE type II IGF receptor can mediate potential growth-promoting effects of circulating IGF II.

In summary, we observed gene expression of IGF I and the types I and II IGF receptors in cultured human RPE cells. Further work is now required to understand regulation of this expression as well as the physiological roles of the IGFs and their receptors in the RPE and eye.

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