Production of IGF-I and IGF binding proteins by retinal cells in vitro

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Abstract

Insulin-like growth factor I (IGF-I) and its associated binding proteins (IGFBPs) have been identified in retinal tissues but their precise cellular origin remains unclear. The aim of this study was to examine the ability of three retinal cell types (microvascular endothelial cells, pericytes, and pigment epithelial cells) and a non-retinal cell type (Tenon's fibroblasts) to produce IGF-I and cell specific IGFBPs in vitro. Using a radioimmunoassay we demonstrated that all four cell types produce IGF-I and that this production continued over a 72 hour period. In addition, western ligand blotting revealed that each cell type produced at least one IGFBP and that each cell type produced a different IGFBP profile. Endothelial cells produced a 24 kDa band only, pericytes produced both a 24 kDa and a 34 kDa band, retinal pigment epithelial cells produced a 38-41 kDa band, while fibroblasts produced both a 24 kDa and a 30 kDa band. Laser scanning densitometry demonstrated that in the majority of experiments the IGFBPs accumulated in the culture medium over a 72 hour period. Neither IGF-I nor IGFBPs were observed in cell lysates indicating de novo synthesis and secretion in vitro. These results suggest an autocrine/paracrine function for IGF-I and its associated binding proteins which may play a significant role in both retinal physiology and disease.

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Insulin-like growth factor I (IGF-I) is a polypeptide which stimulates the growth, differentiation, and metabolism of a variety of cell types.¹ Its activity is controlled in a complex manner – for example, IGF-I expression in most tissues is known to be influenced by hormonal, nutritional, tissue specific, and developmental factors.²⁻⁵ In addition it appears that the growth promoting effects of many circulating hormones may be mediated via the stimulation of local IGF-I production.¹

IGF-I activity in extracellular fluids is carefully controlled by its association with insulin-like growth factor binding proteins (IGFBPs).⁶ Like IGF-I, the IGFBPs appear to be produced from a variety of cell types⁵; six distinct binding proteins have been cloned, sequenced, and given a uniform nomenclature.^{7,8} Their principal functions range from acting merely as transport vehicles for IGF-I/IGF-II to playing an integral role in overall IGF-I physiology.^{1,7} A further determinant of IGF-I function is its binding to specific cell surface receptors which include the closely related insulin and IGF-I receptors and the IGF-II/mannose-6-phosphate receptors,

each of which exhibit varying affinities for IGF-I. Thus, the interaction of IGF-I, IGFBPs, and a family of closely related receptors is responsible for the net effect of IGF-I on target cell function.

Evidence suggests that IGF-I may play an important role in retinal development and physiology. The adult neural retina from a variety of species has been shown to express both the IGF-I and the IGF-I receptor gene⁹⁻¹¹ and IGF-I receptors have been localised to the inner and outer segments of photoreceptor cells.¹²⁻¹⁴ Large numbers of IGF-I binding sites have been identified in the embryonic chick retina^{15,16} and it is also becoming apparent that the whole spectrum of IGFBPs are present in ocular tissues.¹⁷⁻²⁰ While the origin of these binding proteins remains largely unknown, recent evidence has demonstrated the expression of two major IGFBPs, IGFBP-2 and IGFBP-3, in the cornea, iris/ciliary body complex, and the retina.^{18,21}

The characterisation of both IGF-I and their associated binding proteins in ocular cells remains incomplete. The aim of this study was, therefore, to investigate the production of both IGF-I and IGFBP isoforms by four ocular cell types in vitro.

Materials and methods

CELL CULTURE

Retinal microvascular endothelial cells, retinal pericytes, and Tenon's capsule fibroblasts (a control non-intraocular cell type used throughout this study) were isolated from bovine eyes as previously described.²² Endothelial cells were grown on gelatin (Sigma Chemical Co, St Louis, MO, USA) coated 35 mm dishes (Falcon) and maintained in a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) plus 7.5% (v/v) human platelet-poor plasma with pericyte conditioned medium²² supplemented with 20 µl/ml bovine retinal crude extract²³ and 90 µg/ml heparin (Sigma). Pericytes and fibroblasts were routinely grown in 25 cm³ tissue culture flasks (Falcon) and maintained in DMEM supplemented with 20% heat inactivated fetal calf serum (FCS) (Northumbria Biologicals) and antibiotics.

Retinal pigment epithelial (RPE) cells were isolated from donor human eyes as previously described.²⁴ RPE cells were routinely grown in 75 cm³ tissue culture flasks (Costar) and maintained in Ham's F10 supplemented with 20% FCS, antibiotics, and 0.4% glucose (BDH). All cells were routinely incubated at 37°C in a standard 5% carbon dioxide/95% air incubator. In all experiments, each cell type was used between second and fifth passage. Confirmation

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of the purity of the cultures was carried out as previously described.^{22,25}

GROWTH FACTOR PROFILES OF RETINAL CELLS

Cells were detached using 0.25% (v/v) trypsin (Difco) and plated into 24 well plates in their respective media (in the case of endothelial cells the wells were precoated with gelatin). Upon confluence, cells were washed twice in Dulbecco's phosphate buffered saline (PBS) and the medium was replaced with either DMEM supplemented with 7.5% (v/v) human platelet-poor plasma or plasma-free DMEM. Cells were incubated in the two types of media till the following time points: 0, 6, 12, 24, 48, and 72 hours. After each time point the 'conditioned' media were removed and the cells washed twice with PBS and lysed with 0.5% Triton X-100, 20 mM NH_4OH , and 0.1% serum albumin in PBS. Both sets of samples were centrifuged at 3000 g to remove cell debris and the supernatants stored at -20°C before assessment for IGF-I and IGF-BPs. Additional wells were used for cell counts at each time point using a haemocytometer. All experiments were performed with confluent monolayers, using six wells per time point for each cell type and the experiments repeated at least three times for each cell type.

EXTRACTION AND RADIOIMMUNOASSAY FOR IGF-I

Before the assay of culture medium for IGF-I, IGF-BPs were removed from the media samples by acid-ethanol extraction.²⁶ Briefly, media samples (200 μl) were incubated for 30 minutes at room temperature with 800 μl of an extraction mixture (87.5% ethanol/12.5% 2 M HCl, v/v). Extracted media samples were then centrifuged at 1850 g for 30 minutes. A 500 μl aliquot of the supernatant was then transferred into fresh tubes and neutralised with 0.855 M Tris (pH 11) at a ratio of 5:2. IGF-I within the culture media extracts was assayed against human IGF-I (International Reference Preparation coded 87/518; National Institute of Biological Standards and Control, Potters Bar, Herts), serially diluted over the range 0.1–125 ng/ml in radioimmunoassay (RIA) buffer (PBS plus 0.1% (v/v) Tween-20, and 0.1% (w/v) bovine serum albumin) and extracted in an identical fashion to the media samples. The RIA reaction mixture comprised 100 μl ^{125}I -IGF-I, 100 μl unlabelled IGF-I (in the case of standard/test samples) and 100 μl polyclonal rabbit anti-human IGF-I antiserum (donated by Dr Helen Beere, Manchester University) at a dilution of 1:37 500. Reagent additions were carried out on ice and the tubes were incubated overnight at 4°C . After incubation, 100 μl of anti-rabbit IgG coated with cellulose (IDS) was added to each reaction tube except those for total counts. The mixtures were incubated for 1 hour at 37°C . Thereafter, 0.5 ml of RIA buffer was added and the tubes were centrifuged at 1850 g for 15 minutes. The supernatant was discarded, and the pellet was assayed on a gammacounter. In addition, endogenous levels of IGF-I were determined in plasma-containing media which had not been exposed to cells.

WESTERN LIGAND BLOTTING

Media IGF-BPs were identified using methods previously described.²⁶ Media samples were subjected to non-reducing SDS-PAGE (12.5% gel) under 100 V (continuous voltage) and then transferred to nitrocellulose filters (0.45 μm pore size).

Ligand blot analysis was performed at 37°C essentially as described by Hossenlopp,²⁷ in brief, the nitrocellulose membranes were sequentially blocked for 30 minutes in Tris-buffered saline (TBS; 10 mM Tris/HCl and 0.15 M NaCl, pH 7.4) containing 3% Nonidet P-40 (Sigma); 2 hours in TBS containing 1% bovine serum albumin, and, finally, 10 minutes in TBS containing 0.1% Tween 20. Binding of ^{125}I -IGF-II (1×10^6 cpm/5 ml) to the blot was conducted for 3 hours in a sealed plastic bag in TBS containing 1% bovine serum albumin and 0.1% Tween 20. Thereafter, the nitrocellulose membrane was washed twice in TBS plus 0.1% Tween 20, and then three times in TBS alone. The membranes were air dried. A range of prestained molecular weight markers were run in a parallel lane to confirm the molecular weights of each band. Bands were visualised by autoradiography and the relative signals were quantitated by scanning densitometry. Comparative studies were also carried out using ^{125}I -IGF-I (1×10^6 cpm/5 ml) as the radioligand.

Results

All cell types maintained under plasma-free conditions released detectable levels of IGF-I throughout the time course of this study (Fig 1). However, IGF-I production in plasma-containing media was restricted to the RPE cells and only at the 72 hour time point by which time IGF-I levels were greater than the endogenous level in plasma-containing medium (15 (SD 1) ng/ml). The release of IGF-I into plasma-free medium varied between cell types (Fig 1). Both pericytes and RPE cells continually produced IGF-I throughout the time course of the study.

Figure 1 Production of IGF-I by retinal microvascular endothelial cells, pericytes, RPE cells, and Tenon fibroblasts in plasma-free and plasma-containing media over a period of 72 hours. Plasma-free medium: retinal microvascular endothelial cells (□), pericytes (▲), RPE cells (▼), and Tenon fibroblasts (◆). Plasma-containing medium: retinal microvascular endothelial cells and Tenon fibroblasts (○), RPE cells (●). For plasma containing media the endogenous IGF-I levels in 'unconditioned' media (15 (SD 1) ng/ml) were subtracted from the total IGF-I present at each time point. The values presented are the mean of three experiments. The vertical bars represent SEM.

Figure 2 Western ligand blot of IGFBPs produced by the four ocular cell types in the presence (+) or absence (-) of plasma. NC=no cells; R=retinal pigment epithelium cells; P=pericytes; F=Tenon's fibroblasts; E=retinal microvascular endothelial cells. The estimated Mr (kDa) of each form was derived by comparison with defined Mr standards. This autoradiograph was representative of three separate experiments.

By contrast, production of IGF-I by endothelial cells and fibroblasts was continuous up to 48 hours and began to level off after 48 hours. Retinal microvascular endothelial cells produced the highest levels of IGF-I at each time point. Pericytes and fibroblasts produced similar levels of IGF-I over the 72 hour period. However, while RPE cells demonstrated similar levels of IGF-I to pericytes and fibroblasts over the first 48 hours the levels of IGF-I were approximately threefold higher at the 72 hour time point.

Cell numbers for retinal microvascular endothelial cells, pericytes, and RPE cells remained constant in both plasma-containing and plasma-free medium throughout the time course of the experiment. Cell numbers for fibroblasts remained constant in plasma-containing medium throughout the experimental period but those in plasma-free medium showed a 50% reduction in cell numbers after 48 hours.

Each cell type studied produced a distinct IGFBP profile which was identical in both the plasma-containing and plasma-free media (Fig 2), however, the production of IGFBPs in plasma-free medium was greatly reduced compared with that in plasma-containing medium. In the case of plasma-containing media IGFBP profiles were clearly distinguishable from the endogenous IGFBPs in the plasma (predominantly a 38–41 kDa band). No bands were observed in the plasma-free media that had not been exposed to cells.

Endothelial cells produced a 24 kDa band only. Pericytes produced two IGFBPs, one at 34 kDa and a faint band at 24 kDa. RPE cells produced a 38–41 kDa band only while fibroblasts produced a 30 kDa and a faint band at 24 kDa. Identical IGFBPs profiles were observed using ^{125}I -IGF-I as the ligand.

Results from both western ligand blotting and subsequent densitometric analysis depicted differential production of IGFBPs with respect to time, media type, and IGFBP type (Figs 3–6; Table I). In all cases IGFBP accumulation in the culture media was greatest at the 72 hour time point, but the rate of accumulation varied with cell type. Ultrascan data for endothelial cell production of the 24 kDa IGFBP showed a slight variation between the two types of media; production in the plasma-containing media was continuous and similar to that observed for their production of IGF-I (Fig 1), while production in the plasma-free media increased steadily from 12 to 48 hours whereupon the levels of IGFBP remained constant. By contrast, retinal pericyte production of the predominant 34 kDa and the 24 kDa IGFBP depicted similar trends of accumulation in both media types (that is, the production of both increased steadily as a function of time).

Production of the 38–41 kDa IGFBP by RPE

cells appeared to follow no trend in the plasma-containing media, whereas in the plasma-free media production increased rapidly with time (identical to RPE production of IGF-I). Fibroblast production of both the 30 kDa and 24 kDa IGFBPs peaked at 48 hours in the plasma-containing media, after which time it decreased dramatically (indicative of proteolytic degradation). By contrast, production of the 30 kDa IGFBP remained relatively constant after 12 hours of production in the plasma-free medium (although a peak of production was observed at 72 hours); after a 6 hour lag period production of the 24 kDa IGFBP was continuous with time in the plasma-free media. The 24 kDa IGFBP

Figure 3 Western ligand blot showing the production of the 24 kDa IGFBP by endothelial cells over a 72 hour time course. The time course experiments were carried out in the presence (+ plasma) and the absence (- plasma) of plasma. This autoradiograph was representative of three separate experiments.

Figure 4 Western ligand blot showing the production of both the 24 and 34 kDa IGFBPs by pericytes over a 72 hour time course. The time course experiments were carried out in the presence (+ plasma) and the absence (- plasma) of plasma. This autoradiograph was representative of three separate experiments.

Figure 5 Western ligand blot showing the production of the 38–41 kDa IGFBP produced by RPE cells over a 72 hour time course. The time course experiments were carried out in the presence (+ plasma) and the absence (- plasma) of plasma. This autoradiograph was representative of three separate experiments.

Figure 6 Western ligand blot showing the production of both the 30 and 24 kDa IGFBPs by Tenon's fibroblasts over a 72 hour time course. The time course experiments were carried out in the presence (+ plasma) and the absence (- plasma) of plasma. This autoradiograph was representative of three separate experiments.

Table 1 Densitometric analysis of the IGFBP bands generated by western ligand blotting as shown in Figures 3–6. Densitometric measurements are given in arbitrary units

Cell type	IGFBP (kDa)	Media type (+/- plasma)	Time (hours)						
			0	6	12	24	48	72	
Endothelial	24	+	0.06	0.10	0.15	0.19	0.21	0.24	
		–	ND	ND	0.07	0.11	0.12	0.11	
Pericyte	34	+	ND	0.08	0.34	0.40	0.80	0.90	
		–	ND	ND	0.01	0.02	0.03	0.10	
	24	+	ND	ND	ND	0.02	0.03	0.08	
		–	ND	ND	0.02	0.04	0.06	0.15	
RPE	38–41	+	3.6	4.7	4.3	6.3	7.8	7.0	
		–	ND	0.05	0.08	0.1	0.4	8.6	
Fibroblast	30	+	ND	0.52	0.53	0.90	1.1	0.30	
		–	ND	0.02	0.03	0.03	0.03	0.06	
	24	+	ND	0.04	0.05	0.35	0.90	0.71	
		–	ND	ND	0.01	0.04	0.07	0.20	

ND=Not detectable. The data presented are the mean of one experiment. It was impossible to combine the data from different experiments owing to between-experiment variation in labelling for specific activity for probes in ligand-binding analysis and different exposure times for x ray films. However, the same trend in band intensity was observed in each experiment.

appeared to demonstrate a lag period of up to 24 hours before its accumulation could be observed in the media for those cell types responsible for its production.

No detectable IGF-I or IGFBPs were observed in the cell lysates for each of the four cell types studied at any time point.

Discussion

This study has demonstrated (a) that retinal cells simultaneously secrete both IGF-I and IGFBPs, and (b) that the profiles of IGFBPs secreted are dependent on cell type.

Our results support and extend those of Brooks and associates²⁸ and Waldbillig and associates^{12,29} who have demonstrated that both retinal microvascular endothelial cells and RPE cells produce IGF-I in vitro. It is difficult to make comparisons between the levels of IGF-I produced in the various studies owing to the different extraction procedures used before radioimmunoassay and the different cell culture conditions (it is likely that the levels of IGF-I produced by individual cell types are media dependent). While observing different levels of IGF-I production between human and bovine cells this study does not take into account possible species specific, post-translational modification of IGF-I that may occur in bovine cells when compared with human cells.

The apparent lack of production of IGF-I in serum/plasma-containing media is supported by (a) Brooks *et al.*²⁸ who observed IGF-I production by bovine microvascular cells in serum-free medium but failed to observe IGF-I production in serum-containing medium, and (b) Lowe *et al.*³⁰ who concluded that serum factors may inhibit IGF-I production. This discrepancy between IGF-I levels in plasma-free versus plasma-containing medium can be explained in one of two ways. Firstly, there may be negative feedback on the production of IGF-I by retinal cells owing to the presence of endogenous IGF-I in the plasma (15 ng/ml) as postulated by Brooks *et al.* However, this is unlikely since IGF-I production was observed in plasma-free medium at IGF-I levels greater than 40 ng/ml. It is possible, however, that other endogenous factors in plasma are capable of inhibiting IGF-I production; transforming growth factor β has been

shown to downregulate IGF-I production in vitro.³¹ Secondly, the inability to measure IGF-I in plasma-containing media may be due to the effect of plasma on the extraction procedure. The sensitivity of the RIA is reduced 15-fold when IGF-I extraction is undertaken in the presence of plasma as opposed to plasma-free medium (results not shown). Thus it is plausible that the cells were indeed secreting IGF-I in the plasma-containing medium but that it remained below the sensitivity of the RIA. Support for this explanation comes from the fact that IGFBP secretion was seen in both the plasma-containing and plasma-free media.

This study is the first to demonstrate that retinal cells secrete cell-specific IGFBP profiles in a time-dependent manner in vitro. Although western ligand blotting enables the identification of IGFBPs by molecular weight, this technique does not have the positive advantages of immunoblotting or RIA which categorically identify each IGFBP isoform. However, the recent discovery of IGFBP proteases present in a variety of biological fluids necessitates a comprehensive re-evaluation of techniques for assaying IGFBPs.^{4,29} Nevertheless, on the basis of molecular weight the following IGFBP isoforms can be tentatively identified: 38–41 kDa=IGFBP-3, 34 kDa=IGFBP-2, 30 kDa=IGFBP-1, and 24 kDa=IGFBP-4.^{4,27} The only previous reports on IGFBP production by retinal cells in vitro relate to RPE cells. Waldbillig and associates²⁹ reported that monkey RPE secreted a broad band of IGFBPs between 36 and 49 kDa which failed to accumulate in the culture medium beyond 12 hours' incubation indicative of IGFBP degrading activity in the culture system. Ocran and associates²⁰ observed a 31 kDa band for bovine RPE. By contrast, we observed a continuous production of a well defined 38–41 kDa band (tentatively identified as IGFBP-3) by human RPE cells throughout the time course of the experiment. While species variation cannot be ruled out, only definitive sequence data can explain the reasons for these differences.

That IGF-I and its associated binding proteins accumulate in the conditioned media of ocular cells does not necessarily indicate the release of previously internalised IGF-I or IGFBPs (a phenomenon known to occur in endothelial cells³²) since cell lysates did not demonstrate detectable levels of either IGF-I or IGFBPs. However, the production of both IGF-I and the IGFBPs may be significantly higher than the values reported here since internalisation and degradation may have been occurring during the time course of the experiments.

The individual roles that IGFBPs play in overall IGF-I physiology is still unclear. While some reports suggest an inhibitory role by restricting access to receptors, others have suggested that IGFBPs facilitate growth by augmenting interaction with receptors, thus potentiating a mitogenic response.^{4,7} Nearly all binding proteins have been shown to be capable of exerting both stimulatory and inhibitory effects, depending on the cell culture conditions, cell type, etc.^{1,4}

This study demonstrates that the retina contains all the components of a self-contained IGF-

I/IGFBP autocrine/paracrine system which may play an important role in retinal physiology and disease – for example, in proliferative diabetic retinopathy (PDR).^{17–33} All the retinal cell types studied produced IGF-I at concentrations in excess of those reported to stimulate their proliferation,³⁴ their migration,³⁵ and the release of enzymes in the plasminogen cascade,³⁶ which are associated with the cellular events in diabetic neovascularisation.³⁷ It is becoming increasingly apparent that both the type and amount of IGFBPs will variously affect the ability of IGF-I to associate with cell-surface binding sites.⁴ The observation that autocrine/paracrine-derived IGF-I has a decreased affinity for its associated binding proteins³⁸ compared with endocrine derived IGF-I may imply that the autocrine/paracrine production of IGFBPs is of limited significance under normal conditions but may have a role in disease states such as PDR where the contribution from serum-derived IGF-I is more prominent.

In conclusion, the data presented here clearly demonstrate that all three retinal cell types studied are capable of producing both IGF-I and cell-specific IGFBP(s) in vitro. However, exactly how this IGF-I/IGFBP system operates to maintain the homeostasis of the retina, and how its role changes in retinal diseases, requires further investigation.

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