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Discovery and characterization of IGFBP-mediated endocytosis in the human retinal pigment epithelial cell line ARPE-19

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ABSTRACT

Insulin-like growth factor binding proteins (IGFBPs) are prime regulators of IGF-action in numerous cell types including the retinal pigment epithelium (RPE). The RPE performs several functions essential for vision, including growth factor secretion and waste removal via a phagocytic process mediated in part by vitronectin (Vn). In the course of studying the effects of IGFBPs on IGF-mediated VEGF secretion and Vn-mediated phagocytosis in the RPE cell line ARPE-19, we have discovered that these cells avidly ingest synthetic microspheres (2.0 μm diameter) coated with IGFBPs. Given the novelty of this finding and the established role for endocytosis in mediating IGFBP actions in other cell types, we have explored the potential role of candidate cell surface receptors. Moreover, we have examined the role of key IGFBP structural motifs, by comparing responses to three members of the IGFBP family (IGFBP-3, IGFBP-4 and IGFBP-5) which display overlapping variations in primary structure and glycosylation status. Coating of microspheres (FluoSpheres®, sulfate modified polystyrene filled with a fluorophore) was conducted at 37 °C for 1 h using 20 μg/mL of test protein, followed by extensive washing. Binding of proteins was confirmed using a microBCA assay. The negative control consisted of microspheres treated with 0.1% bovine serum albumin (BSA), and all test samples were post-treated with BSA in an effort to coat any remaining free protein binding sites, which might otherwise encourage non-specific interactions with the cell surface. Serum-starved cultures of ARPE-19 cells were incubated with microspheres for 24 h, using a ratio of approximately 100 microspheres per cell. Uptake of microspheres was quantified using a fluorometer and was confirmed visually by confocal fluorescence microscopy. The ARPE-19 cells displayed little affinity for BSA-treated microspheres, but avidly ingested large quantities of those pre-treated with Vn (ANOVA; $p < 0.001$). Strong responses were also observed towards recombinant formulations of non-glycosylated IGFBP-3, glycosylated IGFBP-3 and glycosylated IGFBP-5 (all $p < 0.001$), while glycosylated IGFBP-4 induced a relatively minor response ($p < 0.05$). The response to IGFBP-3 was unaffected in the presence of excess soluble IGFBP-3, IGF-I or Vn. Likewise, soluble IGFBP-3 did not induce uptake of BSA-treated microspheres. Antibodies to either the transferrin receptor or type 1 IGF-receptor displayed slight inhibitory effects on responses to IGFBPs and Vn. Heparin abolished responses to Vn, IGFBP-5 and non-glycosylated IGFBP-3, but only partially inhibited the response to glycosylated IGFBP-3. Our results demonstrate for the first time IGFBP-mediated endocytosis in ARPE-19 cells and suggest roles for the IGFBP-heparin-binding domain and glycosylation status. These findings have important implications for understanding the mechanisms of IGFBP actions on the RPE, and in particular suggest a role for IGFBP-endocytosis.

1. Introduction

The insulin-like growth factor binding proteins (IGFBPs) are a family of 6 structurally related peptides which modulate the bioavailability and action of the insulin-like growth factors (IGF-I and IGF-II) (Clemmons, 2001; Firth and Baxter, 2002). These actions of IGFBPs are mediated systemically via binding to

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circulating IGF peptides secreted by the liver. Further interactions between IGFBPs and IGFs are enabled within tissues via cellular secretion and binding of IGFBPs to the extracellular matrix (ECM) and cell surface. The resulting interactions between ECM proteins, IGFBPs, IGFs and the major IGF receptor (type 1 or IGF-1R) produce variable effects according to cell type, cellular activity and IGFBP structure. IGFBPs can also exert direct effects on a variety of cellular activities including migration, proliferation and apoptosis (Yamanaka et al., 1999; Firth and Baxter, 2002; Mochizuki et al., 2006). While the mechanisms underlying these non-IGF mediated actions of IGFBPs are less well understood, evidence points towards a role for IGFBP-receptors (Leal et al., 1997; Lee et al., 2004), IGFBP-endocytosis (Goda et al., 2008) and nuclear localisation (Jaques et al., 1997). Our current studies of IGF/IGFBP action when applied in conjunction with the ECM molecule vitronectin (Vn) have led to the discovery that members of the IGFBP family promote phagocytosis of synthetic microspheres by ARPE-19 cells, a spontaneously immortalised cell line derived from human retinal pigment epithelial cells (Dunn et al., 1996). This novel finding provides valuable evidence towards understanding the mechanisms of IGFBP action in the RPE.

The retinal pigment epithelium (RPE) is a single layer of highly-specialised epithelial cells which separates the photoreceptor cells of the retina from the adjacent choroidal blood supply (Strauss, 2005). Movement of nutrients and waste products between the blood stream and the photoreceptor cells is dependent upon the RPE transport mechanisms. In particular, vision is maintained in part via the phagocytosis and recycling of outer segments referred to as POS shed daily from the tips of adjacent photoreceptor cells. Attachment and coordinated phagocytosis of POS is mediated in part via $\alpha_v\beta_5$ integrins expressed by the RPE (Nandrot et al., 2004, 2006). The major ligand for $\alpha_v\beta_5$ is vitronectin (Vn), a 70 kDa glycoprotein which while principally regarded as an ECM molecule deposited from serum (Schvartz et al., 1999), is secreted by retinal cells including the RPE (Anderson et al., 1999; Hageman et al., 1999) and has been shown to stimulate phagocytosis of POS by the RPE cells *in vitro*. All six classical IGFBPs along with IGF-I and the IGF-1R are also expressed by the RPE (Yang and Chaum, 2003) and important links have been found between IGF action, hypoxia and retinal pathology via secretion of vascular endothelial growth factor (VEGF) (Lambooij et al., 2003; Rosenthal et al., 2004). To the best of our knowledge however, no studies have been made of the potential of the IGF system to directly influence the phagocytic activity of the RPE via interactions with Vn or other means.

Select IGFBPs, including IGFBP-3, facilitate binding of IGF-I to Vn (Kricker et al., 2003). The resulting Vn-IGFBP-IGF-I complexes stimulate migratory responses which are significantly above those observed towards the individual proteins (Hyde et al., 2004; Ainscough et al., 2006). In other studies, impaired cellular responses to IGF-I have been demonstrated when access to the Vn receptor is blocked (Jones et al., 1996; Zheng and Clemmons, 1998; Upton et al., 2008). Such observations have led to the conclusion that optimal responses to IGF-I are dependent upon co-activation of integrin receptors and there is increasing evidence to support this view (Van Lonkhuyzen et al., 2007; Hollier et al., 2008). This knowledge led us to consider that the RPE cell responses to IGF-I including VEGF secretion might be similarly affected by a requirement for co-activation of integrins. Likewise, integrin mediated responses such as Vn-activated phagocytosis may in turn be affected by co-activation of IGF signalling pathways. We therefore hypothesised that certain IGFBPs might alter the phagocytic and VEGF secretion activities of the RPE based upon their ability to form complexes with IGF-I and Vn (Upton et al., 1999; Kricker et al., 2003). More specifically, we have examined the individual and combined effects of Vn, IGFBP-3 and IGF-I on VEGF secretion and phagocytosis by ARPE-19 cells. Given that it is difficult to control the surface composition of POS extracted from retinal tissue we have utilised a phagocytosis assay based upon the endocytosis of fluorescent polystyrene microspheres which support coating with single or defined combinations of test proteins. Our results have demonstrated for the first time an endocytic response to substrate-bound IGFBPs by ARPE-19 cells. We have explored this discovery in conjunction with measures of VEGF secretion, and have investigated the potential role of key structural motifs including the IGFBP-heparin-binding domain, glycosylation status and candidate receptors.

2. Materials and methods

2.1. Reagents

Vitronectin purified from human plasma (Cat. No. G538) was purchased from Promega Corporation, Annandale, NSW, Australia. Non-glycosylated, recombinant human IGFBP-3 was obtained from Professor Zee Upton, Queensland University of Technology (QUT). Glycosylated forms of recombinant human IGFBP-3 and IGFBP-5, as well as recombinant human IGF-I, were acquired from Novozymes-Gropep Ltd, Adelaide, SA, Australia (Cat. Nos. BP3BU020, BP5BU020 and IU100 respectively). Glycosylated IGFBP-4 from R&D Systems (Cat. No. 804-GB) was provided by Dr Brett Hollier (QUT). Fluorescent polystyrene microspheres (2.0 μm diameter; Cat. No. F8853) were obtained from Molecular Probes/Invitrogen Australia Pty Ltd, Mount Waverley, VIC, Australia. The AccuKine Human VEGF^{165} ELISA kit (Cat. No. E3075) was purchased from Apollo Cytokine Research, Sydney Australia. Bovine serum albumin (BSA, Cat No. A7030), cobalt chloride (Cat. No. 15862-1ML-F) and heparin (Cat. No. H4784) were obtained from Sigma–Aldrich, Castle Hill, NSW, Australia. Monoclonal antibodies raised against the type 1 IGF receptor (clone αIR3) and transferrin receptor (clone 42/6) were purchased from Merck Pty Ltd, Kilsyth, VIC, Australia. Isotype control antibodies were obtained from BD Biosciences (IgA isotype; Cat No. 553476), and Sigma–Aldrich (IgG1 isotype; Cat No. M9269). Foetal bovine serum was from Thermo Trace, Noble Park, VIC, Australia (Cat. No. 15-010-0500V).

2.2. Maintenance of ARPE-19 cells

The ARPE-19 cell line (Dunn et al., 1996) (ATCC CRL-2302, Lot# 4052078, passage number 20) was purchased from Cryosite Distribution Pty Ltd, Lane Cove, NSW, Australia. Upon receipt the cells were expanded in the recommended growth medium consisting of DMEM:F12 (1:1) supplemented with 2.5 mM L-glutamine, 15 mM HEPES, 10% foetal bovine serum and 1% penicillin/streptomycin solution. The expanded stocks tested negative for mycoplasma and were stored in liquid nitrogen. Experiments were conducted using cells derived from passage numbers less than 30.

2.3. VEGF secretion assay

Secretion of VEGF was measured using an AccuKine Human VEGF^{165} ELISA kit. ARPE-19 cells were harvested from near confluent flasks using trypsin/EDTA, and plated in serum-supplemented medium at a density of 4×10^4 cells per well into a 24-well plate. The cells were cultured to confluency for three days and then serum-starved for 24-h prior to addition of test reagents in 500 μL of serum-free culture medium. Control treatments consisted of serum-free medium (SFM) and serum-supplemented growth medium (SSM). After treatment, the culture medium was removed and stored at −80 °C for up to 2 weeks until assay.