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REVIEW

Recent insights into the actions of IGFBP-6

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Abstract IGFBP-6 is an O-linked glycoprotein that preferentially binds IGF-II over IGF-I. It is a relatively selective inhibitor of IGF-II actions including proliferation, survival and differentiation of a wide range of cells. IGFBP-6 has recently been shown to have a number of IGF-independent actions, including promotion of apoptosis in some cells and inhibition of angiogenesis. IGFBP-6 also induces migration of tumour cells including rhabdomyosarcomas by an IGF-independent mechanism. This chemotactic effect is mediated by MAP kinases. IGFBP-6 binds to prohibitin-2 on the cell surface and the latter is required for IGFBP-6-induced migration by a mechanism that is independent of MAP kinases. IGFBP-6 may enter the nucleus and modulate cell survival and differentiation. IGFBP-6 expression is decreased in a number of cancer cells and it has been postulated to act as a tumour suppressor. IGFBP-6 expression is increased in a smaller number of cancers, which may reflect a compensatory mechanism to control IGF-II actions or IGF-independent actions. The relative balance of IGF-dependent and IGF-independent actions of IGFBP-6 in vivo together with the related question regarding the roles of IGFBP-6 binding to IGF and non-IGF ligands are keys to understanding the physiological role of this protein.

Keywords Insulin like growth factor · Insulin like growth factor binding protein-6 · Migration · Proliferation · Survival · Structure

Abbreviations

BAP	B-cell receptor-associated protein
cAMP	cyclic AMP
Hh	hedgehog
HIF	hypoxia-inducible factor
IGF	insulin-like growth factor
IGFBP	IGF binding protein
LMP	LIM mineralization protein
MAP kinase	mitogen activated protein kinase
MMP	matrix metalloprotease
NF-κB	nuclear factor-κB
PHB	prohibitin
REA	repressor of estrogen receptor activity
RMS	rhabdomyosarcoma
SEMA	semaphorin
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

Introduction

The family of six high affinity insulin-like growth factor binding proteins (IGFBPs) is a major regulator of IGF actions. In addition, most IGFBPs have been reported to have IGF-independent actions. IGFBP-6, the focus of this article, is distinctive for its ~50-fold binding preference for IGF-II over IGF-I and relatively specific inhibition of IGF-II actions (reviewed in (Bach 1999, 2005)). Recent studies indicate that IGFBP-6 also inhibits angiogenesis and promotes cell migration by IGF-independent mechanisms, and enters the nucleus

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where it modulates differentiation and survival (Bach, et al. 2013).

Structure

IGFBPs 1–6 share a three-domain structure (Bach, et al. 2005). The N-domain contains a highly conserved high-affinity IGF binding subdomain that has two disulphide linkages stabilising a globular structure containing a three-stranded anti-parallel β-sheet (Kalus, et al. 1998). The N-terminal subdomains of IGFBPs 1–5 contain a conserved GCGCC motif and a ladder-like structure stabilised by four disulphide bonds (Sitar, et al. 2006). IGFBP-6 differs from other IGFBPs as it lacks this cysteine-rich motif and only has three disulphide bonds (Neumann and Bach 1999). Further, a peptide based on this subdomain is predominantly extended with minimal secondary structure (Chandrashekaran, et al. 2007).

The C-domains of IGFBPs 1–6 share sequence homology and a common structure. Each contains three homologous disulphide linkages, a conserved CWCV sequence and a thyroglobulin type 1 fold comprising an α -helix followed by a loop, a three-stranded antiparallel β-sheet incorporating a second loop, and finally a disulphide-bonded flexible third loop (Bach et al. 2005; Headey, et al. 2004a). The C-domain binds IGFs through a largely hydrophobic surface involving the α-helix, the first β-strand, and the first and second loops. Many IGF-independent actions of IGFBPs are mediated by interaction of the C-domain with other proteins or glycosaminoglycans (Bach et al. 2005).

In contrast to their N- and C-domains, the linker domains of the six IGFBPs have no sequence homology. They vary in length and are sites of post-translational modifications including glycosylation, phosphorylation and proteolysis. They do not directly bind IGFs but may contribute by optimising orientation of the N- and C-domains around the IGF molecule. IGFBP-6 is O-glycosylated within this domain, which has no effect on IGF binding but modulates its stability and localisation (Bach, et al. 1992; Marinaro, et al. 2000; Neumann, et al. 1998; Shalamanova, et al. 2008). IGFBP-6 may also be phosphorylated and sulphated although the effects of these modifications on its properties are unknown (Shalamanova et al. 2008).

Specific limited proteolysis of individual IGFBPs is a potential mechanism by which complexed IGFs are released for binding to IGF receptors. Indeed, there are a number of IGFBP-6 proteases, including a cathepsin-D-like acid protease (Marinaro, et al. 1999), a neutral serine protease (Shalamanova, et al. 2001), and MMP-7 (Nakamura, et al. 2005). IGFBP-6 is also a substrate for a number of other MMPs, including MMP-2, which may modulate its anti-angiogenic properties (Dean, et al. 2007), and MMP-9 and MMP-12, which may regulate its capacity to inhibit myelination (Larsen, et al. 2006).

IGFBP-6 is highly conserved across species, and human IGFBP-6 shares 70–85 % sequence identity with rat, mouse, bovine and pig IGFBP-6 (Fig. 1). In zebrafish, the duplicated *IGFBP6* genes have distinct temporal and spatial expression profiles (Wang, et al. 2009). They are 52 % identical to each other and 35–37 % identical with human IGFBP-6. Both gene products retain important N-domain residues that are essential for IGF binding, as well as C-domains containing six conserved cysteines and the CWCV motif, together with most of the amino acids involved in IGF binding. This suggests that zebrafish IGFBPs bind IGFs with high affinity, although this has not been empirically tested. IGFBP-6 genes have been detected in several other fish species but not birds or opossum (Daza, et al. 2011).

Regulation

Serum levels of IGFBP-6 increase gradually with age and are higher in men than in women (Baxter and Saunders 1992; Van Doorn, et al. 1999), but there are conflicting studies of the direct effects of sex steroids on IGFBP-6 expression in different tissues (Bach et al. 2013). Serum levels are decreased during pregnancy, and increased in renal failure and in patients with the rare condition of non-islet cell tumour hypoglycaemia. In different cells, IGFBP-6 is regulated by factors including cAMP, IGFs, retinoic acid, vitamin D, p53 and glucocorticoids in vitro (Bach et al. 2013).

In desmoid tumours, *IGFBP6* transcription was significantly downregulated by β–catenin, an important component of the Wnt signaling pathway (Denys, et al. 2004). In cells from these tumours, transforming growth factor (TGF)-β increased β-catenin and both of these independently inhibited *IGFBP6* promoter activity (Amini Nik, et al. 2007). In contrast, TGF-β increased *IGFBP6* expression in fibroblasts (Ong, et al. 2009), suggesting that its effect is cell-type specific.

The hedgehog (Hh) pathway is critical for development, and stimulates both IGF-II and IGFBP-6 expression (Ingram, et al. 2002; Yoon, et al. 2002). Specifically, sonic hedgehog increased IGFBP-6 expression during fetal prostate development (Lipinski, et al. 2005). IGFBP-6 levels were higher in prostate cancer-associated fibroblasts than in normal prostate fibroblasts, and levels were regulated by Hh signalling (Wilkinson, et al. 2013). In pancreatic cancer cells, inhibition of Hh signalling decreased IGFBP-6 and Bcl2 expression and increased apoptosis, and it was suggested that IGFBP-6 may thereby modulate survival of these cells (Xu, et al. 2009).

Fig. 1 Sequence alignment of IGFBP-6 from rat, mouse, human, cow, pig and zebrafish (2 gene products). Small residues are shown in red, acidic residues in blue, basic residues in magenta, and others in green. * fully conserved residue; : strongly conserved residue with similar properties; . weakly conserved residue. Alignment was performed using Clustal Omega v 1.2.1, EMBL-EBI (www.ebi.ac.uk) (Sievers, et al. 2011)

Functions of IGFBP-6

IGF-II inhibition

The principal function of IGFBP-6 is inhibiting IGF-II actions, which has been demonstrated in many cell lines (Bach 1999, 2005). IGFBP-6 inhibited IGF-II-induced cell proliferation, differentiation, migration and survival, but, in contrast, has little or no effect on IGF-I actions, at least in part due to its lower binding affinity.

IGFBP-6 inhibits growth of IGF-II-dependent cancer cells in vivo. IGFBP-6 inhibited the growth of neuroblastoma cell xenografts in vivo (Grellier, et al. 1998). IGFBP-6 also inhibited anchorage-dependent and -independent proliferation and survival of rhabdomyosarcoma cells in vitro, and IGF-II addition partially overcame these effects (Gallicchio, et al. 2001). Xenografts of rhabdomyosarcoma clones overexpressing IGFBP-6 were ~80 % smaller than control clones after 18 days (Gallicchio et al. 2001), and treatment with CCI-779, a rapamycin analogue, additively delayed tumour formation in IGFBP-6-overexpressing xenografts (Gallicchio, et al. 2003).

IGF-independent actions

Although a number of studies have shown that IGFBP-6 decreases cell proliferation and survival by inhibiting IGF-II actions (Bach 1999, 2005), there is increasing evidence that IGFBPs, including IGFBP-6, also have IGF-independent actions. A recent study suggested that IGFBP-6 inhibited fibroblast proliferation by both IGF-dependent and –independent mechanisms (Raykha, et al. 2013). Additionally, IGFBP-6-induced apoptosis appeared to be IGF-dependent (Hale, et al. 2000) and IGF-independent (Iosef, et al. 2008; Sueoka, et al. 2000b) in different cell lines.

Intracellular actions

A series of studies in osteoblasts suggested that IGFBP-6 has intracrine actions by interacting with LIM mineralization protein-1 (LMP-1), a protein that shuttles between the cytoskeleton and nucleus (Strohbach, et al. 2008; Yan, et al. 2001). Intracrine IGFBP-6 inhibited differentiation of these cells by binding LMP-1 and modulating shuttling, and coexpression of LMP-1 prevented inhibition of the promoter for type I procollagen, a differentiation marker, by IGFBP-6.

Nuclear actions

IGFBPs may enter the nucleus and modulate cellular actions by interacting with transcription factors. IGFBP-6 inhibited vitamin D and liothyronine-mediated differentiation of osteoblasts by interacting with their cognate nuclear receptors (Cui, et al. 2011; Qiu, et al. 2012). IGFBP-6 entered the nucleus in

rhabdomyosarcoma cells via a C-domain nuclear localisation sequence that interacted with importin-α, and deletion of the sequence prevented IGFBP-6-mediated apoptosis (Iosef et al. 2008). Nuclear localisation of IGFBP-6 was not altered by the presence of IGF-II (Iosef et al. 2008). IGFBP-6 interacted with and regulated the availability of Ku80, a DNA repair and stability protein, and it was postulated that IGFBP-6 may modulate cell survival by perturbating DNA repair (Iosef, et al. 2010). IGFBP-6 acted as a tumour suppressor in nasopharyngeal cancer cells via its role as a transcription factor that directly bound the EGR-1 promoter and modulated its expression (Kuo, et al. 2010).

Senescence

Senescent cells remain metabolically active but are unresponsive to growth stimuli due to cell cycle arrest. Senescence is associated with ageing and atherosclerosis, and may be induced by processes including inflammation and oxidative stress. In contrast, it has been also linked to tumour suppression. The IGF system, including IGFBP-6, has been implicated in this process. Senescence induced by hydrogen peroxide or in physiological oxygen conditions increased IGFBP-6 levels in fibroblasts (Coppe, et al. 2010; Xie, et al. 2005) and doxorubicin-induced senescence in colon cancer cells was also associated with increased IGFBP-6 levels (Chang, et al. 2002). Additionally, serum IGFBP-6 levels were higher in ageing mice (Xie et al. 2005) and humans (Micutkova, et al. 2011). One study suggested that IGFBP-6 inhibits senescence, but it also showed that IGFBP-6 increased cell proliferation and survival (Micutkova et al. 2011), which contradicts essentially all other studies of this protein. Additionally. other IGFBPs (IGFBP-3 and IGFBP-5) promote senescence, so the role of IGFBP-6 clearly requires further study.

Inhibition of angiogenesis

Angiogenesis, the formation of new capillaries from existing blood vessels, is a physiological process that is important for tissue repair following injury. It also plays key roles in cancer, both by being essential for ongoing solid tumour growth (Potente, et al. 2011; Roodink and Leenders 2010) and by enhancing metastasis through ingress of cancer cells into abnormal, tumour-related blood vessels (Valastyan and Weinberg 2011). Hypoxia is a major regulator of angiogenesis via hypoxia-inducible transcriptional factors (HIFs). Vascular endothelial growth factor (VEGF) pathways are the major mediators of angiogenesis, but IGFs also play a role (Carmeliet and Jain 2000) by stimulating HIF-1α expression (Hoeben, et al. 2004), and inducing VEGF synthesis (Hoeben et al. 2004; Stearns, et al. 2005; Warren, et al. 1996) via HIF-1-dependent and -independent pathways (Slomiany and Rosenzweig 2006). The human *IGFBP6* promoter contains multiple hypoxia response elements and HIF-1 ancillary sequences, and IGFBP-6 expression was upregulated by hypoxia in endothelial cells via HIF-1α (Zhang, et al. 2012).

IGFBP-6 overexpression inhibited angiogenesis in rhabdomyosarcoma xenografts and zebrafish embryos, and prolonged hypoxia increased IGFBP-6 expression via HIF-1α (Zhang et al. 2012). These findings suggest that IGFBP-6 contributes to a negative feedback mechanism limiting hypoxia-induced angiogenesis (Messmer-Blust, et al. 2009). IGFBP-6-induced inhibition of tube formation by human umbilical vein endothelial cells, an in vitro model of angiogenesis, was IGF-independent (Zhang et al. 2012). SEMA3B is a tumour suppressor that has VEGF-dependent and independent actions, and it increased IGFBP-6 expression in lung cancer cells (Koyama, et al. 2008). IGFBP-6 mediated the antiproliferative effect of SEMA3B, but its effect on angiogenesis was not studied (Koyama et al. 2008). Vasohibin-2, an angiogenic factor that promoted breast cancer cell proliferation, also increased IGFBP-6 expression, but the functional role of IGFBP-6 was not explored (Min et al. 2014).

Cell migration

IGFBP-6 promoted migration of two rhabdomyosarcoma cell lines and a colon cancer cell line by IGF-independent mechanisms (Fu, et al. 2007, 2010). It was further shown that rhabdomyosarcoma migration was due to chemotaxis rather than chemokinesis (Fu et al. 2010).

MAP kinase pathways are implicated in cell migration and invasion (Huang, et al. 2004). IGFBP-6 increased p38 MAP kinase phosphorylation in one rhabdomyosarcoma cell line, and inhibition of p38 MAP kinase prevented IGFBP-6-induced migration (Fu et al. 2007). In contrast, IGFBP-6 increased phosphorylation of ERK and JNK1 but not p38 MAP kinase in another rhabdomyosarcoma cell line (Fu et al. 2010). In both cell lines, cross-talk between the MAP kinase pathways was involved in the migratory response (Fu et al. 2007, 2010). Interestingly, JNK activation increased IGFBP-6 expression in oral cancer cells (Cacalano, et al. 2008), suggesting the possibility of a positive feedback loop between IGFBP-6 and JNK activation in these cells. We recently found that IGFBP-6 had opposing effects on migration of two ovarian cancer cell lines (Yang and Bach 2015). Further, this difference was observed despite similar MAP kinase pathway activation in both cell lines, indicating that other pathways are also involved in the migratory response.

Prohibitin-2

One of the major recent challenges in understanding IGFBP biology has been identification of cell surface receptors that mediate their IGF-independent actions. A proteomic approach was used to identify rhabdomyosarcoma cell membrane

proteins that may mediate the promigratory effect of IGFBP-6 (Fu et al. 2013). Prohibitin-2 (PHB2), a single-span membrane protein, was found to bind IGFBP-6 by multiple techniques. The binding affinity for this interaction was in the nanomolar range via the C-domain but not the N-domain of IGFBP-6. IGFBP-6 increased tyrosine phosphorylation of cell membrane PHB2, although the effect was indirect as IGFBP-6 has no kinase activity. PHB2 knockdown completely abolished IGFBP-6-mediated rhabdomyosarcoma cell migration, but it had no effect on MAP kinase activation, suggesting that PHB2 either acts as a downstream effector of these pathways or acts via independent pathways (Fig. 2). These results indicate that PHB2 plays a key role in IGF-independent inhibition of cancer cell migration by IGFBP-6.

The biology of prohibitins (PHB1 and −2) suggest a number of ways in which they might mediate actions of IGFBP-6. They are highly conserved eukaryotic proteins that contain a SPFH or PHB domain that is important for membrane association (Mishra et al. 2006). Although most studies emphasise their role in mitochondria, prohibitins are also found on plasma membranes and in nuclei. PHB1 and −2 form high molecular weight, functionally active ring complexes in mitochondria and plasma membranes. PHBs modulate mitochondrial function by acting as chaperones for newly synthesised enzymes and/or acting as scaffolding proteins (Osman et al. 2009). Nuclear prohibitins modulate transcription, but the functions of plasma membrane prohibitin complexes are not well understood. PHB1 and −2 are expressed in a range of cancers, including colon cancer, and are found on plasma membranes of colon cancer cells in vitro (Mengwasser et al. 2004). It has been suggested that prohibitins also act as chaperones at the cell surface (Sievers, et al. 2010).

Information about the specific functions of PHB2, which is also known as 'repressor of estrogen receptor activity' (REA) or 'B-cell receptor-associated protein' (BAP37), is limited. PHB2 knockout resulted in early embryonic lethality (Park et al. 2005), while PHB2 deletion impaired mouse embryonic fibroblast proliferation and increased their susceptibility to apoptotic stimuli (Merkwirth et al. 2008). In breast cancer cells, nuclear PHB2 repressed the activity of estrogen receptor-α (Kim et al. 2009).

Although prohibitins were initially identified as antiproliferative genes, their actions are cell- and context-specific. Thus, PHB1 or −2 deficiency shortened the lifespan of wild-type *C. elegans*, but lengthened it in diapause mutants (involving insulin/IGF and TGF-β pathways) or with dietary restriction (Artal-Sanz and Tavernarakis 2009). Further, knockdown

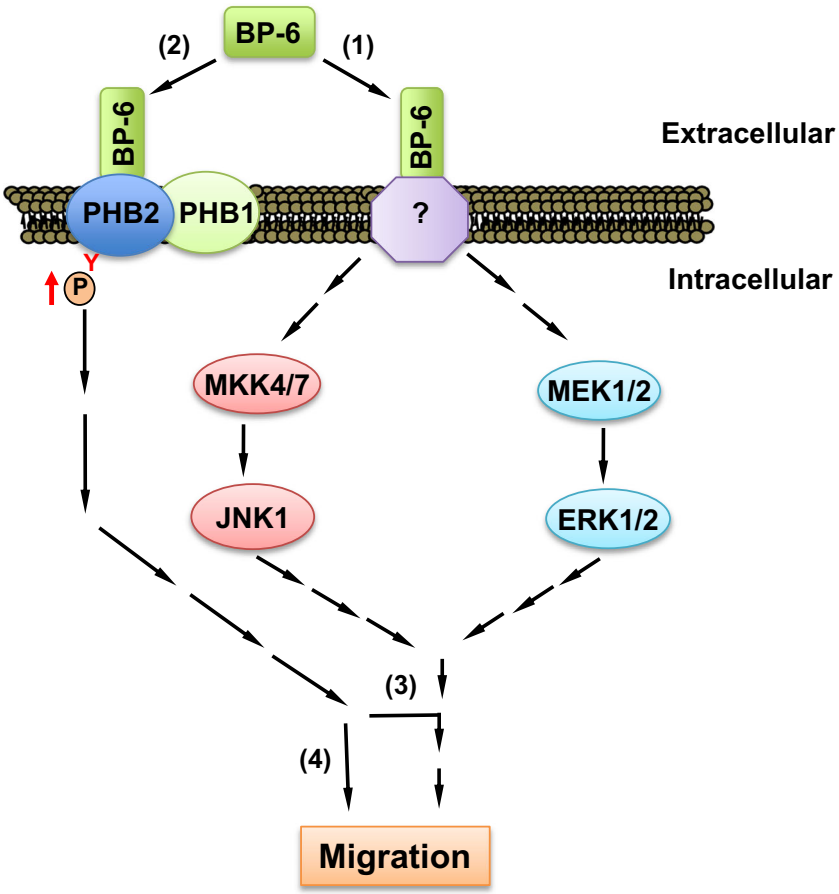

Fig. 2 Potential roles of prohibitin-2 (PHB2) in IGFBP-6-induced rhabdomyosarcoma cell migration. IGFBP-6 binds to (1) an unknown cell surface receptor, leading to MAP kinase pathway activation. (2) IGFBP-6 also binds to PHB2 on the cell surface and indirectly increases its tyrosine phosphorylation. PHB2 is essential for IGFBP-6-induced migration either by (3) acting downstream of MAP kinases, or (4) regulating migration independently of MAP kinase activation. This figure and research were originally published in J Biol Chem. Fu P, Yang Z, Bach LA. Insulin-like growth factor binding protein-6 (IGFBP-6)-induced rhabdomyosarcoma cell migration is modulated by binding to prohibitin-2. J Biol Chem. 2013; 288: 29890–900. © the American Society for Biochemistry and Molecular Biology

of PHB1 or −2 decreased HeLa cancer cell proliferation and adhesion to extracellular matrix proteins (Sievers et al. 2010).

As mentioned above, IGFBP-6 induced phosphorylation of PHB2 (Fu et al. 2013). Phosphorylation of prohibitins modulated their effects on cell signalling and function (Mishra et al. 2010). Insulin phosphorylated PHB1 at Tyr114, which is conserved in PHB2, resulting in SHP1 recruitment and decreased AKT phosphorylation (Ande, et al. 2009).

Plasma membrane prohibitins have been implicated in PI3 kinase/AKT and Ras/MAPK/ERK signaling (Chowdhury, et al. 2014; Mishra et al. 2010). For example, PHB1 bound c-Raf and was required for its membrane targeting and activation, leading to cell migration (Rajalingam, et al. 2005). PHB2 but not PHB1 bound AKT2, and it reciprocally regulated AKT2 levels in myoblasts (Heron-Milhavet, et al. 2008). PHBs therefore have a broad range of biological roles and the consequences of the IGFBP-6/PHB2 interaction are worthy of further study.

IGFBP-6 and cancer

The role of IGFBP-6 has been investigated in many cancers, which may be especially relevant since IGF-II is frequently an autocrine cancer growth factor (Bach et al. 2013). In most studies, IGFBP-6 expression was lower in malignant cells than in normal cells, which is consistent with the idea that it acts as an inhibitor of tumorigenic processes, including those driven by excess IGF-II activity. However, a smaller number of studies have shown the opposite, which may represents a compensatory response to increased IGF-II activity or may reflect IGF-independent actions of IGFBP-6. A number of brief examples in diifferent cancers follow.

Adrenocortical cancer

A number of recent studies have shown increased expression of IGFBP-6 in adrenocortical tumours. *IGFBP6* was one of several IGF system genes that was higher in human adrenocortical carcinomas than in adenomas (Velazquez-Fernandez, et al. 2005). Increased *IGFBP6* expression and gene hypomethylation were also seen in adrenocortical neoplasms induced by gonadectomy in mice (Schillebeeckx, et al. 2014).

Breast cancer

IGFBP-6 was expressed in estrogen-receptor negative and positive breast carcinoma cell lines (Figueroa, et al. 1993; Martin, et al. 1995; Sheikh, et al. 1993) and was also expressed at low levels in breast cancer specimens (Figueroa et al. 1993). The IGF system is implicated in the development of resistance to HER2 inhibitors, which is an important clinical problem in breast cancer. IGFBP-6 expression was higher in resistant breast cancer cells than in sensitive cells, but its functional consequences were not studied (Oliveras-Ferraros, et al. 2010).

Colon cancer

IGF2 was the most highly overexpressed gene in colorectal tumours (Zhang, et al. 1997). IGFBP-6 may be anti-tumorigenic for colon cancer as evidenced by lower levels in a metastatic than a non-metastatic cell line (Futschik, et al. 2002), and in a multidrug-resistant than in a sensitive colon cancer cell line (Fan, et al. 2004). Exogenous IGFBP-6 inhibited IGF-II-induced proliferation and adhesion of colon cancer cells (Leng, et al. 2001), and IGFBP-6 expression was increased in colon cancer cells by (n-3) fatty acids and retinoic acid, both of which inhibit proliferation (Kim, et al. 2000, 2001). Coculture of colon cancer cells with normal colonic epithelial cells decreased IGFBP-6 secretion, and IGFBP-6 expression was dramatically decreased in normal colonic epithelial cells undergoing epithelial-mesenchymal transdifferentiation, a process that is implicated in tumorigenesis (Zeng, et al. 2013).

Gastric cancer

Epigenetic silencing may contribute to cancer development. The *IGFBP6* promoter was abnormally methylated in a quarter of 152 gastric cancers, and hypermethylation was associated with decreased IGFBP-6 expression in cancer cell lines (Jee, et al. 2009). Conversely, an inhibitor of DNA methylation increased IGFBP-6 expression in immortalized fibroblasts (Kulaeva, et al. 2003).

Head and neck cancer

IGFBP-6 expression was lower in metastatic head and neck squamous cell carcinomas than in primary disease (Liu, et al. 2008). IGFBP-6 was down-regulated in nasopharyngeal cancer cells and it acted as a tumour suppressor by its effects on transcription factor EGR-1 expression (Kuo et al. 2010). In oral carcinoma cells, IGFBP-6 decreased migration but not proliferation (Liu et al. 2008), whereas apoptosis and IGFBP-6 expression were both increased by JNK activation and NF-κB inhibition (Cacalano et al. 2008).

Lung cancer

IGFBP-6 appears to have an inhibitory role in lung cancer. Although commonly expressed in human lung cancers (Wegmann, et al. 1993), its expression was lower in cancers than in normal lung in mice (Yao, et al. 2002). IGFBP-6 inhibited proliferation of human bronchial epithelial cells (Sueoka, et al. 2000a) and increased apoptosis of non-small cell lung cancer cells (Sueoka et al. 2000b). In lung cancer

cells, IGFBP-6 levels were increased by the tumour suppressor SEMA3B, which may mediate its antiproliferative effect (Koyama et al. 2008). IGFBP-6 levels were also increased by the tumour suppressor p53 (Kannan, et al. 2001).

Neuroblastoma

Neuroblastoma is an IGF-II-dependent tumour that is commonest in children (Toretsky and Helman 1996). N-myc oncogene overexpression, which is associated with poor prognosis, decreased IGFBP-6 levels (Chambery, et al. 1999), whereas FGF-2, which stimulates neuronal differentiation, increased IGFBP-6 expression (Russo, et al. 2004). IGFBP-6 has functional effects in neuroblastoma cells. Constitutive IGFBP-6 overexpression inhibited neuroblastoma xenograft growth and promoted apoptosis in vivo (Grellier, et al. 2002; Grellier et al. 1998). IGFBP-6 infusion also delayed neuroblastoma xenograft growth in vivo, probably by inhibiting IGF-II actions (Seurin, et al. 2002).

Ovarian cancer

IGF-II mRNA levels were >300-fold higher in cancer tissues than in normal ovary, and IGF-II levels predicted poor survival in advanced stage ovarian cancer (Sayer, et al. 2005). IGFBP-6 was detected in 38 of 41 ovarian cancers (Walker, et al. 2007), but a microarray study showed that IGFBP-6 mRNA levels were lower in ovarian cancer tissue than in non-cancerous tissue (Bahrani-Mostafavi, et al. 2008). Our recent finding that IGFBP-6 had opposing effects on migration of two ovarian cancer cell lines suggests heterogeneous responsiveness in this cancer (Yang and Bach 2015).

Prostate cancer

IGFBP-6 and other IGF system components were upregulated during prostate epithelial cell differentiation (Massoner et al. 2011), whereas expression of IGFBP-6 and other IGFBPs was progressively lower in more tumorigenic transformed human prostate epithelial cells (Plymate, et al. 1996). Vitamin D, which inhibits proliferation of prostate carcinoma cells, increased IGFBP-6 levels (Drivdahl et al. 1995). In androgen-independent prostate cancer cells, IGFBP-6 may mediate the antiproliferative effects of diethylstilbestrol (Koike et al. 2005).

Rhabdomyosarcoma

IGF-II is an autocrine growth factor in rhabdomyosarcoma, which is a tumour of childhood and adolescence (Foulstone et al. 2005; Toretsky and Helman 1996). In animal models, IGF-II was required for development and malignant behaviour of rhabdomyosarcoma (Hahn, et al. 2000) and it increased angiogenesis (Wang et al. 1998). IGFBP-6 inhibited anchorage-dependent and –independent proliferation and survival of rhabdomyosarcoma cells in vitro; these effects appeared to be at least partly IGF-dependent (Fu et al. 2007; Gallicchio et al. 2001). Intranuclear actions were also implicated in apoptosis induced by IGFBP-6 (Iosef et al. 2008, 2010). As described above, IGFBP-6 overexpression inhibited rhabdomyosarcoma xenograft growth and angiogenesis in vivo (Fu et al. 2013; Gallicchio et al. 2001; Zhang et al. 2012), and IGFBP-6 promotes rhabdomyosarcoma cell migration via pathways dependent on PHB2 and MAP kinase signalling (Fu et al. 2007, 2010, 2013).

Clinical relevance of serum IGFBP-6 levels

A number of studies have investigated circulating IGFBP-6 levels in various diseases. As mentioned above, a small study showed that serum IGFBP-6 levels were lower in women with breast cancer than in those with benign breast disease (Kaulsay et al. 1999). However, two studies of serum IGFBP-6 levels in women with ovarian cancer had opposing results (Gunawardana et al. 2009; Lin et al. 2009). Higher plasma IGFBP-6 levels were associated with good prognosis in patients with malignant glioma (Lin et al. 2013), whereas serum IGFBP-6 levels were lower in patients with prostate cancer than those with benign prostatic hypertrophy (Xu et al. 2014). Plasma IGFBP-6 levels were also significantly lower in patients with hepatocellular cancer than in those with hepatitis (Sun et al. 2008).

With regard to non-malignant disease, serum IGFBP-6 levels were substantially higher in children with chronic renal failure, which may contribute to decreased growth in these patients (Powell et al. 1997). Serum IGFBP-6 levels were higher in patients with type 1 diabetes and its complications, although there was substantial overlap with control subjects (Lu et al. 2012). IGFBP-6 was also recently proposed as a candidate serum biomarker of proliferative vitreoretinopathy (Yu et al. 2014).

Many of these studies are limited by small sample size and results have not been validated in independent cohorts or by different laboratories. There is also considerable overlap in levels between normal and disease states in many of these studies. Further studies are therefore required to determine a clear clinical indication for measurement of plasma IGFBP-6 levels.

Conclusions and future directions

IGFBP-6 clearly has a physiological role as an inhibitor of IGF-II actions, and recent evidence indicates that it also has IGF-independent actions including inhibition of angiogenesis. This combination of properties would appear to be ideal for a cancer inhibitor. However, promotion of migration is

generally held to be pro-tumorigenic, and the association with PHB2 binding and MAP kinase pathway activation provides some direction in furthering our understanding of this action.

There are a number of important questions regarding IGFBP-6 that remain unanswered. One of these is the structural basis for the IGF-II binding preference of IGFBP-6. The N-domain high affinity IGF binding subdomain is highly conserved between the IGFBPs, making it unlikely to contribute to this preference. Studies of isolated N- and C-domains of IGFBP-6 suggested that the C-domain of IGFBP-6 is responsible for its IGF-II binding preference (Headey et al. 2004b), but structural studies did not reveal a single key C-domain determinant for this preference (Headey et al. 2004a). A peptide based on the N-terminal subdomain of IGFBP-6 preferentially bound IGF-II, but its affinity for both IGFs was very low (Chandrashekaran et al. 2007). It is possible that both N- and C- domains cooperatively contribute to the binding preference since the binding sites for the N-terminal subdomain and C-domain of IGFBP-6 on IGF-II are adjacent.

Regarding IGF-independent actions, the mechanism whereby IGFBP-6 inhibits angiogenesis requires further study. IGFBP-6 inhibited basal and VEGF-induced angiogenesis in vitro as well as angiogenesis during development and xenograft growth in vivo (Zhang et al. 2012). The underlying molecular interactions as well as the relative roles of IGFBP-6 in endothelial and tumour cells are worthy of study. The role of PHB2 in IGFBP-6-induced cancer cell migration also requires further study at the molecular and cellular level. For example, the effects of IGFBP-6 binding and phosphorylation of PHB2 on PHB1/PHB2 ring complex formation and intracellular localisation are of interest. The molecular mechanisms whereby IGFBP-6 increases PHB2 phosphorylation and the consequent pathway leading to cell migration are also important questions. In this respect, the dissociation between IGFBP-6-induced migratory responses and MAP kinase pathway activation in some circumstances (Fu et al. 2013) (Yang and Bach 2015) indicates that other pathways are involved.

The relative balance of IGF-dependent and IGF-independent actions of IGFBP-6 in vivo is clearly a key question in understanding the physiological role of this protein. Related to this, the roles of non-IGF binding partners such as PHB2 on IGFBP-6 actions such as proliferation, survival, angiogenesis and migration are also important. Further studies of the structural determinants of binding of IGFBP-6 to IGF-II, PHB2 and other key molecules implicated in the angiogenic and migratory responses could lead to the design of IGFBP-6 mutants with specific binding characteristics that will assist in answering these critical questions.

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