Insulin-like growth factor-binding protein-6 and cancer

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Abstract
The IGF (insulin-like growth factor) system is essential for physiological growth and it is also implicated in a number of diseases including cancer. IGF activity is modulated by a family of high-affinity IGF-binding proteins, and IGFBP-6 is distinctive because of its marked binding preference for IGF-II over IGF-I. A principal role for IGFBP-6 is inhibition of IGF-II actions, but recent studies have indicated that IGFBP-6 also has IGF-independent effects, including inhibition of angiogenesis and promotion of cancer cell migration. The present review briefly summarizes the IGF system in physiology and disease before focusing on recent studies on the regulation and actions of IGFBP-6, and its potential roles in cancer cells. Given the widespread interest in IGF inhibition in cancer therapeutics, increasing our understanding of the mechanisms underlying the actions of the IGF ligands, receptors and binding proteins, including IGFBP-6, will enhance our ability to develop optimal treatments that can be targeted to the most appropriate patients.

Key words: binding protein, cancer, insulin-like growth factor (IGF), insulin-like growth factor-binding protein-6 (IGFBP-6), therapeutic

INTRODUCTION
IGF (insulin-like growth factor)-I and IGF-II are polypeptides that share significant sequence and structural homology with pro-insulin. They promote proliferation and survival of a wide range of cell types, as well as having insulin-like metabolic effects. IGFs are critical for normal mammalian development [1,2], and dysregulation of the IGF system is implicated in a range of diseases, including cancer, diabetes, atherosclerosis and neurodegeneration. The family of six high-affinity IGFBPs (IGF-binding proteins) is a key regulator of IGF actions, as well as having some actions that are IGF-independent [3,4]. IGFBP-6, which has a distinctive IGF-II binding preference, is the major focus of the present review [5]. A brief overview of the IGF system (Table 1) will provide important context.

BIOCHEMISTRY AND PHYSIOLOGY OF THE IGFs
IGF and insulin genes diverged from a single precursor via a series of gene duplications 500–600 million years ago, so that the protochordate amphioxus has a single insulin/IGF molecule, which predominantly mediates cell proliferation and survival. In contrast, hagfish, a primitive vertebrate, has one insulin and one IGF molecule, allowing the separation of metabolic and growth-related functions. The earliest species to have distinct IGF-I and IGF-II molecules is Squalus acanthias, the spiny dogfish shark [6].

IGF-I is regulated by GH (growth hormone) and mediates many, but not all, of its actions. It is expressed in most tissues, but the predominant source of circulating IGF-I is the liver. IGF-I has endocrine, autocrine and paracrine actions. The roles of circulating and local actions of IGF-I have been extensively studied using a series of organ-specific knockout mice [7]. Mice in which the IGF1 gene is globally deleted are small at birth. Depending on the background strain, they may survive to adult life and remain small, weighing 30% of normal. Liver-specific IGF1 gene deletion markedly decreased circulating IGF-I levels by 65%, but a mild growth phenotype manifested only after puberty, suggesting the importance of local IGF-I. However, loss of circulating IGF-I affects post-pubertal bone accrual. In contrast, bone- and muscle-specific IGF-I gene deletion results in decreased pre-pubertal growth and bone formation.
IGF RECEPTORS

Most IGF actions are mediated by the IGF-I receptor, which consists of two heterodimers that are cross-linked by disulfide bonds. This receptor binds IGF-I with 3-fold higher affinity than IGF-II and insulin with substantially lower affinity [2]. The receptor has tyrosine kinase activity upon ligand binding, resulting in autophosphorylation and recruitment of intracellular mediators such as IRS (insulin receptor substrate)-1 and IRS-2 [9]. A number of signalling cascades including Ras/MAPK (mitogen-activated protein kinase) and P13K (phosphoinositide 3-kinase)/Akt are subsequently activated.

The insulin receptor is closely related to the IGF-I receptor and has two isoforms. IR-B (insulin receptor type B) is the predominant receptor involved in metabolic responses and binds insulin with higher affinity than IGF-I or IGF-II. In contrast, IR-A (insulin receptor type A), which is generated by the skipping of exon 11 resulting in a 12-amino-acid deletion, is more highly expressed in the fetus and in cancer cells. This isoform binds insulin and IGF-II with higher affinity than IGF-I and predominantly mediates growth responses [10].

Insulin and IGF-I receptors form hybrids with one heterodimer from each receptor [2,9]. These hybrids may form a significant proportion of receptors in some situations. Hybrids involving IR-A bind IGF-I and IGF-II with similar affinity to the IGF-I receptor but bind insulin with significantly higher affinity than the IGF-I receptor. Hybrids with IR-B bind IGF-I and IGF-II with lower affinity than the IGF-I receptor and they have low affinity for insulin. The physiological significance of hybrid receptors remains uncertain.

The IGF-II/mannose 6-phosphate receptor is structurally distinct from the IGF-I and insulin receptors [11]. It binds a large range of ligands including IGF-II and proteins containing man-
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Figure 1  Domain structure of IGFBP-6
In common with IGFBP-1–IGFBP-5, IGFBP-6 has three domains. The N-terminal domain (N-domain) has IGF-binding sub-domains. Both the N- and C-terminal domains (C-domain) are cysteine-rich (conserved disulfide linkages in all IGFBPs shown in green, whereas those that differ in IGFBP-6 are shown in red) and mediate high-affinity IGF binding, whereas the linker domain is a site of post-translational modification. The C-terminal domain also subserves IGF-independent actions. Structures shown from left to right are: (i) the proximal region of the N-domain of IGFBP-6 (PDB code 2JM2 [44]); (ii) the binding interface of IGF-I (yellow) and the high-affinity IGF-binding domain of IGFBP-4 (cyan), which is conserved in other IGFBPs (PDB code 2DSP [16]); and (iii) the C-terminal domain of IGFBP-6 (PDB code 1RMJ [48]). Structures were prepared using UCSF Chimera.

of specific proteases predominantly in their linker domains. Proteolysis is thought to be an important local mechanism for release of free IGFs from IGFBPs, allowing subsequent IGF receptor binding and activation [3,4]. IGFBP-1, IGFBP-3 and IGFBP-5 may be serine-phosphorylated in their linker domains, resulting in altered IGF-binding affinity, as well as modulation of proteolysis and cell-surface binding [3,4]. Non-phosphorylated IGFBP-1 may enhance the actions of IGF in some cells, whereas the phosphorylated forms are inhibitory [3]. IGFBP-3 and IGFBP-4 are N-glycosylated, whereas IGFBP-5 and IGFBP-6 are O-glycosylated. Glycosylation does not directly affect IGF binding, but decreases IGFBP binding to glycosaminoglycans, inhibits their proteolysis and increases their circulating half-lives [17,18].

IGFBPs play a number of roles in IGF physiology. IGFBP-3 and IGFBP-5 are low-affinity binders that form a high-molecular-mass ternary complex [3]. IGFs within this complex cannot leave the vasculature and this accounts for more than 75% of circulating IGFs. Sequestration of IGFs in the 150 kDa complex is essential for glucose homoeostasis, as the insulin-like activity of the high plasma concentrations of IGFs would otherwise cause hypoglycaemia. Breakdown of this homoeostatic mechanism is seen in the syndrome of non-islet cell tumour hypoglycaemia, in which tumours secrete a partially processed IGF-II precursor that does not efficiently form high-molecular-mass complexes. It therefore forms binary complexes with IGFBPs that can cross the vascular permeability barrier resulting in hypoglycaemia [19]. In normal physiology, binary complexes may facilitate IGF transport from the vasculature and target them to specific tissues [3].

IGFBPs are differentially regulated. For example, IGFBP-1 expression is rapidly inhibited by insulin, and IGFBP-3 expression is stimulated by IGF-I and therefore indirectly by GH [3]. Different IGFBPs have differing relative affinities for IGFs, the most striking example of which is the IGF-II binding preference of IGFBP-6 [20]. IGFBP expression is cell-type-specific and different IGFBPs are susceptible to digestion by specific proteases. For example, IGFBP-4 is cleaved by pregnancy-associated plasma protein-A, which has a major role in regulating its activity [21]. The IGFBPs therefore form a highly regulated and flexible extracellular system for fine tuning the actions of IGF in specific tissues.

In recent years, there has been increasing interest in IGF-independent actions of IGFBPs [3]. The evidence for IGF-independent actions comes from several approaches: actions in cell lines that do not express IGFs or IGF receptors, actions that are unaffected by IGF mutants that do not bind IGFBPs or bind poorly, and, more recently, actions of IGFBPs that are mutated to abrogate IGF binding. Most studies demonstrating IGF-independent actions of IGFBPs have been performed in vitro. However, IGFBP-3 IGF-independently inhibited late prostate cancer progression in a mouse model [22].

Different IGFBPs exert IGF-independent actions via distinct mechanisms. IGFBP-1 and IGFBP-2 have Arg-Gly-Asp integrin recognition sequences in their C-terminal domains, and IGFBP-1 stimulates cell migration through integrin engagement [23]. The role of integrin interaction in the actions of IGFBP-2 is less clear, with different studies having contrasting results [24,25]. The C-terminal domains of IGFBPs interact with a large number of proteins and other biomolecules, and these interactions may also contribute to IGF-independent actions [4]. Identification of specific cell-membrane ‘IGFBP receptors’ has been more problematic, although a number of candidates have emerged [3,26].
Some IGFBPs (initially IGFBP-3 and IGFBP-5, but subsequently IGFBP-2 and IGFBP-6) have also been shown to localize to the nucleus in vitro via specific NLs (nuclear localization sequences), and IGFBPs may IGF-independent bind to transcription factors [3].

Deletion of individual IGFBP genes has not resulted in major phenotypes, presumably due to functional redundancy between the IGFBPs with respect to regulation of the actions of IGFs [27]. However, triple knockout of IGFBP-3–IGFBP-5 decreased growth and increased glucose disposal, suggesting that IGFBPs are required to maintain post-natal IGF-mediated growth and glucose homeostasis [28]. Furthermore, more detailed studies of IGFBP-2-knockout mice showed effects on bone turnover [29]. High-fat feeding of IGFBP-3-knockout mice revealed a role for this IGFBP in metabolic regulation [30], and crossing IGFBP-3-knockout mice with a c-myc model of prostate cancer indicated that this IGFBP is a metastasis suppressor [31].

IGFs AND CANCER

There is considerable evidence linking dysregulation of the IGF system with cancer. Epidemiological studies have shown that higher circulating IGF-I levels within the normal range are associated with an increased subsequent risk of developing many tumours, including prostate, colorectal and breast cancer [32]. Animal studies support this relationship, as colon and breast cancer growth is decreased in mice with low circulating IGF-I levels due to liver-specific deletion of the IGF1 gene [33,34].

There is also a strong biological basis supporting this relationship. The IGF-I receptor facilitates malignant transformation by a number of oncogenes in vitro [35]. A large number of studies have shown that IGFs promote survival, proliferation, migration and invasion of many cancer cell types [35–37], consistent with a role in cancer cell biology [37]. Additionally, IGFs promote hypoxia-induced tumour angiogenesis, a process that is necessary for solid tumours to survive as they increase in size [38]. This action is mediated at least in part by IGF-induced stimulation of VEGF (vascular endothelial growth factor) synthesis [38].

Many cancer cells express IGF-I receptors in vitro and in vivo and are therefore responsive to IGFs, which may derive from autocrine, paracrine and/or endocrine sources. In particular, there is evidence that many cancers overexpress IGF2 [39] by mechanisms including LOI and LOH (loss of heterozygosity) [8,40]. Recent evidence also indicates that many cancers express IR-A, which is preferentially activated by insulin and IGF-II, but not IGF-I, resulting in tumorigenic effects including mitogenesis, survival, motility, and invasiveness [10].

The pre-clinical evidence indicating a role for a dysregulated IGF system in cancer has resulted in the development of a range of potential therapeutic agents targeting the IGF-I receptor or IGF ligands. These include monoclonal antibodies and small-molecule TKIs (tyrosine kinase inhibitors), as well as an antibody directed against IGF-1 and IGF-II [32,39,41]. A large number of clinical studies have been conducted using these agents alone and in combination with other therapeutic modalities. Although some small Phase 2 studies have shown responses that are occasionally dramatic, early results from a Phase 3 study did not demonstrate efficacy. Toxicity has also been reported, with hyperglycaemia being a major side effect. For TKIs, this may be due to cross-reactivity and partial inhibition of the insulin receptor, whereas antibody-mediated IGF-I receptor inhibition may lead to GH hypersecretion and consequent insulin resistance. A current challenge is to identify subgroups of patients who respond to IGF-based therapeutics by developing appropriate biomarkers. A successful example of this approach is the use of Her2 status to identify patients with breast cancer who will benefit from trastuzumab, a monoclonal antibody targeting the Her2 receptor. Refined approaches to the use of IGF-based therapies will also result from understanding the mechanisms of resistance, which may include properties of the cancer cells themselves or systemic responses to therapy, such as increased IGF and insulin levels, leading to increased signalling through the insulin receptor [41].

IGFBP-6

The distinctive feature of IGFBP-6 is its ~50-fold binding preference for IGF-II over IGF-I [5,20]. In contrast, IGFBP-1–IGFBP-5 bind IGF-II with slight preference or with equal affinity to IGF-I. Because of this IGF-II-binding preference, IGFBP-6 is a relatively specific inhibitor of the actions of IGF-II, and most studies have failed to show IGF-I inhibition by IGFBP-6.

The C-terminal domains of all IGFBPs contain a thyroglobulin type 1 fold, three homologous disulfide linkages and a conserved CWCV sequence [4]. The high-affinity IGF binding subdomain of their N-terminal domains have two conserved disulfide linkages [4,42] (Figure 1). However, there are a number of structural differences between IGFBP-6 and the other IGFBPs in their N-terminal subdomains (Figure 1). This subdomain of IGFBP-1–IGFBP-5 contains a conserved GCGCC motif and a ladder-like structure stabilized by four disulfide bonds [16], and it has been proposed that the first few N-terminal amino acids of IGFBP-4 contribute to IGF binding [16]. This subdomain of IGFBP-6 lacks the GCGCC motif and only has three disulfide bonds [43], and a peptide based on this region is predominantly extended with little secondary structure [44].

IGFBP-6 is O-glycosylated within its linker domain, which modulates its stability and localization [18,45]. More recently, it was shown that IGFBP-6 may also be phosphorylated and sulfated, although the functional consequences of these modifications are unknown [46].

The structural basis for the IGF-II-binding preference remains unresolved. The N-terminal high-affinity IGF-binding domain is highly conserved between the IGFBPs. On the basis of IGF-binding studies of isolated N- and C-terminal domains of IGFBP-6, we postulated that the C-terminal domain of IGFBP-6 is responsible for its IGF-II-binding preference [47]. However, the three-dimensional structure of the C-terminal domain of IGFBP-6 did not reveal an obvious structural determinant for this preference [48]. A peptide based on the N-terminal subdomain
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Figure 2  Actions of IGFBP-6
IGFBP-6 inhibits IGF-II-mediated actions, including proliferation, survival and myoblast differentiation, by preventing binding to the IGF-I receptor. IGF-independent actions of IGFBP-6 have also been described. Migration is associated with MAPK pathway activation, whereas apoptosis and osteoblast differentiation are linked to nuclear localization and interaction with transcription factors. The pathways underlying IGFBP-6 induced inhibition of angiogenesis and the nature of cell-membrane receptors for IGFBP-6 are unknown.

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ACTIONS OF IGFBP-6 (Figure 2)

As mentioned above, the predominant well-documented action of IGFBP-6 is inhibition of the actions of IGF-II, including IGF-II-induced proliferation, differentiation, migration and survival in many cell lines [5,20]. These actions are clearly relevant to cancer biology. This section will focus on more recent studies and those suggesting novel actions.

In vivo
Global deletion of the IGFBP6 gene did not result in a dramatic phenotype, presumably because of functional redundancy with the other IGFBPs. Transgenic mice overexpressing IGFBP-6 in the brain under the control of the glial fibrillary acidic protein promoter were smaller during the first post-natal month [50]. In these mice, cerebellar size and the number of differentiated astrocytes were substantially decreased, and reproductive abnormalities suggested a possible hypothalamo–pituitary disorder. Furthermore, these mice gained more weight than control mice following a high-fat diet [51]. Although their food intake was unchanged, they were more glucose-intolerant and insulin-resistant, and had higher leptin levels. UCP-1 (uncoupling protein-1) expression was down-regulated in brown adipose tissue and there were subtle changes in energy expenditure, suggesting that brain IGFBP-6 influences the regulation of energy homeostasis. In zebrafish, overexpression of either of the duplicated IGFBP6 genes inhibited embryonic growth and development [49].

Nervous system
In addition to the above studies in transgenic mice, many studies support a role for IGFBP-6 in the nervous system. IGFBP-6 is expressed in the CNS (central nervous system) and is developmentally regulated [52]. It is expressed at higher levels in non-neurogenic than neurogenic astrocytes and it inhibits differentiation of multipotent adult neural stem/progenitor cells [53].

IGFBP-2, IGFBP-5 and IGFBP-6 levels are increased in spinal motor neurons of patients with amyotrophic lateral sclerosis [54]. These increased IGFBP levels may enhance neuronal apoptosis, as they may sequester IGFs, which are neuronal survival factors. This is of particular interest, since IGF-I is an experimental therapeutic agent for patients with this disease. IGFBP-6 levels are also increased following axotomy or avulsion of spinal...
motor neurons, and levels are increased further by BDNF (brain-derived neurotrophic factor) [55].

A number of studies suggest that IGFBP-6 regulates myelination. IGFBP-6 inhibited rat oligodendrocyte survival and myelin protein expression [56]. Mice deficient in MMP (matrix metalloproteinase)-9 and MMP-12 had increased IGFBP-6 levels and delayed myelination [57]. Studies of post-mortem CNS tissue also showed that IGFBP-6 levels are increased in oligodendrocytes at the edge of demyelinated plaques from patients with multiple sclerosis [58]. The authors therefore suggest that this, together with other changes in the IGF system, may play a pathogenic role in the demyelination process.

**Intracellular and nuclear actions**

Some studies have suggested that IGFBP-6 may have intracrine actions. In particular, inhibition of osteoblast differentiation by IGFBP-6 was suggested to be an intracrine action [59], and this effect may be due to binding of IGFBP-6 to LMP-1 (LIM mineralization protein-1), a protein that shuttles between the cytoskeleton and nucleus and is involved in differentiation [60]. LMP-1 is regulated by protein–protein interactions that regulate subcellular compartment movement. IGFBP-6 modulated this process, and LMP-1 prevented IGFBP-6-induced inhibition of pro-collagen promoter activity.

Nuclear localization of IGFBP-6 in RMS (rhabdomyosarcoma) cells is dependent on a C-terminal domain LNS via importin-α [61]. IGFBP-6 inhibited vitamin D-mediated osteoblast differentiation by interacting with the vitamin D receptor in the nucleus and inhibiting its transcriptional activity [62]. More recently, the same group showed that IGFBP-6 also bound thyroid hormone receptor α1 and inhibited T3, 3,3’,5-tri-iodothyronine-induced osteoblast differentiation [63]. As discussed below, nuclear IGFBP-6 may play a role in RMS cell survival [64] and as a tumour suppressor in nasopharyngeal cancer [65].

**IGFBP-6 REGULATION IN VITRO AND IN VIVO**

IGFBP-6 is developmentally regulated and serum levels are higher in men than in women [20]. Levels are decreased during pregnancy and in active acromegaly, and increased in renal failure. In vitro, IGFBP-6 is regulated in a cell-specific manner by cAMP, IGFs, retinoic acid, vitamin D and glucocorticoids [20,66]. Gene regulation is a critical component of cancer biology and IGFBP-6 expression is regulated by a number of pathways that are important in cancer.

**Transcriptional regulation**

β-Catenin

β-Catenin is a key component of the Wnt signalling pathway that is implicated in tumour formation. In desmoid tumours, IGFBP-6 expression was significantly down-regulated by β-catenin via interaction with two functional β-catenin/TCF (T-cell factor)-responsive elements in the human IGFBP6 promoter [67]. TGF (transforming growth factor)-β increased β-catenin in these cells, and both of these independently decreased IGFBP-6 promoter activity [68].

**Hh (Hedgehog) (Figure 3)**

The Hh pathway is critical for development, and mutations leading to its overactivity have been found in a number of cancers [69]. Hh ligands bind to Ptc (Patched), which derepresses Smo (Smoothened) and results in transcriptional activation via zinc-finger transcription factors Gli1–Gli3. There is considerable interest in interactions between this pathway and the IGF system, and Hh stimulates expression of a number of IGF system genes, including IGF2 [70]. Overexpression of Gli1, which mediates some actions of Shh (Sonic Hedgehog) in development, increased IGFBP-6 expression through Gli consensus sequences in the IGFBP6 promoter [71]. Shh increased IGFBP-6 expression during fetal prostate development, and cyclopamine, an Shh inhibitor, decreased IGFBP-6, indicating a role for the Shh pathway in the developmental regulation of IGFBP-6 [72]. Cyclopamine had a similar inhibitory effect on IGFBP-6 expression in testicular seminiferous tubules [73]. In pancreatic cancer cells, cyclopamine or Gli1 knockdown increased apoptosis and decreased IGFBP-6 and Bcl2 expression [74]; the authors suggested that IGFBP-6 may modulate survival of these cells, although this was not directly demonstrated. The potential role of the Hh pathway in RMS is discussed below.

**Hypoxia**

Hypoxia is a major regulator of angiogenesis. A family of HIFs (hypoxia-inducible transcriptional factors) are key regulators of angiogenesis-related genes, including VEGF. IGFBP-6 expression was up-regulated by hypoxia in endothelial cells and the human IGFBP6 promoter contains multiple hypoxia-response elements and HIF-1 ancillary sequences [75].

p53

IGFBP-6 levels are increased by p53, which is a tumour suppressor, in lung cancer cells [76], and p53-dependent IGFBP-6 accumulation is associated with copper-mediated neuronal apoptosis [77].

**Sex steroids**

Circulating IGFBP-6 levels are higher in males than females, suggesting that sex steroids may regulate its expression. However,
there are conflicting studies in this regard. Oestrogens increased IGFBP-6 levels in breast [78] and prostate [79] cancer cells. In contrast, oestradiol had no effect on IGFBP-6 levels in oestrogen-responsive ovarian cancer cells and inconsistent effects in osteoblasts [80,81].

A 5α-reductase inhibitor decreased IGFBP-6 expression in the distal epididymis, suggesting that dihydrotestosterone increases IGFBP-6 expression [82], and pharmacological doses of testosterone, but not ethinyloestradiol, increased plasma IGFBP-6 levels in adolescents with constitutional tall stature [83].

Progesterone decreased IGFBP-6 levels in endometrial cancer cells [84] but increased levels in oligodendrocytes [85]. Myometrial IGFBP-6 levels were correlated with plasma progesterone levels during pregnancy in the rat [86]. Administration of RU486, a progesterone inhibitor, during late pregnancy decreased IGFBP-6 levels, whereas progesterone supplementation increased them [86]. These findings suggest considerable complexity in the regulation of IGFBP-6 expression by sex steroids and further studies are needed.

TGF-β
As mentioned above, TGF-β decreased IGFBP-6 promoter activity in desmoid tumours [68]. In contrast, TGF-β as well as MCP-3/CCL7 (monocyte chemoattractant protein-3/CC chemokine ligand 7) additionally increased IGFBP-6 expression as determined by microarray in fibroblasts [87], suggesting that the effect of TGF-β may be cell-type-specific.

Other modes of regulation
Senescence
Cellular senescence is a process resulting in cell-cycle arrest but not cell death. It is associated with aging and may be induced by a number of factors including oxidative stress. Senescence has been linked to tumour suppression and it may also be induced by chemotherapeutic agents. Components of the IGF system, including IGFBP-6, have been implicated in this process [5]. IGFBP-6 levels are increased in fibroblasts following senescence induced by H2O2 and other agents [88], and in mouse fibroblasts that senesced under physiological oxygen conditions [89]. Consistent with these findings, IGFBP-6 levels were higher in sera from aging mice [88] and humans [90]. The latter study suggested that IGFBP-6 is a negative regulator of senescence, but, in contrast with almost all other studies, it surprisingly showed that IGFBP-6 overexpression increased proliferation and decreased apoptosis [90]. It has also been shown that IGFBP-3 and IGFBP-5 promote senescence, so these provocative findings for IGFBP-6 require further study.

Proteolysis
Limited proteolysis of IGFBPs is believed to be a mechanism whereby IGFs are released for receptor binding and a number of proteases have been identified for specific IGFBPs. Indeed, a number of IGFBP-6 proteases have been described, including a cathepsin-D-like acid protease [91], a neutral serine protease [92] and MMP-7 [93]. Proteases are linked to tumorigenic processes such as cell migration and invasion, as well as angiogenesis. IGFBP-6 was identified as a substrate for MMP-2 in a proteomic screen for candidate angiogenic inhibitors that may be neutralized by the protease [94], and as a substrate for MMP-9 and MMP-12, which may regulate its potential role as an inhibitor of myelination [57].

IGFBP-6 AND CANCER
As with other components of the IGF system, the role of IGFBP-6 has been investigated in a considerable number of cancer cells and models [5]. In most studies, IGFBP-6 expression is lower in malignant than normal cells, suggesting that it has an inhibitory effect, although there are some exceptions. Studies of IGFBP-6 in a range of common adult and paediatric tumours will be highlighted.

RMS
RMS is the most common soft tissue sarcoma of childhood and adolescence, and it arises from primitive skeletal myoblasts. There are two principle RMS subtypes, embryonal (ERMS) and alveolar (ARMS). ERMS mainly affects children younger than 10 years of age and is associated with LOH or LOI at the chromosome 11p15.5 locus, which contains a number of imprinted genes including IGF2 [95–97]. ARMS is predominantly found in adolescents and young adults. The PAX3–FKHR (forkhead in rhabdosarcoma) or PAX7–FKHR fusion protein, in which the DNA-binding domain of PAX3/PAX7 is fused with the transactivation domain of FKHR, is present in the majority of ARMS, and is associated with increased aggressiveness and poor prognosis [97,98].

Although mutations in components of the IGF system have not been detected in RMS, dysregulation of IGF signalling is important in mediating RMS transformation and progression [12,99]. In particular, IGF-II has been implicated as an autocrine growth factor in RMS [100,101]. LOI and LOH of IGF2 result in increased IGF2 expression in ERMS [102]. In ARMS, the PAX3–FKHR fusion protein can induce IGF-I receptor expression [103] and co-operate with IGF-II [99] to enhance IGF signalling [104,105]. IGF-II may also be overexpressed in RMS following loss of p53 function due to mutation [106] or enhanced levels of the transcription factor AP-2 (activator protein-2) [107]. As a result, consistently increased IGF-II levels have been identified in both RMS subtypes. This autocrine IGF-II secretion results in increased RMS cell proliferation and migration [108,109]. In animal models, IGF-II is indispensable for the development and malignant behaviour of RMS [110], and IGF-II is also associated with RMS angiogenesis [111]. Furthermore, IGF-I receptor overexpression increases proliferation and protects against stress-induced apoptosis, which are associated with poor survival in patients with RMS [112].

RMS is associated with mutations that increase activity of the Hh pathway [69]. A role for Hh signalling in RMS is suggested by its development in mice deficient in the cell-membrane tumour suppressor Ptc1 (Figure 3), which also have increased levels of Gli1 and IGF-II [113]. Indeed, Shh and IGF signalling synergized to induce medulloblastoma formation in a mouse model.
Betulinic acid, an apoptosis-inducing agent, inhibited Hh signalling and IGF-II expression in Gli1-overexpressing RMS13 RMS cells; however, it had no effect on IGF-II levels and less effect on survival in Gli1-negative RD and Rh30 RMS cells [115].

**RMS and IGFBP-6**

IGFBP-6 inhibits the tumorigenic properties of IGF-II-dependent cancer cell lines including RMS in vitro and in vivo. IGFBP-6 inhibited anchorage-dependent and anchorage-independent proliferation and survival of RMS cells in vitro and IGF-II addition overcame these effects to some extent [116]. Xenografts of Rh30 RMS clones overexpressing IGFBP-6 were ~80% smaller than vector control after 18 days, although tumour growth subsequently ‘escaped’ to parallel control tumours [116]. Tumour formation was delayed further when IGFBP-6-overexpressing mice were treated with the rapamycin analogue CCI-779 [117], suggesting that modulation of multiple components of the IGF pathway may enhance inhibitory efficacy [118]. These results show that IGFBP-6 is a potent inhibitor of RMS growth, at least in part by inhibiting the autocrine effects of IGF-II.

We have also identified IGF-independent actions of IGFBP-6 in these cells using a non-IGF-binding IGFBP-6 mutant (mIGFBP-6) [119]. WT (wild-type), but not mIGFBP-6, inhibited RMS cell proliferation, suggesting that IGFBP-6 acts by inhibiting autocrine IGF-II actions. Surprisingly, however, mIGFBP-6 and WT IGFBP-6 both promoted RD and Rh30 RMS cell migration, indicating that this action of IGFBP-6 is IGF-independent [119,120]. ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAPKs are implicated in cell migration and invasion [121]. WT and mIGFBP-6 both increased p38 MAPK phosphorylation in RD cells, and inhibition of p38 MAPK by a chemical inhibitor or siRNA (small interfering RNA) prevented IGFBP-6-induced migration [119]. In contrast, WT and mIGFBP-6-induced phosphorylation of ERK1/2 and JNK1, but not p38 MAPK, in Rh30 cells [120]. Furthermore, cross-talk between the MAPK pathways appeared to be involved in IGFBP-6-induced chemotaxis in both cell lines [119,120]. These findings clearly show that IGFBP-6 promotes migration in an IGF-independent manner and that MAPK pathways are involved. Interestingly, as mentioned below, IGFBP-6 expression was increased by JNK activation in oral cancer cells [122], raising the intriguing possibility that there may be a positive-feedback loop between IGFBP-6 and JNK activation in some cancer cells.

IGFBP-6 may localize to the nucleus of RMS cells, and this is mediated via a C-terminal domain NLS that interacts with importin-α [61]. Overexpression of IGFBP-6 increased RMS cell apoptosis, an effect abrogated by deletion of the NLS, suggesting that nuclear translocation may play a role. Furthermore, IGFBP-6 interacts with Ku80, which is involved in DNA repair and stability [64]. Overexpression of IGFBP-6 altered the nuclear localization of Ku80, and knockdown of the latter enhanced the apoptotic effect of IGFBP-6, suggesting that IGFBP-6 may modulate RMS cell survival by regulating the availability of Ku80 for the DNA-repair process.

Angiogenesis, the formation of new capillaries from existing blood vessels, is critical for solid tumours to grow beyond 1–2 mm³ [123,124]. Angiogenesis also enhances metastasis by facilitating cancer cell entry into abnormal, tumour-induced, leaky vessels [125]. Although VEGF pathways are central mediators of angiogenesis, IGF-II also plays a role in RMS angiogenesis [111], at least in part by inducing VEGF synthesis [38,126,127]. RMS cell lines express VEGF [128], and angiogenesis inhibitors impair RMS xenograft growth [129,130]. IGFBP-6 overexpression inhibited angiogenesis in RMS xenografts and zebrafish embryos, and IGFBP-6 inhibited tube formation by HUVECs (human umbilical vein endothelial cells) in vitro in an IGF-independent manner [75]. IGFBP-6 expression in HUVECs was increased by prolonged hypoxia via HIF-1α, and the slow response and its inhibitory effect are consistent with a role for IGFBP-6 as part of a negative-feedback mechanism that regulates hypoxia-induced angiogenesis [131]. These findings raise the possibility that IGFBP-6 may be a dual IGF-II/VEGF inhibitor, which would be expected to enhance its anticaner activity. As mentioned below, SEMA3B (sema domain, Ig domain, short basic domain, secreted 3B), a semaphorin, is a tumour suppressor that competes with and inhibits VEGF activity and also has actions independent of VEGF [132]. In lung cancer cells, SEMA3B increased the expression of IGFBP-6, which mediated its antiproliferative effect, suggesting another level at which IGFBP-6 may be involved in dual pathway inhibition involving angiogenesis [132].

The efficacy of IGF-I receptor inhibition is highly variable in different cancers and ongoing clinical development requires an understanding of factors that contribute to resistance to these inhibitors. In a panel of sarcomas (including RMS) and neuroblastomas, increased IGFBP-6 and IGFBP-3 expression were associated with resistance [133], possibly because endogenous IGF activity was already suppressed by high IGF levels.

**Lung cancer**

IGFBP-6 was expressed in nine out of 11 human lung cancer specimens, as well as in normal lung [134]. Its expression was lower in lung adenomas and adenocarcinomas than in normal lung tissue in mice [135]. IGFBP-6 inhibited proliferation of human bronchial epithelial cells and its expression was induced by retinoic acid, so that it may contribute to the inhibitory effects of the latter [136]. IGFBP-6 increased apoptosis of non-small-cell lung cancer cells [137]. In lung cancer cells, IGFBP-6 levels were also increased by the tumour suppressors p53 [76] and SEMA3B [132]; knockdown of IGFBP-6 suggested that it mediates the antiproliferative effect of the latter.

**Breast cancer**

IGFBP-6 is expressed at low levels in breast cancer specimens [138], and a small study has shown that serum IGFBP-6 levels are lower in women with breast cancer than in those with benign breast disease [139]. IGFBP-6 is also expressed in oestrogen-receptor-negative breast carcinoma cell lines [138,140], and oestradiol increased IGFBP-6 levels in oestrogen-receptor-positive breast cancer cells [78]. In an in vivo model of sex-steroid-responsive breast cancer, ovariectomy induced tumour regression and also increased stromal IGFBP-6 expression [141]. Tumour growth was restored by hormone repletion, which also resulted in increased IGF-II and decreased IGFBP-6 expression [141].
Retinoids and retinoids, which have inhibitory effects on breast cancer growth, also increase IGFBP-6 levels [20,66,140,142]. Development of breast cancer resistance to HER2 inhibitors is an important clinical problem and the IGF system has been implicated in its development. IGFBP-6 expression was higher in breast cancer cells resistant to HER2 inhibition by trastuzumab than in sensitive cells, but its potential contribution to the resistance has not been explored [143].

**Prostate cancer**
IGFBP-6 is expressed by benign and malignant prostate epithelial and stromal cells [144–146]. IGFBP-6 and other IGF system components are up-regulated during prostate epithelial cell differentiation [146], whereas the expression of IGFBP-6 and other IGFBPs decreased as a series of transformed human prostate epithelial cells became more tumorigenic [147]. In contrast, IGFBP-6 levels are increased by 1,25dihydroxyvitamin D3, which may be antiproliferative in prostate carcinoma cells [148]. DES (diethylstilboestrol), a synthetic oestrogen, also increased the expression of IGFBP-6, which may mediate its antiproliferative effects, in androgen-independent prostate cancer cells [80].

**Colon cancer**
IGF-II was the most highly overexpressed gene in colorectal tumours compared with normal colorectal tissue [149]. IGFBP-6 levels were lower in a multidrug-resistant colon cancer cell line than in a sensitive line [150], and were also lower in a metastatic than in a non-metastatic cell line [151], both suggesting that IGFBP-6 may be antitumorigenic. n-3 fatty acids and retinoic acid, inhibitors of colon cancer cell proliferation, stimulated IGFBP-6 expression in cancer cells [152,153], whereas the antiproliferative effects of vitamin D analogues on colon cancer cells may be due to decreased IGF-II and IGFBP-2 levels and increased IGFBP-6 levels [154]. IGFBP-6 levels were also increased by doxorubicin, a chemotherapeutic agent that increases cellular senescence in colon cancer cells [155]. In support of an antitumorigenic role, exogenous IGFBP-6 inhibited IGF-II-induced proliferation and adhesion of colon cancer cells [156] and IGFBP-6 overexpression also inhibited their growth.

**Head and neck cancer**
IGFBP-6 is down-regulated in nasopharyngeal cancer cells and it acts as a tumour suppressor gene by acting within the nucleus to regulate the expression of the transcription factor EGR-1 (early growth-response gene product-1) [65]. IGFBP-6 expression in metastatic head and neck squamous cell carcinoma was lower than in primary disease; IGFBP-6 decreased migration but not proliferation of oral carcinoma cells [157]. Apoptosis and IGFBP-6 expression were both increased by JNK activation and NF-κB (nuclear factor κB) inhibition in oral cancer cells, although the direct effect of IGFBP-6 on apoptosis in these cells was not studied [122].

**Ovarian cancer**
IGF-II appears to have an important role in ovarian cancer. IGF-II transcript levels were more than 300-fold higher in cancer tissues compared with normal ovaries [158]. Furthermore, IGF2 gene expression may predict poor survival, since levels were 7-fold higher in advanced-stage ovarian cancer with survival of <45 months compared with survival of >45 months [158]. IGFs have also been shown to stimulate ovarian cancer cell invasion, proliferation and angiogenesis [159].

IGFBP-6 was detected in 38 out of 41 ovarian cancers by immunohistochemistry, but it was not regulated by oestrogen in oestrogen-responsive cancer cells in vitro [79]. A single microarray study showed that IGFBP-6 mRNA levels were lower in ovarian cancer tissue compared with normal tissue [160], this may result in increased IGF-II action, but IGFBP-6 levels were not confirmed in an independent assay. Two studies of women with ovarian cancer showed discrepant results with one showing increased serum IGFBP-6 levels [161] and the other showing lower levels [162]. These findings obviously require further study.

**Neuroblastoma**
Neuroblastoma is the most common malignancy of infancy and the most common extracranial solid cancer of childhood. It is an IGF-II-dependent tumour [100], and overexpression of the N-myc oncogene, which is associated with poor prognosis in neuroblastoma, decreased IGFBP-6 and increased IGFBP-2 and IGF-I receptor levels [163]. IGFBP-6 expression coincided with decreased proliferative responsiveness of neuroblastoma cells to IGFs and insulin [164], and FGF (fibroblast growth factor)-2, which stimulates neuronal differentiation, increased IGFBP-6 expression [165]. Constitutive IGFBP-6 overexpression inhibited neuroblastoma xenograft growth in vivo [166], an effect that may be attributable to increased apoptosis [167]. Furthermore, infusion of IGFBP-6 also delayed neuroblastoma xenograft growth in vivo, probably by inhibiting the actions of IGF-II [168].

**Other cancers**
A couple of studies have suggested a possible role for IGFBP-6 as a tumour marker. A small study of thyroid cancer showed that IGFBP-6 expression is increased in papillary cancer, but decreased in follicular cancer, and it was suggested that it is part of a five-gene signature that can distinguish between these cancers [169]. This study requires confirmation in a larger cohort. Plasma IGFBP-6 levels were significantly lower in patients with hepatocellular cancer than in those with hepatitis B/C, also suggesting a role as a tumour marker [170].

Epigenetic silencing of tumour suppressor genes is an important contributor to cancer development. Abnormal methylation of the IGFBP-6 promoter was detected in ~23% of 152 primary gastric cancer samples, and cancer cell-line-specific hypermethylation was associated with decreased IGFBP-6 expression [171]. Consistent with these findings, 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, increased IGFBP-6 expression in immortalized fibroblasts [172].

As demonstrated above, many studies have shown decreased IGFBP-6 levels in cancer, but a smaller number have shown the opposite. Recent examples including pancreatic cancer [74] and adrenocortical cancer [173]. It is possible that this increase represents a compensatory response to increased IGF-II activity or it may reflect IGF-independent actions of IGFBP-6.
CONCLUSIONS

The present review has summarized the role of the IGF system in normal physiology and disease before focusing on IGFBP-6 with its unique IGF-II-binding specificity and the more recent findings that it inhibits angiogenesis but promotes migration in an IGF-independent manner. Given the widespread interest in IGF inhibition in cancer therapeutics, increasing our understanding of the mechanisms underlying the actions of the IGF ligands, receptors and binding proteins such as IGFBP-6 will enhance our ability to develop optimal treatments that can be targeted to the most appropriate patients. In particular, an IGF-II-specific inhibitor may have utility in paediatric and adolescent patients where it may be advantageous to inhibit high levels of autocrine IGF-II without perturbing normal growth mediated by circulating IGF-I.

ACKNOWLEDGEMENT

We thank Dr Anne Mc Robert (Department of Medicine, Monash University) for her insightful comments.

FUNDING

Our own work is supported by the National Health and Medical Research Council of Australia, the Australian Research Council, the Cancer Council Victoria and the Alfred Research Trust.

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Received 28 June 2012/14 August 2012; accepted 12 September 2012
Published on the Internet 31 October 2012, doi: 10.1042/CS20120343