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Insulin-like growth factor binding proteins and angiogenesis: from cancer to cardiovascular disease

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Short title: Angiogenesis and the insulin-like growth factor binding proteins

Abstract

Angiogenesis is a tightly regulated activity that is vital during embryonic development and for normal physiological repair processes and reproduction in healthy adults. Pathological angiogenesis is a driving force behind a variety of diseases including cancer and retinopathies, and inhibition of angiogenesis is a therapeutic option that has been the subject of much research, with several inhibitory agents now available for medical therapy. Conversely, therapeutic angiogenesis has been mooted as having significant potential in the treatment of ischemic conditions such as angina pectoris and peripheral arterial disease, but so far there has been less translation from lab to bedside.

The insulin-like growth factor binding proteins (IGFBP) are a family of seven proteins essential for the binding and transport of the insulin-like growth factors (IGF). It is being increasingly recognised that IGFBPs have a significant role beyond simply modulating IGF activity, with evidence of both IGF dependent and independent actions through a variety of mechanisms. Moreover, the action of the IGFBPs can be stimulatory or inhibitory depending on the cell type and environment. Specifically the IGFBPs have been heavily implicated in angiogenesis, both pathological and physiological, and they have significant promise as targeted cell therapy agents for both pathological angiogenesis inhibition and therapeutic angiogenesis following ischemic injury. In this short review we will explore the current understanding of the individual impact of each IGFBP on angiogenesis, and the pathways through which these effects occur.

Key words:

Insulin like growth factor binding protein; IGFBP; angiogenesis; ischemia

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a fundamental, tightly regulated activity in many biological contexts including development, reproduction and tissue repair [1]. Angiogenesis can also become a pathological process critical to the development and progression of several diseases and may potentially be harnessed therapeutically to improve tissue perfusion in ischemia [2].

During development there are two distinct processes that form the vascular network: vasculogenesis and angiogenesis. Vasculogenesis refers to the initial differentiation of endothelial precursors in the embryonic period that occurs in order to establish a population of endothelial precursor cells. These cells can then release angiogenic factors and cytokines as well as being able to differentiate into a population of endothelial cells, which establish the rudimentary vasculature [3]. Angiogenesis is the process by which new blood vessels sprout from pre-existing vasculature. This occurs via a stepwise process that involves endothelial cell proliferation and migration to form vascular sprouts, followed by degradation and invasion of the extracellular matrix and finally vascular tube formation [4]. Angiogenesis is a complex process, orchestrated by the local production of a range of growth factors and cytokines. These activate cellular signalling pathways and gene transcription to modulate the sprouting, proliferation and migration of endothelial cells and their interaction with the extracellular matrix [5]. Although vascular endothelial growth factors (VEGF) are the central mediators of angiogenesis, others growth factors including fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and insulin-like growth factors (IGFs) are known to play important roles. Understanding how these growth factors and their regulatory partners are involved in angiogenesis in critical to scientific advancement in the fields of development, cancer and ischemic disorders.

In pre- and post-natal development, angiogenesis is essential for organ growth but in healthy adults the vasculature is largely quiescent, with the exception of the female reproductive

system. However, the need for normal physiological repair processes such as wound healing and continual remodelling of capillary beds requires angiogenesis to be a tightly regulated process that can be 'switched' on and off as needed, depending on the balance between stimulatory and inhibitory signals [6].

'Pathological' angiogenesis is involved in the progression of several diseases including age-related macular degeneration, diabetes retinopathy and rheumatoid arthritis [7] [2]. Survival of tumour cells also relies on pathological angiogenesis: when cancer cells first develop they are initially dormant until they develop a vascular network that allows them to grow and metastasise. Tumour cells grow at an accelerated rate and in doing so cannot maintain an adequate supply of glucose and oxygen, thereby creating the perfect stressors for the release of pro-angiogenic factors and the activation of angiogenesis [8].

In ischemic states secondary to atherosclerosis or thrombosis, local release of angiogenic factors endeavours to promote perfusion of the ischemic tissues, but often the physiological process is inadequate to restore sufficient tissue perfusion [9]. A pertinent example is peripheral arterial disease (PAD), which presents clinically as intermittent claudication or critical limb ischemia, the latter of which poses a major risk of limb loss and is associated with high mortality. In some patients, revascularisation either fails or is not an appropriate option, leaving amputation as the only alternative [10].

Over the past decades, efforts have been undertaken to modulate angiogenesis as a therapeutic strategy to either promote revascularization of ischemic tissues or inhibit angiogenesis in cancer, ocular, joint or skin disorders. Angiogenesis inhibitors have been successfully developed which are now used clinically in the treatment of certain cancers and forms of macular degeneration [11]. Manipulating upregulation of angiogenesis in order to repair damaged organs and regrow blood vessels in ischemic disorders, termed 'therapeutic angiogenesis', has proved to be more challenging. Potential approaches include the systemic

or local delivery of pro-angiogenic factors (as recombinant proteins or gene therapy) or cell-based strategies using autologous or ex vivo modified stem/progenitor cells [12].

Initial exploration of therapeutic angiogenesis in animal models and Phase 1 clinical trials appeared very promising. VEGF and FGF recombinant protein were used in both animal and human models to successfully induce angiogenesis and collateralisation in myocardial ischemia, with demonstrably improved perfusion of the target organ [13] [14] [15]. The delivery of autologous bone marrow derived stem cells in PAD and myocardial infarction also appeared to successfully induce angiogenesis and improve perfusion in initial trials [16] [17]. The VIVA trial was the first larger scale randomised control trial (RCT) and examined the effects of intra-coronary rhVEGF administration in patients with refractory stable angina. No improvement was seen in myocardial perfusion or exercise tolerance, with only a marginal improvement in self-reported angina symptoms seen in the treated group [18]. Meta-analyses of autologous cell therapy in PAD also showed that despite promising results in several trials, in placebo controlled RCTs with a low risk of bias no benefit with cell therapy was observed [19].

Gene therapy has been mooted as a potential method to overcome the shortcomings of recombinant protein administration, with direct delivery of vectors containing VEGF-A or FGF genes to areas of ischemia. As with the other methods this also appeared to be effective in animal models and small human trials, both for PAD and myocardial ischemia [20] [21] [22]. However, larger placebo controlled studies again failed to show any significant improvement in perfusion or clinical symptoms [23] [24].

Given these shortcomings when examined on a large scale, a pro-angiogenic therapy has not yet been licensed for routine clinical use. Nevertheless, the clinical need to improve tissue perfusion in ischemic disorders remains; therefore therapeutic angiogenesis remains an important research area, and new pro-angiogenic therapies need to be identified. The majority

of studies so far have only examined VEGF and FGF administration, but there are several other cytokines and growth factors with significant therapeutic potential.

The insulin-like growth factor (IGF) system comprises two insulin-like growth factors (IGF-I and IGF-II), their receptors (IGF1R and IGF2R) and seven insulin-like growth factor binding proteins (IGFBPs), with 99% of all circulating IGFs bound to a member of the IGFBP family [25]. The IGFs are growth factors with molecular structural homology to proinsulin and significant overlap in signalling pathways and receptor interaction [26]. They act as circulating factors but also possess important autocrine and paracrine actions dependent on local release. Circulating IGF-I is predominantly released in response to stimulation by growth hormone (GH) and its primary role is to promote cellular growth and proliferation, although it is also involved in blood glucose regulation and plays an active role in promoting angiogenesis [25]. IGF-II expression is independent of GH and has historically been considered to have a prominent role only in pre-natal growth and development, due to negligible expression of the protein post-natally in adult rodents [27]. In humans, however, expression of IGF-II persists postnatally and it continues to play a role in cell growth, proliferation and angiogenesis [28]. Most cellular actions of IGF-I and IGF-II are mediated by the IGF1 receptor (IGF1R), a receptor tyrosine kinase which activates several signalling pathways including phosphatidylinositol 3-kinase and protein kinase B (PI3K/AKT) and Ras/Raf/ERK. The IGF2 receptor (IGF2R) is thought to be involved with IGF-II clearance but may have a limited signalling role.

A family of seven IGFBPs confers spatial and temporal regulation to IGF activity. Several IGFBPs also possess important IGF-independent effects. Three distinct structural regions are shared by all IGFBPs: an N-terminal cysteine rich region; a C-terminal cysteine rich region; and a linker region. The N-terminal and C-terminal regions contribute to IGF binding and are highly conserved across the IGFBPs; while the linker region is variable and can contain a variety of functional motifs and binding sites [29]. The linker domain is susceptible to post-translational modification and contains sites for proteolysis by a range of proteases. In the

circulation, the majority of IGFs are bound to IGFBPs to form binary complexes or form large molecular mass complexes with IGFBP-3 and IGFBP-5 and an acid labile subunit (ALS) which are unable to cross to endothelial barrier. Binary complexes allow IGFBPs to localise within tissues where their action is predominantly to inhibit IGF actions, although this is context-dependent and IGFBPs may potentiate IGF actions in certain situations.

All members of the IGFBP family have been studied in the context of angiogenesis, where inhibitory and stimulatory actions have been ascribed to the different binding proteins. Local production of IGFBPs in the blood vessel wall [30], ischemic tissues or tumours [31] is of particular relevance. The predominant effects of IGFBPs on angiogenesis are mediated through modulation of IGF bioactivity, although important IGF-independent actions are emerging.

IGFBP-1

IGFBP-1 is a 30 kDa protein, with circulating levels produced predominantly in the liver and kidneys [32]. IGFBP-1 is expressed locally within the blood vessel, including the endothelium [33]. The predominant action of IGFBP-1 is thought to be dynamic regulation of IGF bioavailability, although an integrin binding domain (RGD) found in the C-terminus region of IGFBP-1 has been found to be an important mediator of IGF independent actions [34] [35].

IGFBP-1 levels are dynamically regulated in relation to nutritional intake: fasting levels are four to five times higher than non-fasting levels and IGFBP-1 production in the liver is inhibited directly by the increase in insulin levels in the acute post-prandial state [36]. IGFBP-1 accounts for only a small proportion of overall IGF binding capacity and so is thought to be mainly involved in acute IGF-I regulation [36] [37].

Influences of IGFBP-1 on cellular processes including migration and proliferation have been extensively researched and both IGF-dependent and IGF-independent activities have been

reported. Both stimulation and inhibition of IGF actions by IGFBP-1 have been demonstrated depending on cell type and environment [35] [38]. Although several studies implicate IGFBP-1 in vascular pathophysiology and endothelial function, investigation of IGFBP-1 in the setting of angiogenesis is limited.

In the context of glioblastoma, proteomic analysis identified IGFBP-1 as the key mediator of angiogenesis secreted by microglial cells in response to macrophage colony stimulating factor (MCSF). When conditioned medium from glioma cells was added to human umbilical vein endothelial cells (HUVEC), silencing of MCSF prevented tube formation. Upregulation of IGFBP-1 was identified in the microglial cell secretome in response to MCSF, and silencing of IGFBP-1 in microglial cells blocked angiogenesis in HUVECs treated with the conditioned media [39]. The mechanism by which IGFBP-1 promoted angiogenesis was not elucidated in this study.

In human chondrocytes, lysophosphatidic acid (LPA) was shown to activate the NF-kB pathway and to enhance tube formation and cell migration of HUVECs treated with conditioned media [40]. IGFBP-1 was identified in the chondrocyte secretome along with known pro-angiogenic factors including VEGF, interleukin-8 (IL-8), matrix metalloproteinase (MMP)-9 and monocyte chemoattractant protein 1 (MCP-1). Although IGFBP-1 was found to be up-regulated, the direct contribution of IGFBP-1 to angiogenesis was not determined.

We previously reported that over-expression of IGFBP-1 in transgenic mice improved vascular endothelial function through increased basal nitric oxide (NO) production. Endothelial nitric oxide synthase (eNOS) mRNA expression was upregulated [37]. IGFBP-1 stimulated eNOS phosphorylation via the PI3K/AKT pathway – an effect independent of the IGF1 receptor. Over-expression of IGFBP-1 was also found to have an anti-atherosclerotic effect in the same transgenic mice [41]. NO is an important mediator of angiogenesis [42], and although in vitro angiogenesis was not examined in cells from these mice, it could be hypothesised that

upregulation of the PI3K/AKT pathway by IGFBP-1 could stimulate angiogenesis as described elsewhere [43]. The integrin-binding RGD domain of IGFBP-1 is potentially of interest in this context, recognising that signalling downstream of integrins, for example through focal adhesion kinase, stimulates angiogenesis [44].

Amniotic membrane is developmentally avascular and has potential to inhibit neovascularisation of the cornea following ocular injury. The anti-angiogenic properties of the amniotic membrane have been shown to be mediated by a complex of hyaluronan and the heavy chain of inter-α-inhibitor. This complex significantly inhibited tube formation in HUVEC and reduced neo-vascularisation in a chorioallantoic membrane (CAM) model [45]. IGFBP-1 and the anti-angiogenic platelet factor 4 (PF4 or CXCR4) were identified in the complex by a screen of potential angiogenesis-related proteins, although the contribution of each was not specifically demonstrated.

In summary, IGFBP-1 has been shown to have both stimulatory and inhibitory effects on cells, through IGF dependent and independent pathways. Limited evidence supports a pro-angiogenic effect of IGFBP-1 but confirmatory studies are needed.

IGFBP-2

IGFBP-2 is a 31.4kDa protein and the second most abundant IGFBP in the circulation [46]. It contains an RGD integrin binding domain within the C-terminus similar to IGFBP-1, and in addition contains a heparin binding domain (HBD) and nuclear localisation sequence (NLS) within the link region, both of which act as functional motifs to facilitate extracellular matrix (ECM) binding and nuclear localisation respectively [29] [47]. Although IGFBP-2 levels are not as acutely regulated by insulin levels as IGFBP-1 [26], plasma IGFBP-2 levels are inversely correlated with insulin resistance [48] and over-expression of IGFBP-2 has been demonstrated to be protective against obesity and insulin resistance [49].

IGFBP-2 appears to play an important role in vascular development, as knockdown led to impaired vascular sprouting in zebrafish embryos [50]. In adult animals, IGFBP-2 expression is increased in ischemic stroke [51] and upregulation of IGFBP-2 is a signature of several types of cancer in which it has been implicated in tumour angiogenesis. IGFBP-2 over-expression activates pro-tumorigenic gene expression in neuroblastoma cells, including significantly up-regulated VEGF mRNA transcription. Increased in vivo angiogenesis was observed when IGFBP-2 overexpressing neuroblastoma cells were studied in a quail embryo CAM assay [52]. Interestingly, upregulation of VEGF transcription was only seen in the presence of intra-cellular IGFBP-2, with nuclear translocalisation of IGFBP-2 shown to be mediated by the functional nuclear localization sequence within the link region [53]. No role was seen for IGF-I, suggesting these were IGF independent effects.

A separate study by Russo et al. also examined neuroblastoma cells and found over-expression of IGFBP-2 enhanced proliferation, migration and invasion in vitro, and through the use of IGFBP-2 mutants determined this was facilitated through ECM binding by the HBD. Within the same study IGFBP-2 inhibited exogenous IGF-I mediated proliferation, suggesting a dual role through differing, competing pathways [47]. A further study examining glioma progression linked IGFBP-2 to upregulation of NF-κB through integrin binding and activation of integrin-linked kinase (ILK) pathways. Although this study didn't examine angiogenesis directly; cell migration, invasion and overall glioma progression were shown to be positively up-regulated by IGFBP-2 through IGF-independent integrin binding [54], highlighting the potential for IGFBP-2 to act through several different pathways.

IGFBP-2 has also been identified as a pro-angiogenic factor in melanoma. Das et al. investigated the role of melanoma differentiation associated gene-9 (mda-9/syntenin) in tumour angiogenesis, and found that mda-9/syntenin augmented angiogenesis in CAM assays and increased tube formation in co-cultured HUVECs. IGFBP-2 was identified as a mda-9/syntenin induced factor, and was shown to independently augment tube formation in

HUVECs, with knockdown of IGFBP-2 associated with both reduced in vitro tube formation and CAM neovascularisation [55]. The induction of IGFBP-2 expression by mda-9/syntenin was shown to be a consequence of AKT and hypoxia-inducible factor 1 α (HIF-1α) activation. The same authors found that integrin binding by IGFBP-2 in HUVECs caused activation of the PI3K/AKT pathway and VEGF-A up-regulation, and hypothesised that endothelial recruitment and subsequent activation of this pathway by IGFBP-2 was the mechanism through which angiogenesis in melanoma occurred [55]. Interestingly, although integrin binding was seen to be key to activation of the PI3K/AKT pathway, inhibition of the IGF-1R abrogated this response, suggesting a significant role for the IGF-1R, although the mechanism of this role was not defined.

Png et al. also described an IGF-I dependent pro-angiogenic role for IGFBP-2. They had previously identified the micro-RNA miR-126 as a suppressor of breast cancer metastasis [56]. They went on to find that this was achieved through the suppression of IGFBP-2 induced metastatic angiogenesis in endothelial cells and that IGFBP-2 secreted by breast cancer cells positively modulated IGF-I mediated activation of IGF-1R on endothelial cells, promoting their recruitment for metastatic angiogenesis [57]. The involvement of the IGF-1R in the pro-angiogenic role of IGFBP-2 was also seen by Shen et al., who found binding of IGFBP-2 to receptor protein tyrosine phosphatase β (RPTPβ) caused inactivation of RPTPβ and subsequently inhibited transcription of the tumour suppressor gene PTEN. Inhibition of PTEN allowed increased activation of the PI3K/AKT pathway by IGF-I, promoting vascular smooth muscle proliferation, and raising the potential for a pro-angiogenic action as well. Inhibiting IGF-1R expression however prevented the inactivation of RPTPβ, suggesting the effects of IGFBP-2 on PTEN phosphorylation required coordination of IGFBP-2, IGF-I and IGF-1R [58].

In summary, although IGFBP-2 has been shown in some environments to inhibit the mitogenic actions of IGF-I [59], the overwhelming evidence suggests a pro-angiogenic role for IGFBP-2. The effects of IGFBP-2 have been proven to occur through a variety of both IGF dependent

and independent pathways, creating the possibility of dual, competing roles. Further understanding of these pro-angiogenic pathways could allow the use of IGFBP-2 as an agent for therapeutic angiogenesis after ischemic insult, and conversely the inhibition of IGFBP-2 may be a novel avenue in the treatment of various cancers.

IGFBP-3

IGFBP-3 is a 28.7kDa protein primarily produced in the liver and is the most abundant IGFBP, responsible for over 80% of IGF-I and IGF-II binding in the circulation. It binds IGF-I in a ternary complex with an acid-labile subunit, forming a stable 150kDa complex and preventing transport of IGF-I into the tissues [25]. Both pro-angiogenic and anti-angiogenic roles of IGFBP-3 have been reported.

Granata et al. demonstrated a pro-angiogenic role for IGFBP-3 in HUVECs both in vitro and in vivo. IGFBP-3 induced the expression of several angiogenesis-related genes including VEGF, MMP-2, and MMP-9. This pro-angiogenic action was mediated by IGFBP-3 induction of the sphingosine kinase (SphK1) pathway and was dependent on IGF-I signalling [60].

IGFBP-3 was released with other angiogenic factors, such as hypoxia inducible factor alpha (HIF-α) and VEGF when human retinal pigmented epithelial cells were stimulated with IGF-I, suggesting that IGFBP-3 may have a pro-angiogenic effect in choroidal neovascularisation [61]. Several in vivo studies have provided further evidence of this. Lofqvist et al. showed that IGFBP-3 deficient mice had a decrease in retinal vessel regrowth following hypoxia and wild type mice treated with IGFBP-3 had a significant increase in vessel regrowth, independent of IGF-I levels. This study also incorporated a prospective clinical study of premature human infants showing an association between IGFBP-3 levels and a decrease in retinal neovascularisation [62]. These findings were corroborated by Chang et al., who injected an IGFBP-3 expressing plasmid into the vitreous of mice and showed that this IGFBP-3 over-expression promoted angiogenic repair of the eye [63].

Fanton et al. demonstrated that cardiac atrial appendage stem cells (CASCS) secrete high levels of IGFBP-3, VEGF and endothelin-1 (ET-1), and promoted microvascular endothelial cell proliferation, tube formation and migration in vitro and stimulated angiogenesis in vivo in a CAM assay. Combined inhibition of the three growth factors was required to inhibit tube formation, with individual inhibition having no effect. The authors postulated that these growth factors acted synergistically to enhance angiogenesis, although a pathway for this was not elucidated, and the authors did not explore the effect of inhibition of VEGF and ET-1 only [64].

In contrast to the previous reports, there is also significant evidence for IGFBP-3 as an anti-angiogenic agent. IGFBP-3 was found to block tumour angiogenesis in both non-small cell lung cancer and squamous cell carcinoma of the head and neck. This was demonstrated to occur via an IGF-independent pathway involving the inactivation of ERK1/2 and Elk-1, leading to inhibition of basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) transcription: growth factors known to have significant roles in angiogenesis and cancer cell survival [65]. IGFBP-3 has also been shown to inhibit endothelial cell adhesion to the extracellular matrix [66], which is important in angiogenesis and endothelial repair. Han et al. demonstrated that a peroxisome proliferator-activated receptor (PPAR-δ) agonist caused endothelial progenitor cells to produce MMP-9, which degraded IGFBP-3, leading to a higher capillary-to-myocyte ratio and faster recovery of blood flow in an ischemic hind-limb mouse model. Muscle architecture was also more intact and no fibrosis was observed, both of which were reversed with the introduction of IGFBP-3. IGFBP-3 degradation has also been shown to induce IGF-1R phosphorylation, resulting in augmented angiogenesis in an in vivo mouse hindlimb ischemia model [67].

It has been shown that in head and neck cancers, IGFBP-3 expression reduces the tumours' angiogenic capacity, thereby limiting lymph node and wider body metastasis, and treatment

with IGFBP-3 above the physiological concentration of 2-7 μg/ml will inhibit VEGF expression [68]. An in vivo study conducted in mouse endothelial progenitor cells showed that the transcription factor Runx1 promoted angiogenesis by down-regulating mRNA expression of IGFBP-3 through directly binding to the mouse IGFBP-3 gene and preventing its transcription [69]. Increasing expression of IGFBP-3 has already been exploited therapeutically by way of SCH6633, a farnesyl transferase inhibitor, which is an anti-angiogenic oral agent designed to block tumour growth [70].

To summarise, a pro-angiogenic role for IGFBP-3, especially in the context of hypoxia, has been revealed, with potential for its utilisation as a therapeutic agent in the context of ischemic insult. However, this needs to be balanced against its anti-angiogenic function that has also been demonstrated to occur through a variety of pathways, which could potentially be exploited as a novel cancer therapeutic inhibitor.

IGFBP-4

IGFBP-4 is a 24kDa protein produced in the liver and is the smallest IGFBP [71]. Unlike the other IGFBP, IGFBP-4 inhibits the action of IGF-I and IGF-II in almost all cellular environments [25]. Several studies have investigated how the inhibitory effect of IGFBP-4 on IGF-I can be manipulated to restrict tumour size through a reduction in angiogenesis. Colon cancer cells implanted into mice to create subcutaneous tumours had significantly fewer micro-vessels per area when treated with IGFBP-4 compared to controls. This increased IGFBP-4 exposure led to a concomitant rise in IGF-1R levels, potentially indicating the anti-angiogenic effects of IGFBP-4 were secondary to inhibition of IGF-I and IGF-1R interaction [72].

Contois et al. also showed that IGFBP-4 inhibited IGF-I induced angiogenesis in vivo, but interestingly did not have an effect on VEGF-induced angiogenesis [73]. The continued stimulatory effects of VEGF were dependent on sustained p38/MAPK activity, indicating IGFBP-4 may have pathway specific anti-angiogenic properties. An in vivo model of murine

breast cancer also demonstrated that a protease-resistant IGFBP-4 mutant restricted the actions of IGF-I by preventing binding to its receptor, subsequently decreasing tumour growth through reduced angiogenesis [74].

A recent study by Smith et al. highlighted a potential mechanism through which the interaction between IGFBP-4, IGF-I and the IGF-1R could be exploited therapeutically. IGF-I is cleaved from IGFBP-4 by the protease pregnancy-associated plasma protein-A (PAPP-A), allowing the free IGF-I molecule to bind to IGF-1R. A PAPP-A resistant IGFBP-4 mutant was shown to prevent binding of IGF-I to IGF-1R through competitive antagonism. This in turn reduced IGF-I mediated activation of the PI3K/AKT pathway; inhibiting angiogenesis, migration and invasion both in vitro and in vivo [75].

An IGF-independent role for IGFBP-4 in the inhibition of angiogenesis has also been seen. Human glioblastoma cells exposed to Dibutyryl cAMP (dB-cAMP) have been found to be less aggressive, invasive and have a reduced ability to stimulate angiogenesis in human brain endothelial cells. A study by Moreno et al. found that this response appeared to be mediated by high levels of IGFBP-4 secreted by the glioblastoma cells after they had by exposed to dB-cAMP. An IGFBP-4 neutralising antibody reversed the anti-angiogenic effect seen. It was speculated that this inhibitory effect of IGFBP-4 on angiogenesis was independent of IGF-I, as the levels of IGF-I secreted by the cells were undetectable [76]. This IGF-independent effect appeared to be mediated by the C-terminal protein fragment of IGFBP-4 (CIBP-4). This fragment contains a thyroglobulin type 1 domain and a recombinant fragment was found to inhibit tubulogenesis, cathepsin activity and glioblastoma tumour growth to the same extent as the complete IGFBP-4 protein [77].

In summary, although the molecular mechanisms underlying the anti-angiogenic effect of IGFBP-4 are yet to be fully determined, it is clear that IGFBP-4 is an anti-angiogenic molecule with significant potential as an inhibitor in novel cancer therapies.

IGFBP-5

IGFBP-5 is a 28.6kDa protein and binds the IGFs in a ternary complex with ALS, in a similar manner to IGFBP-3 [46]. Association with extracellular matrix proteins results in a reduced affinity to the IGFs, releasing them from the ternary complex that IGFBP-5 forms with IGF and the acid labile subunit [78]. Through this mediation IGFBP-5 modulates IGF action, but IGF-independent actions have also been seen.

In vitro, VEGF-induced proliferation, tube formation and migration in HUVEC were directly inhibited by over-expression of IGFBP-5. This inhibitory effect was reversed using siRNA to silence IGFBP-5 over-expression. IGFBP-5 has also been shown to significantly inhibit blood vessel formation in vivo in the CAM assay and in an in vivo tumour growth model in which SKOV-3 ovarian cancer cells were xenografted into mice. Subcutaneous injection of IGFBP-5 markedly inhibited tumour growth and decreased the number of blood vessels in IGFBP-5 treated mice compared to controls. Reduced expression of phosphorylated AKT and phosphorylated eNOS was observed, indicating this anti-angiogenic activity may be mediated through inhibition of the PI3K/AKT pathway [79]. These findings have been echoed recently, with a truncated IGFBP-5 peptide derived from the c-terminus inhibiting angiogenesis and ovarian tumour growth in vivo and ex vivo. Both the AKT/ERK and NF-kB-VEGF/MMP pathways were down-regulated by this c-terminal region in an IGF-independent manner, as no inhibition was seen with a peptide containing the IGF-binding site only [80].

In contrast to the inhibitory actions described above, IGFBP-5 has been found to stimulate proliferation in intestinal smooth muscle cells [81] and prostate cancer cells [82]. In summary, although these findings support a primarily anti-angiogenic role, any future therapeutic option will need to be assessed with caution.

IGFBP-6

IGFBP-6 is a 22.8kDa protein produced in the liver, and uniquely has a 50-fold increased preference to IGF-II compared to IGF-I [83]. The primary role of IGFBP-6 is to inhibit the actions of IGF-II, such as cell proliferation, migration and differentiation, but IGF independent actions have also been reported [84].

HUVECs treated with IGFBP-6 showed impaired tube formation in vitro and addition of IGFBP-6 negated VEGF-induced HUVEC tube formation. Angiogenesis inhibition was also examined in vivo by injection of human IGFBP-6 mRNA into flk1:GFP zebrafish, resulting in impaired embryonic angiogenesis. Furthermore, an IGFBP-6 mutant with a 10,000 times lower affinity for IGF-II exerted the same anti-angiogenic effect, suggesting an IGF-independent mechanism [85]. Exposing HUVECs to hypoxic conditions over a prolonged time period induced IGFBP-6 mRNA expression, likely through HIF-1α mediated activation. The authors therefore hypothesised that IGFBP-6 could be a negative regulator of hypoxia induced angiogenesis [85].

A recent study by Qiu et al. also showed an inhibitory role for IGFBP-6 with dose-dependent IGFBP-6 inhibition of proliferation, invasion and migration of colorectal cancer cells seen in vitro [86]. However, Fu et al. demonstrated IGFBP-6 promoted migration in rhabdomyosarcoma cell lines. This was demonstrated to be IGF independent, as a non-IGF-binding IGFBP-6 mutant also stimulated cell migration, with p38/MAPK activation implicated as the causative pathway [87]. Furthermore, opposing actions of IGFBP-6 have been seen in two different ovarian cancer cell lines, with promotion of SKOV3 cell migration and inhibition of HEY cell migration, despite upregulation of MAPK pathways in both [88].

In summary, evidence for the involvement of IGFBP-6 in angiogenesis is limited, and both inhibitory and stimulatory roles have been observed in different cellular environments. Further research is required before we have a definitive understanding of IGFBP-6 and its place in angiogenesis regulation.

IGFBP-7

IGFBP-7, also known as insulin like growth factor binding protein related protein [89], has an amino acid sequence with high similarity to the other human IGFBPs [90]. IGFBP-7 (formally known as mac25) meets structural criteria as a member of the IGFBP family and affinity cross-linking data has shown that IGFBP-7 specifically binds IGF-I and IGF-II, indicating that it is a bona fide IGFBP [90]. IGFBP-7 also binds to unoccupied IGF-1R and suppresses downstream signalling, thereby inhibiting protein synthesis, cell growth, and survival [91].

In endothelial cells, IGFBP-7 is stored in Weibel-Palade Bodies, suggesting that on its release it is involved in vascular homeostasis [92]. Evidence to support a role for IGFBP-7 in angiogenesis is conflicting, although the majority of data suggest IGFBP-7 is anti-angiogenic. A study in glioblastoma indicated that IGFBP-7 was strongly expressed in tumour endothelial cells, was pro-angiogenic and enhanced tube formation in brain endothelial cells [93]. Subsequent reports, however, have shown that IGFBP-7 treatment reduced angiogenesis in hepatocellular carcinoma, when nude mice received xenografts from human hepatocellular carcinoma cells [94] [95]. In human endothelial cells and vascular endothelial cells from rat corpus luteum, IGFBP-7 reduced VEGF tube formation, proliferation and the phosphorylation of MEK/ERK 1/2 [96] [97]. Not only did IGFBP-7 down regulate VEGF signalling, it also down regulated VEGF expression [96] [97]. Recent data from a murine angiogenesis model show that IGFBP-7 inhibited retinal angiogenesis by blocking ERK signalling pathway and down-regulating VEGF expression [98]. This is supported in vitro using a retinal endothelial cell line which showed that IGFBP-7 can inhibit the stimulatory effect of VEGF on retinal angiogenesis in vitro by inhibiting expression of B-Raf to induce apoptosis [99]. In epithelial cells, knock down of IGFBP-7 upregulates the MAPK signalling pathway further strengthening studies from endothelial cells that suggest IGFBP-7 down regulates MAPK signalling [100].

In summary, although IGFBP-7 has been ascribed pro-angiogenic effects in glioblastoma, other reports collectively suggest a robust anti-angiogenic effect of IGFBP-7.

Conclusion

The actions of the different members of the IGFBP family are widely varied and their role in angiogenesis is summarised in table 1. Furthermore, the IGFBP family have been shown to have both IGF dependent and independent actions, with their role in angiogenesis especially appearing to have both IGF-independent and IGF-dependent mechanisms.

Recombinant proteins are one of the fasting growing classes of therapeutic compounds and several of the IGFBPs have pro- or anti-angiogenic actions that could potentially be explored clinically. Amongst IGFBP family members, IGFBP-1 and IGFBP-2 have consistent pro-angiogenic effects that could potentially be exploited in the future for therapeutic angiogenesis during times of ischemia. However, more information is required regarding the specific pathways through which these effects occur, and there is need for a greater volume of in vivo data demonstrating a beneficial effect. IGFBP-3 and IGFBP-7 appear to have both pro and anti-angiogenic properties, whilst IGFBP-4 and IGFBP-5 have predominantly anti-angiogenic properties, and could provide potential new targets and avenues for research in developing novel cancer therapeutics.

Over the last twenty years we have developed a much greater understanding of the individual structure and actions of the IGFBPs and the influence they can exert within distinct cellular environments. However, a great deal more investigation is required to fully elucidate the mechanisms through which they exert their effects, and to understand their tissue distribution and the proteases which regulate their local activity before their promise can be translated into therapeutic agents.

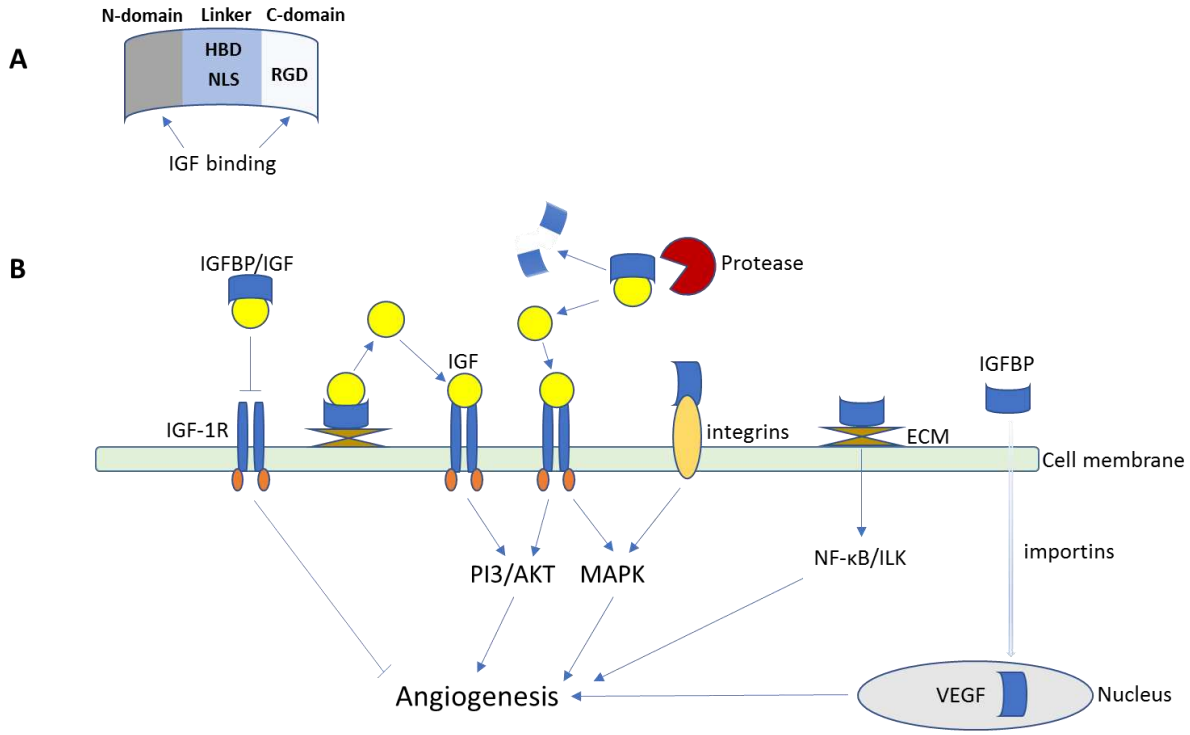

Figure 1. (A) Demonstration of potential domains and functional motif locations within the Insulin-like growth factor binding proteins (IGFBP). IGF binding domains are demonstrated to be present in the N and C domains, and are highly conserved across the IGFBPs. The linker domain is variable between the IGFBPs, and can contain a range of functional motifs. The heparin binding domain (HBD) and nuclear localisation sequence (NLS) demonstrated here are most well described in IGFBP-2, but both are also present in IGFBP~~s~~-3 and 5, with IGFBP-6 possessing an NLS but not an HBD. The RGD motif demonstrated within the C-domain is present in both IGFBP-1 and IGFBP-2. (B) Schematic of the different potential modes of action of the IGFBPs and how they can influence angiogenesis. Both stimulatory and inhibitory interactions with the IGF-1R are evident, as well as IGF-independent interaction with various cell surface receptors, upregulation of pro-angiogenic signalling pathways and direct upregulation of VEGF mRNA transcription via nuclear localisation.

Binding Protein	Pro- angiogenic	Anti-angiogenic
IGFBP-1	✓	✓
IGFBP-2	✓	×
IGFBP-3	✓	✓
IGFBP-4	×	✓
IGFBP-5	×	✓
IGFBP-6	×	✓
IGFBP-7	✓	✓

Table 1: Summary of Insulin-like growth factor binding proteins' (IGFBPs) role in angiogenesis

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Bios

Thomas Slater

Thomas Slater graduated in Medicine from Newcastle University in 2011. During his medical studies he completed an intercalated MRes in Medical and Molecular Biosciences, graduating with Distinction. He currently is undertaking a PhD with Dr Stephen Wheatcroft as part of a BHF Clinical Research Training Fellowship at the University of Leeds, examining the role of IGFBP-2 in vascular function, repair and regeneration.

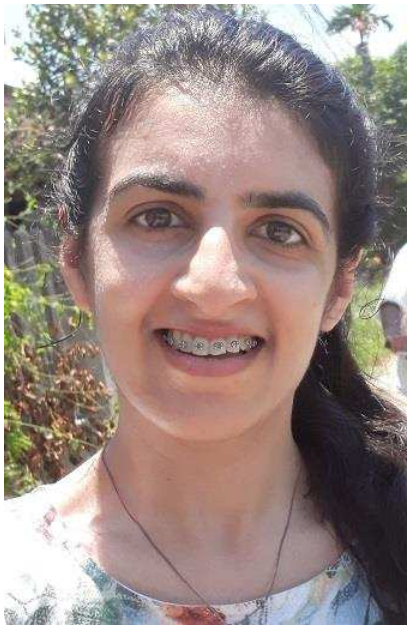
Natalie Haywood

Natalie Haywood graduated from the University of Nottingham with a first class BSc (Hons) in Biotechnology. Following this, she undertook an MSc in Bioscience Specialising in Human disease and graduated from the University of Leeds with Distinction. She undertook her PhD research in the laboratory of Dr Stephen Wheatcroft and was awarded her PhD in 2015 also from the University of Leeds, UK.

Connor Matthews

Connor is a final year medical student at the University of Leeds. He completed a BSc in Cardiovascular medicine at the University of Leeds in 2017, investigating developmental angiogenesis in an in vivo model of murine neovascularisation at the Leeds institute of cardiovascular and metabolic medicine (LICAMM).

Harneet Cheema



Harneet Cheema obtained a Bachelor's degree in Medicine and Surgery from the University of Birmingham, UK, in 2017. She obtained an intercalated BSc (Hons) in Cardiovascular Medicine from the University of Leeds, UK, in 2016, during which she conducted research into the angiogenic properties of insulin-like growth factor binding proteins 1, 2 and 3. She currently works as a doctor for the National Health Service in the UK.

Stephen Wheatcroft

Stephen Wheatcroft graduated in Medicine from the University of Birmingham and completed a PhD in Cardiovascular Medicine at King's College London. He developed a research interest in IGF binding proteins in vascular biology through a British Heart Foundation Intermediate Clinical Research Fellowship and a European Research Council Starting Grant. He leads a cardiometabolic research group focussing on the IGF binding proteins.