BEYOND CARRIER PROTEINS

Specifying the cellular responses to IGF signals: roles of IGF-binding proteins

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Abstract

Regulation of peptide growth factor/hormone activities by secreted hormone-binding proteins has emerged as a common theme in cell-cell signaling. Among the best-studied examples are members of the IGF-binding protein (IGFBP) gene family. These secreted proteins bind the IGF ligands with equal or even greater affinities than do the IGF receptors, and therefore are placed in a critical regulatory position between IGFs and their cell surface receptors. The circulating IGF/IGFBP complexes prolong the half-lives of IGFs and buffer the potential hypoglycemic effects of IGFs. Locally expressed IGFBPs provide a means of localizing IGFs in specific cells and can alter the IGF biological activity. While some members of

the IGFBP gene family have been consistently shown to inhibit IGF actions by preventing them from gaining access to the IGF receptors, others potentiate IGF actions by facilitating the ligand-receptor interaction. Furthermore, recent studies indicate that some IGFBPs can regulate several cellular processes through ligand-independent mechanisms. This review will focus on the roles of IGFBPs in vascular smooth muscle cells. A conceptual model of the molecular mechanisms by which IGFBPs act to determine the specific physiological outcomes of IGF stimulation is proposed and discussed.

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Introduction

Insulin-like growth factors (IGFs), including IGF-I and IGF-II, are a family of polypeptide growth factors structurally related to proinsulin. IGF-I mediates many of the growth-promoting effects of growth hormone during post-natal life. Studies utilizing 'knock-out' mice have demonstrated that both IGF-I and IGF-II are essential for fetal growth (Baker *et al.* 1993, Liu *et al.* 1993). Recent genetic studies in *Xenopus* indicate that the IGF signaling pathway is also required for head formation during vertebrate embryogenesis (Pera *et al.* 2001, Richard-Parpaillon *et al.* 2002). The biological actions of IGFs are mediated by the IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase. In mammals, a second transmembrane IGF receptor, the IGF-II/cation-independent mannose-6-phosphate receptor exists. It has higher binding affinity for IGF-II over IGF-I. This receptor has no known signal transduction function, and binding of IGF-II to this receptor has been shown to cause internalization and degradation of IGF-II (LeRoith *et al.* 2001). Comparative studies indicate that the mannose-6-phosphate receptors of non-mammalian species do not possess the capacity to

bind IGF with high affinity. Therefore, the IGF-II-binding property of this receptor as well as any of its physiological functions with regards to IGFs might have been a later acquisition during evolution (Duan 1998). The IGFs in serum and other extracellular environments are bound to specific IGF-binding proteins (IGFBPs). IGFBPs are a family of secreted proteins that specifically bind IGF-I and IGF-II with affinities that are equal to or greater than those of the IGF receptors. Six distinct IGFBPs, designated IGFBP-1 to -6, have been isolated and characterized from a variety of vertebrate species ranging from mammals to fish (Duan *et al.* 1999a, Hwa *et al.* 1999, Baxter 2000, Clemmons 2001, Maures & Duan 2002). These IGFBPs share a common domain organization. They all have a highly cysteine-rich N-terminal domain (12 cysteine residues), a cysteine-rich C-terminal domain (six cysteine residues), and a central (L) domain with no cysteine residue except in IGFBP-4. The N- and C-domains are highly conserved among various members of this gene family in a given species. These two domains also exhibit strong homology across vertebrate species. The L-domain is highly variable. These proteins bind IGF with high affinity and act as carrier

proteins in the bloodstream and control the efflux of IGFs from the vascular space. The IGF/IGFBP complexes prolong the half-lives of IGFs and buffer the potential hypoglycemic effects of high concentrations of IGFs in the circulation. Since each of the soluble forms of IGFBPs possesses a higher affinity for IGFs than the IGF receptors, locally expressed IGFBPs may provide a means of localizing IGFs in specific cells and can alter IGF biological activity by modulating their interaction with the IGF receptors. In addition, some IGFBPs have been shown to have biological actions that are IGF-independent.

During the past two decades, there has been a large and growing body of information supporting the concept that these evolutionarily conserved proteins play vital roles in IGF actions in a wide variety of cell types. The primary goal of this article is to review recent advances in our understanding of the roles of IGFBPs in one specialized cell type, the vascular smooth muscle cells (SMCs). Readers are referred to several recent reviews for general information on IGF and IGFBP biology (Hwa *et al.* 1999, Baxter 2000, Clemmons 2001, LeRoith *et al.* 2001, Schnieder *et al.* 2002). The following aspects of the IGF signaling pathway will be discussed: the diverse actions of IGFs in SMCs, the multiple and redundant intracellular IGF signaling pathways, the major forms of IGFBPs produced in SMCs and how they are regulated. The distinct biological actions of these IGFBPs are described. Finally, we will discuss the molecular basis of IGFBP actions and propose a model to explain the molecular mechanisms by which IGFBPs act to determine the specific physiological responses to the IGF stimulation.

The diverse cellular actions of IGFs in SMCs

Vascular SMCs are located in the arterial tunica media and are important for regulating vascular tone and blood pressure. These cells are normally maintained in a non-proliferative and contractile state in adult animals. Injury of the arterial wall results in accelerated proliferation and directed migration of SMCs into the intimal layer of the artery wall, leading to intimal hyperplasia and thickening (Ross 1993). This process contributes to the pathogenesis of several cardiovascular disorders, including atherosclerosis, in which local IGFs play a critical role (Clemmons 1997, Sowers 1997, Bayes-Genis *et al.* 2000, Zaina *et al.* 2002). IGFs are mitogens for human, porcine, bovine and rat SMCs *in vitro* and *in vivo* (Clemmons 1985, Bornfeldt *et al.* 1994, Thommes *et al.* 1996, Motomura *et al.* 1997, Wang *et al.* 1997, Duan & Clemmons 1998, Duan *et al.* 2000). IGFs are also very potent chemoattractants for SMCs (Bornfeldt *et al.* 1994, Duan *et al.* 2000). In addition to the mitogenic and chemotactic activity, IGFs have been shown to regulate SMC apoptosis, differentiation, glucose uptake, protein synthesis, and contractility (Sowers 1996, Bai *et al.* 1999, Patel *et al.* 2001). These

actions are mediated through the IGF-IR (Delafontaine *et al.* 1995, Du & Delafontaine 1995, Bayes-Genis *et al.* 2000), but how activation of the same receptor by the same ligand(s) leads to these diverse, sometimes even mutually exclusive, cellular responses is not clear. This phenomenon is not unique to IGFs or limited to SMCs. Many hormones exert pleiotropic effects in a wide variety of cell types. Understanding how specificity arises in connecting a given hormonal input with the appropriate cellular responses is a central issue in endocrinology.

The multiple and redundant intracellular IGF signaling pathways in SMCs

To understand how the IGF signaling pathway leads to the diverse actions in SMCs, we will first discuss the intracellular IGF signaling network in these cells. Because the signaling pathways initiated by the IGF-IR are very complex, studies on the IGF signal transduction mechanisms have been performed predominantly in immortalized cell lines such as 3T3 cells. Studies using these 'model' systems indicate that one of the earliest steps in signal transduction initiated by the IGF-IR is the phosphorylation of adapter/docking proteins such as insulin receptor substrate (IRS)-1 or -2, Shc, Grb2 and Grb10 (LeRoith *et al.* 2001). These molecules then interact with downstream signal transducers and effectors, resulting in activation of the MAPK (also known as ERK, extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase) signaling pathways. Activation of the MAPK pathway is considered to be critical for cell proliferation, while the PI3K pathway is important for mediating the metabolic and anti-apoptotic signals of IGF-I. The intracellular biochemical signaling pathway(s) leading to the chemotactic action of IGFs are largely unknown. Although these 'model' systems are ideal for demonstrating protein-protein interactions, they are less suited for elucidating the physiological outcome of the activation of these signaling pathways. Furthermore, intracellular signaling pathways induced by the IGF-IR are cell-type specific. Diploid, normal SMCs in culture, which are untransformed, may respond differently from 3T3 cells or other immortalized cell lines often used for signal transduction studies.

What are the major biochemical signaling pathways activated by the IGF-IR in SMCs? Are the signal transduction pathways activated by the IGF-IR that lead to directed migration different from those that lead to proliferation? Early studies using human SMCs suggested that IGF-I stimulation did not activate MAPK in this cell type (Takagi *et al.* 1995, Graves *et al.* 1996, Koyama *et al.* 1996). However, others reported an increase in MAPK activity after IGF-I treatment in rat SMCs (Thommes *et al.* 1996). These conflicting data cannot simply be attributed to species differences because IGF-I is a strong

activator of MAPK in many human and bovine cell types (Pavlovic-Surjancev *et al.* 1992, Parrizas *et al.* 1997). Using the sensitive, phospho-specific MAPK antibodies made available recently, we have found that IGFs induce a time- and dose-dependent activation of ERKs in cultured porcine SMCs (Duan *et al.* 1999b). Furthermore, inhibition of the MAPK activity by either specific pharmacological inhibitors or antisense oligonucleotides attenuated IGF-I-stimulated DNA synthesis and gene expression, suggesting that the MAPK activation is involved in mediating these IGF actions (Imai & Clemmons 1999, Duan *et al.* 1999b, 2000). It is noted that IGF-I is a relatively weak activator of the MAPK in these cells compared with other growth factors such as platelet derived growth factor (PDGF)-BB. In porcine SMCs, the effect of IGF-I on MAPK activation is transient (~ 5 –10 min) and this activation is only observed when high doses of IGF-I are added (Duan *et al.* 1999b). These variations may explain the previous negative findings. In contrast to its weak and transient effect on MAPK activity, IGF-I is a strong activator of the PI3K pathway. IGF-I stimulation led to a 60-fold increase in PI3K activity. This activation occurs within minutes and lasts for at least 6 h (Duan *et al.* 1999b). In addition to the induction of PI3K, IGF-I stimulation also led to a concentration-dependent and sustained activation of PKB/Akt and p70^{S6k}, two Ser/Thr kinases acting downstream of the PI3K. IGF-I primarily utilizes IRS-1 to transmit its signal to the PI3K in SMCs (Duan *et al.* 2000). The minimum effective concentration of IGF-I needed to activate PI3K, PKB/Akt, and p70^{S6k} is considerably lower than that of IGF-I required to activate MAPK. While IGF-I activates both of these pathways via its binding to the IGF-IR, the activated IGF-IR relays the IGF signal to the two pathways via different docking molecules: it is linked to the PI3K by IRS-1 but to the *ras-raf-Mek* and MAPK pathway via Grb2, Shc and Sos. This may explain the discrepancy in the minimum concentrations of IGF-I required. Using inhibitors specifically interfering with PI3K and MAPK activation, the role(s) of MAPK and PI3K in mediating the growth and chemotactic signals of IGF-I have been studied in SMCs. Specific inhibition of either MAPK or PI3K activation attenuates IGF-I-stimulated DNA synthesis. Likewise, inhibition of MAPK or PI3K significantly reduces the number of cells that migrated towards IGF-I (Duan *et al.* 2000). These findings suggest that IGF stimulation activates both of the MAPK and PI3K pathways in SMCs. At low concentrations, IGF-I only activates the PI3K pathway and this activation is a long-lasting effect. As the concentrations of IGF-I increases, the MAPK pathways are activated transiently. Both signaling pathways are important for mediating the growth and chemotactic signals of IGFs in SMCs.

In addition to the MAPK and PI3K pathways, IGF-I has been shown to stimulate diacylglycerol formation, phosphatidylinositol turnover, intracellular Ca^{2+} flux,

nitric oxide (NO) production, and activity of the Na^+/K^+ -ATPase pump in SMCs (Bornfeldt 1996, Sowers 1996). It has been shown that IGF-I stimulates NO production in an age-related manner, which in turn may mediate IGF-I-induced attenuation of vascular contractility (Walsh *et al.* 1996, Muniyappa *et al.* 1997, Ren *et al.* 1999). Early studies indicated that down-regulation or activation of protein kinase C (PKC) by phorbol myristate acetate (PMA) affects the IGF-I-induced DNA synthesis in SMCs (Dempsey *et al.* 1990, Pukac *et al.* 1998), suggesting that PKC activation is required for IGF actions. PKC is a family of cytoplasmic phosphorylating enzymes consisting of at least 11 members, which can be categorized into conventional PKC (α , β I, β II and γ), novel PKC (δ , ϵ , η , θ) and atypical PKC (ζ , λ , ι) (Newton 1995). These PKC isoforms are subject to differential tissue distribution and may have distinct functions. Out of the 11 members of the PKC family, PKC- α , - β I, - ϵ , - η , - λ , - θ and - ζ are easily detectable in cultured porcine SMCs. Among them, the subcellular distribution of PKC- η , - ϵ , - ζ and - β I but not that of PKC- α and - λ , are regulated by IGF-I (Yano *et al.* 1999). To examine whether PKC activation is required for the IGF-I-regulated biological responses, high doses of PMA and GF109203X (a bisindolylmaleimide that inhibits the activity of all three types of PKCs by competing for the ATP-binding site) were used to down-regulate or inhibit PKC activity. Both PMA and GF109203X suppressed the total PKC activity after a 30 min incubation by more than 90%, and this inhibition lasted for at least 24 h. Down-regulation or inhibition of PKC activity abolished the IGF-I-induced DNA synthesis, migration and IGFBP-5 gene expression. Since the actions of IGF-I on DNA synthesis and migration in SMCs have been shown to be mediated through the PI3K and MAPK signaling pathways, their relationship with PKC has been examined. Inhibition of PKC activity with either PMA or GF109203X did not alter the effects of IGF-I on PI3K activation or PKB/Akt phosphorylation. Likewise, inhibition of PKC by GF109203X did not change the IGF-I-stimulated MAPK activation (Yano *et al.* 1999). These results indicate that the four IGF-I-regulated PKC isoforms, namely PKC- η , - ϵ , - ζ and - β I may be essential components of the IGF signaling network and that these PKC isoforms act either independently of the PI3 kinase and MAPK pathways or further downstream of PKB/Akt or ERK in these pathways in SMCs. The specific role that each of these PKC isoforms plays, however, remains to be determined.

These studies revealed that specific inhibition of MAPK, PI3K or PKC activation all results in a reduction in IGF-induced cell proliferation, as well as directed migration. In other words, the biochemical pathways activated by the IGF-IR that lead to directed migration cannot be separated from those leading to proliferation. These pathways are interconnected and they act redundantly or in concert to transduce the IGF signal. These

findings fit an emerging notion that a number of signaling proteins interact in a combinatorial fashion to form signaling networks of interaction that allow diverse cellular responses. So what then determines whether cells will proliferate or migrate in response to IGF stimulation? This question is addressed in the following section

Local IGFbps and their biological actions in SMCs

The ability of IGF-I to regulate various cellular processes in a defined tissue is not simply dependent on the levels of IGF-I, the IGF-IR, and post-receptor signaling mechanisms, but may also be influenced by the presence of various high-affinity IGFbps in the local microenvironment.

Major forms of IGFbps produced by SMCs and their regulation

Studies using human, porcine, bovine and rat SMCs have shown these cells secrete several IGFbps, including IGFBP-2, IGFBP-4 and IGFBP-5 (Giannella-Neto *et al.* 1992, Cohick *et al.* 1993, 1995, Ververis *et al.* 1993, Kamyar *et al.* 1994, Gockerman *et al.* 1995, Parker *et al.* 1995, Boes *et al.* 1996, Duan *et al.* 1996, Duan 1997, Duan & Clemmons 1998). In porcine SMCs, IGFBP-2 is the most abundant IGFBP. The availability of these IGFbps in SMCs is regulated by an array of hormones and growth factors. The mechanisms by which this regulation occurs are complex, involving regulation at levels ranging from transcriptional activation of IGFBP genes to proteolytic degradation. For instance, PDGF treatment results in moderate increases in IGFBP-2 mRNA expression and biosynthesis in porcine and rat SMCs, while other growth factors such as transforming growth factor- β , fibroblast growth factor and epidermal growth factor have little effect (Cohick *et al.* 1993, 1995). Intriguingly, IGF-I is one of the most potent regulators of its own binding proteins in cultured porcine and rat SMCs. In these cells, IGF-I down-regulates IGFBP-4 levels by activation of its proteolysis (Cohick *et al.* 1993, Kamyar *et al.* 1994, Duan & Clemmons 1998), but increases IGFBP-5 levels by stimulating the IGFBP-5 gene transcription (Duan *et al.* 1996, Duan & Clemmons 1998). This regulation of IGFBP-5 expression by its own ligand is mediated through the IRS-1, PI3K, PKB/Akt and p70^{s6k} signaling pathway (Duan *et al.* 1999b).

In the course of studying the regulated expression of IGFbps in SMCs, we have noticed that different IGFbps are expressed differentially depending on the cellular context. For instance, higher levels of IGFBP-5 mRNA and protein are found in the synthetic and proliferative SMCs as compared with the differentiated and contractile cells (Duan & Clemmons 1998, Zheng *et al.* 1998). In culture, the concentration of IGFBP-5 in the conditioned medium decreased 18-fold when cell density increases from 30 to 100% confluence (Fig. 1A, right panel). In

contrast, the concentrations of IGFBP-4 increased 15-fold (Fig. 1A left panel). No significant change in IGFBP-2 is observed in regards to cell density changes (Fig. 1A, central panel). Therefore, different IGFbps are differentially regulated by cell confluency. The differentiation state of SMCs also influences the local availability of IGFbps. In normal blood vessels, SMCs are attached to a basement membrane that contains type IV collagen and laminin, and are maintained in a quiescent and contractile state. However, in response to vascular injury, SMCs alter their phenotype and are stimulated to proliferate. This phenotypic transition from the contractile to the synthetic state is accompanied by enhanced expression of specific matrix proteins such as fibronectin (Bornfeldt *et al.* 1990). The phenotypic transition can be recapitulated *in vitro* by growing SMCs on different extracellular matrix substrata (Hedin *et al.* 1988). Contractile SMCs can be converted to the synthetic phenotype when cultured on a fibronectin substratum and if maintained on fibronectin, they will proliferate in the presence of appropriate growth factors. In contrast, if the cells are cultured on laminin plus type IV collagen, early passage cells can be maintained in a non-proliferative and contractile state. Compared with cells cultured on laminin and type IV collagen, SMCs cultured on fibronectin synthesize and release more IGFBP-5 (Fig. 1B). The increase in IGFBP-5 synthesis is associated with increased IGFBP-5 mRNA expression. The effect is specific for IGFBP-5 as IGFBP-2 synthesis is unaltered (Fig. 1C). Therefore, the specific forms of IGFbps and their relative abundance in the local microenvironments vary significantly under different cellular context.

The paracrine/autocrine IGFbps are important determinants of IGF actions in SMCs

Since it is known that different IGFbps have different biological effects, and because the availability of IGFBP-2, IGFBP-4 and IGFBP-5 are differentially regulated by the cellular context, it is postulated the locally produced IGFbps may provide a means of controlling the amount of IGF-I accessible to its cell surface receptors and may therefore be important for determining whether SMCs will proliferate and/or migrate in response to the same IGF stimulation. We tested this hypothesis utilizing an IGF-I analog, Des(1-3)IGF-I. Because Des(1-3)IGF-I binds to IGF-IR with normal affinity but has significantly reduced affinity for IGFbps (Francis *et al.* 1992), the contribution of the paracrine/autocrine IGFbps can be determined by examining the difference between native IGF-I and Des(1-3)IGF-I. As shown in Fig. 2A, IGF-I stimulated DNA synthesis in a dose-dependent manner with the maximal concentration being 20 ng/ml. At concentrations of 5, 10 and 20 ng/ml, Des(1-3)IGF-I was more potent than native IGF-I. These data indicate that the autocrine/paracrine IGFbps in SMCs are inhibitory for IGF-I-stimulated DNA synthesis. When the IGFbps

Figure 1 Differential expression and regulation of IGFBP-2, IGFBP-4 and IGFBP-5 in SMCs. (A) Effects of cell confluence on IGFBP expression in SMCs. Conditioned media from sparse, subconfluent and highly confluent SMC cultures were subjected to Western ligand blot analysis using ^{125}I -IGF-I as probe (left panel). The IGFBP-2 (central panel) and IGFBP-5 (right panel) were separated by immunoprecipitation and then subjected to Western ligand blot. (B) Regulation of IGFBP-5 and (C) IGFBP-2 biosynthesis by cellular context. SMCs were cultured on plastic dishes (lanes 1 and 4) on fibronectin (lanes 2 and 5) or laminin plus type IV collagen (lanes 3 and 6) for 48 h (lanes 1–3) and 96 h (lanes 4–6). During the last 8 h of incubation, the cells were exposed to methionine-free medium containing 50 μCi [^{35}S]methionine and 50 $\mu\text{g}/\text{ml}$ heparin (to inhibit IGFBP-5 proteolysis). Culture media were collected and immunoprecipitated using antiserum against IGFBP-5 (B) or IGFBP-2 (C).

in the system were saturated by addition of more IGF-I (50 and 100 ng/ml), this inhibitory effect disappeared. We next determined the chemotactic effects of IGF-I and Des(1–3)IGF-I and found that IGF-I was more potent than Des(1–3)IGF-I in stimulating SMC migration. At concentrations of 10, 20 and 50 ng/ml, IGF-I caused a 4-, 7.3- and 11-fold increase over the control respectively. In comparison, Des(1–3)IGF-I only resulted in a 1.6-, 3- and 3.7-fold increase respectively (Fig. 2B). The difference was even greater at 100 ng/ml. These data indicate that the autocrine/paracrine IGFBPs greatly promote SMC migration to IGF-I while they inhibit IGF-I-stimulated DNA synthesis. These data indicate that the autocrine/paracrine IGFBPs are critical factors in determining the chemotactic or mitogenic responses to IGF stimulation.

Different IGFBPs exhibit different effects on IGF-induced cell migration and proliferation

While the above experiments have demonstrated a critical role of the autocrine/paracrine IGFBPs in specifying IGF

actions in SMCs, they did not provide insight into the specific effects of various IGFBPs in regulating IGF actions. We therefore tested the effects of purified IGFBPs in the presence or absence of IGF-I. When added to SMC cultures, exogenous IGFBP-2 or IGFBP-4 inhibits the mitogenic activity of IGF-I in a dose-dependent manner (Fig. 3A; also see Gockerman *et al.* (1995)). IGFBP-5, on the other hand, has been shown to be capable of both inhibiting and potentiating the IGF-induced DNA synthesis in this cell type (Imai *et al.* 1997, Duan & Clemmons 1998, Zheng *et al.* 1998). When co-incubated with IGF-I for a relatively short time (24 h), IGFBP-5 has an inhibitory effect. When co-incubated for a longer time (48 h), IGFBP-5 modestly potentiates the IGF-I-induced increase of DNA synthesis (Fig. 3A). Both effects require relatively high concentrations and are fairly modest. These seemingly contradictory observations are probably due to the presence of a specific IGFBP-5 protease in these cells (Duan *et al.* 1996, Busby *et al.* 2000). Most, if not all of the added IGFBP-5 is degraded after more than 48 h of incubation, while a substantial proportion of IGFBP-5

Figure 2 The paracrine/autocrine IGFbps are important determinants in specifying the IGF actions in SMCs. (A) Effects of IGF-I and Des(1-3)IGF-I in stimulating DNA synthesis. The values are means $\pm$ s.e. of three separate experiments. (B) Effects of IGF-I and Des(1-3)IGF-I in stimulating SMC migration. The values are means $\pm$ s.e. of three separate experiments. These results indicate that the local IGFbps modestly inhibit IGF-I-stimulated proliferation but strongly promote IGF-I-stimulated SMC migration.

remained intact within the first 24 h of incubation (Duan & Clemmons 1998). Since intact IGFBP-5 binds to IGF-I with a high affinity that is at least 10-fold greater than the

IGF-IR (Gockerman *et al.* 1995), it limits IGF and IGF-IR interaction when present in the culture medium. Under conditions in which long incubation times are used,

Figure 3 Different IGFBPs have distinct biological effects in SMC proliferation and migration. (A) Effects of IGFBP-4 and IGFBP-5 on IGF-I-stimulated DNA synthesis. The results are expressed as the percentage increase over the IGF-I control group (50 ng/ml). Each bar represents the mean $\pm$ s.e. of four separate experiments. (B) Effect of IGFBP-4 and IGFBP-5 on IGF-I-stimulated SMC migration. Each bar represents the mean $\pm$ s.e. of three separate experiments.

the added IGFBP-5 is degraded into a 21 kDa fragment. This fragment has very low affinity for IGF-I (Duan *et al.* 1996). This switch in binding affinity results in the equilibrium favoring binding of IGF-I to IGF-IR. This conclusion is further supported by the observation that a mutant form of IGFBP-5 that is protease-resistant strongly inhibits IGF-I actions in porcine SMCs (Imai *et al.* 1997). Regardless of the underlying mechanism(s), IGFBP-5 does not appear to have any major effect on IGF-I-stimulated cell proliferation, while IGFBP-2 and IGFBP-4 inhibit IGF-stimulated SMC proliferation. This may explain why Des(1–3)IGF-I is more potent than native IGF-I in stimulating SMC proliferation.

When the effects of individual IGFBPs in regulating IGF-I-stimulated directed migration were examined, a different picture emerged. IGFBP-4 alone had no effect on basal SMC migration level, but it significantly inhibited SMC migration towards IGF-I. Likewise, IGFBP-2 acted as an inhibitor of IGF-I actions. It alone had no effect on basal SMC migration level (data not shown). In contrast, IGFBP-5 caused a further increase in the IGF-I-induced migration when co-incubated with IGF-I (Fig. 3B). Intriguingly, IGFBP-5 alone caused a moderate but statistically significant increase in basal migration. The effect of IGFBP-5 on basal cell motility cannot be inhibited by an IGF-I-neutralizing antibody. These results suggest that IGFBP-5 not only potentiates IGF-I-induced cell migration, but itself also regulates the motility of SMCs. The effect of IGFBP-5 was further studied using cells overexpressing IGFBP-5. These cells expressed significantly higher levels of IGFBP-5 compared with the mock-transfected and wild-type cells. There is no significant difference in the growth curves among IGFBP-5-overexpressing, mock-transfected and wild-type control cells; however, cells overexpressing IGFBP-5 appear to grow somewhat more slowly. In contrast, the IGFBP-5-overexpressing cells showed a dramatic increase in cell migration even without IGF-I stimulation. This effect was not seen in mock-transfected cells. Addition of IGF-I to the lower chamber further increases the number of cells that migrate, with the maximal concentration shifted from 50 to 10 ng/ml. These results suggest that overexpression of IGFBP-5 in SMCs may have two effects: while it promotes IGF-I-induced SMC migration towards IGF-I, IGFBP-5 also alters the basal motility of SMCs. The effect of IGFBP-5 on cell motility was further studied using the gold particle cell motility assay. This technique visualizes tracks of cultured cells on glass coated with a layer of gold particles, based on the classic observation that cells, when moving, produce particle-free tracks on a glass substrate that is evenly coated with supra-colloidal gold particles (Albrecht-Buehler 1977). As shown in Fig. 4, the IGFBP-5-overexpressing cells showed significantly higher levels (2.8-fold) of motility over the controls under basal conditions and this increase could not be suppressed by an IGF-I-neutralizing antibody (Sm 1·2).

Taken together, these studies suggest that the three forms of endogenously produced IGFBPs have different, sometimes even opposite biological effects on IGF actions. Both IGFBP-2 and IGFBP-4 act primarily as inhibitors of IGF actions on proliferation and migration. IGFBP-5 has a relatively modest effect on the IGF-regulated cell proliferation, but it strongly influences SMC migration. Furthermore, IGFBP-5 itself regulates the motility of SMCs in an IGF-independent manner.

The molecular basis of IGFBP actions in SMCs

Ligand-dependent actions of IGFBPs

The finding that various IGFBPs have different, and sometimes even opposite biological effects is intriguing but also puzzling. Since the IGF-IR mediates the biological actions of the IGFs, and because soluble IGFBPs bind to IGFs with higher affinity than that of the IGF-IR, the inhibitory effects of IGFBP-2 and IGFBP-4 can be rationally attributed to their competition for the ligand with the cell surface receptors. What makes IGFBP-5 potentiate IGF actions? Recent studies indicate that unlike IGFBP-4, IGFBP-5 is not only present in the extracellular fluid but is also localized on the cell surface by its association with glycosaminoglycans (GAG) and proteoglycans (Jones *et al.* 1993, Arai *et al.* 1994, 1996, Cohen *et al.* 1995, Andress 1996, Parker *et al.* 1996, 1998). This has been attributed to the presence of the heparin-binding motifs in IGFBP-5. In agreement with these previous studies, our affinity cross-linking studies indicate that IGFBP-5 is present on the cell surface of porcine SMCs. It is possible that the cell surface association of IGFBP-5 may provide a mechanism for facilitating IGF-I binding to its cell surface receptors. One possible scenario is that the IGFBP-5 localized on the cell surface serves as a reservoir for IGF-I by binding to and concentrating IGF-I in discrete areas. If this is correct, then increasing the amount of IGFBP-5 on the cell surface by preincubation of SMCs with IGFBP-5 should result in a greater chemotactic response to IGF-I. On the contrary, our results indicate that preincubation of SMCs with IGFBP-5 had minimal effect while adding IGFBP-5 directly to the migration chambers significantly enhanced the chemotactic action of IGF-I, suggesting that IGF-I and IGFBP-5 may form a binary complex first and then the binary complex binds to the cell surface (Hsieh *et al.* 2000). To further test this possibility, we added heparin to the migration chambers with or without IGFBP-5 and then subjected SMCs to migration towards an IGF-I gradient. It has been shown that heparin can inhibit the IGF-I/IGFBP-5 complex formation and separate the pre-formed IGF-I/IGFBP-5 complex (Arai *et al.* 1994). When heparin was present and the IGF-I/IGFBP-5 complex formation was inhibited, the IGFBP-5 had no effect in promoting migration towards IGF-I. Heparin alone at the given concentration had no significant effect in basal or

Figure 4 IGFBP-5 increases SMC motility through a ligand-independent mechanism. A representative gold particle motility assay view showing the effect of IGFBP-5 overexpression on SMC motility. IGFBP-5 transfected (BP5), mock transfected (Mock), and wild-type (WT) porcine SMCs were serum-starved and subsequently subjected to gold particle motility assays. The IGF-neutralizing antibody (Sm 1.2) had no effect on IGFBP-5-induced motility change.

IGF-I-induced SMC migration (Fig. 5). We further investigated the migration-promoting effect of IGFBP-5 using a specific IGFBP-5 antibody. This antibody does not cross-react with IGFBP-2 and IGFBP-4 (Duan *et al.* 1996). Addition of the IGFBP-5 antibody but not pre-immune serum blocked the migration-promoting effect of IGFBP-5. This antibody had little effect on the IGF-I-induced migration. These data suggest that the formation of IGF-I/IGFBP-5 complexes is necessary for the action of IGFBP-5 in promoting SMC migration towards IGF-I. If this is true, then there must be a mechanism of releasing the IGF-I from the IGF-I/IGFBP-5 complex after binding to the cell surface. To this end, several *in vitro* biochemical studies should be noted. It has been shown that binding of IGFBP-5 to heparin reduces its binding affinity to IGFs (Arai *et al.* 1994, 1996). It is therefore possible that binding of the IGF-I/IGFBP-5 complex to cell surface GAG may lead to a reduction in the affinity of

cell surface-associated IGFBP-5 for IGF-I, resulting in the release of IGF-I to its receptors. Based on these previous findings, we propose the following three-step hypothetical model: (i) the IGFBP-5 secreted by SMCs would bind to IGF to form the IGF/IGFBP-5 complex; (ii) the IGF/IGFBP-5 complex would then be 're-localized' to the cell surface by binding to GAG or proteoglycan, thereby 'bringing' IGF-I to the proximity of the IGF-IR; and (iii) binding of the IGF/IGFBP-5 complex to the cell surface would reduce the affinity of IGFBP-5 for IGF, therefore allowing for release of IGF for receptor binding (Fig. 7). The ligand occupancy would activate the intracellular IGF signaling network discussed above and result in elevated cell migration and proliferation.

Ligand-independent actions of IGFBPs

In addition to its ability to bind IGFs with high affinities and modulating the IGF actions, IGFBP-5 possesses

Figure 5 Formation of the IGF-I/IGFBP-5 complex is required for IGFBP-5 to promote SMC migration towards IGF-I. Serum-starved porcine SMCs were subjected to transwell migration assays with or without IGF-I (50 ng/ml) and IGFBP-5 (200 ng/ml) and in the presence or absence of heparin (100 µg/ml). Values are means $\pm$ S.E. of three experiments.

intrinsic biological activity that is ligand-independent. The alternative pathway(s) that IGFBP-5 utilizes to elicit these IGF-independent actions is not yet well understood. It was reported that IGFBP-5 specifically binds to a 420 kDa membrane protein in murine osteoblasts and binding of IGFBP-5 to this protein enhanced its phosphorylation at the serine residue(s), suggesting this protein may serve as the 'receptor' for IGFBP-5 (Andress 1998). However, this finding has not been confirmed in other cell types. Studies by several other groups have shown that fluorescently-tagged IGFBP-5 and IGFBP-3 can be translocated into the nucleus through a yet unidentified pathway in human lung cancer cells, T47D breast cancer cells, and opossum kidney cells (Jaques *et al.* 1997, Li *et al.* 1997, Schedlich *et al.* 1998, 2000, Hsieh *et al.* 2000). These observations are consistent with the presence of a putative nuclear localization signal sequence (NLS) in IGFBP-5 and IGFBP-3 (Radulescu 1995). Mutation of the NLS sequence in IGFBP-5 abolishes its nuclear localization (Li & Duan 2001). The functional significance of the nuclear presence of IGFBP-5 or IGFBP-3 is unknown. A recent report shows that IGFBP-5 interacts with a nuclear protein, FHL2 (a LIM domain-containing protein with unknown function) (Amaar *et al.* 2002) in human osteoblasts, where IGFBP-5 itself is a growth factor with cellular effects not dependent on IGFs (Miyakoshi *et al.* 2001). We have shown with several independent experimental approaches that endogenously produced IGFBP-5 is localized in the nuclei of cultured SMCs. These experimental approaches include: (i) co-localizing IGFBP-5 with DNA by immunocytochemistry; (ii) cell fractionation followed with immunoprecipitation and immunoblot

analysis; (iii) cell fractionation followed with ligand blot analysis; and (iv) transfecting cells with IGFBP-5/enhanced green fluorescence protein (EGFP) fusion constructs. Furthermore, IGFBP-5 labeled with either ^{125}I or Cy3 can be internalized by SMCs with relatively rapid kinetics. Kinetic studies showed that IGFBP-5 internalization started to be detected 1 h after the addition of the protein to the cell culture system and increased during the first 4 h (Li & Duan 2001). This internalization and nuclear translocation is unique to IGFBP-5 since IGFBP-4 is not found in SMC nuclei. To determine the structural basis underlying nuclear localization of IGFBP-5, six IGFBP chimeras (N5L5C4, N5L4C4, N4L5C4, N4L4C5, N5L4C5 and N4L5C5) were generated by exchanging the N-, L- and C-domains between IGFBP-4 and IGFBP-5. After confirming their ability to bind to IGFs, these chimeras were further tagged with EGFP and transfected into SMCs, and their nuclear localization assessed in living cells. When introduced to cells, wild-type IGFBP-5/EGFP is seen exclusively in the nuclei, whereas IGFBP-4/EGFP is observed only in the cytoplasm (Xu & Duan 2001). As shown in Figure 6, all the chimeras lacking the IGFBP-5 C-domain exhibited little or no nuclear presence. Replacement of the IGFBP-4 C-domain with that of IGFBP-5 yielded three chimeric IGFBPs and they were predominantly nuclear. These results suggest that C-domain of IGFBP-5 is both necessary and sufficient for the nuclear localization of IGFBP-5.

If IGFBP-5 were secreted and then subsequently internalized, then it must interact with a cellular receptor(s) or transporter protein in the plasma membrane. Several lines

Figure 6 C-domain of IGFBP-5 is essential for the nuclear localization of IGFBP-5. (A) Schematic diagram showing the structures of various IGFBP/EGFP fusion proteins. In addition to wild-type IGFB-5 (WTBP5) and IGFBP-4 (WTBP4), six IGFBP chimeras (N5L5C4, N5L4C4, N4L5C4, N4L4C5, N5L4C5 and N4L5C5) were generated by exchanging the N-, L- and C-domains between IGFBP-4 and IGFBP-5. (B) Nuclear localization of the IGFBP/EGFP fusion proteins. All the chimeras lacking the IGFBP-5 C-domain exhibited little or no nuclear presence (N5L5C4, N5L4C4, N4L5C4). Replacement of the IGFBP-4 C-domain with that of IGFBP-5 yielded three chimeric IGFBPs (N4L4C5, N5L4C5 and N4L5C5) and they were predominantly nuclear.

of evidence suggest that the cellular entry of IGFBP-5 requires its interaction with heparan sulfate (HS) proteoglycans (HSPG), which are abundant on the SMC surface.

Proteoglycans are proteins characterized by a posttranslational attachment of GAG moieties, each comprising repeating disaccharide units. Cell surface HSPGs are

Figure 7 A proposed model of IGF, IGF-IR and IGFBP interactions in SMCs. (i) The action of IGFs on SMC migration and proliferation is mediated through the IGF-IR; (ii) ligand occupancy of the IGF-IR activates a spectrum of intracellular signaling pathways, including the MAPK and PI3K pathways and several PKC isoforms – activation of MAPK, PI3K and PKC is important for IGF-induced proliferation as well as chemotaxis; (iii) SMCs synthesize and secrete three high-affinity IGFBPs, namely IGFBP-2, IGFBP-4 and IGFBP-5, which in turn regulate IGF bioactivity; (iv) different IGFBPs play distinct roles in determining SMC responses to IGF-I – while IGFBP-2 and IGFBP-4 inhibit IGF actions by competing with receptors for ligand binding, IGFBP-5 can be localized to the cell surface after ligand occupancy, thereby ‘bringing’ IGF-I to the IGF-IR; and (v) IGFBP-5 also stimulates SMC migration independently of IGFs – IGFBP-5 is internalized and translocated into the nucleus and the nuclear IGFBP-5 plays an important role in stimulating SMC motility.

known to act as cellular receptors for several viral proteins, such as the HIV Tat protein. These viral proteins, released from infected cells, can interact with cell surface HSPG to enter the cell and then the nucleus, where they act as transactivators (Vivès *et al.* 1997, Summerford & Samuski 1998, Tyagi *et al.* 2001). If IGFBP-5 binding and internalization initiate through its interaction with cell surface proteoglycans, then one or more of the major GAGs found on membrane-associated proteoglycans should act as competitive inhibitors of IGFBP-5 binding and internalization. Competition experiments using known GAGs revealed

that HS and heparin, but not chondroitin sulfate A, B or C, inhibited IGFBP-5 internalization (Duan *et al.* 2002). More definitive genetic evidence came from our analysis of IGFBP-5 internalization in mutant CHO-K1 cell lines that are defective in HSPG biosynthesis. While IGFBP-5 internalization was clearly detectable in the wild-type CHO-K1 cells, its entry was severely impaired in pgs A-745 mutant cells, pgs B-618 cells, and pgs E-606 cells (Duan *et al.* 2002). A745 cells are defective in the enzyme xylosyltransferase, which initiates GAG synthesis (Esko *et al.* 1987). B-618 cells lack galactosyltransferase-I activity

catalyzing the second step in GAG synthesis and produce about 15% of the amount of GAGs synthesized by wild-type cells (Esko *et al.* 1986). Pgs E-606 cell line produces an N-undersulfated form of HS due to a defect in the HS N-sulfotransferase enzyme (Esko *et al.* 1985). This biochemical and genetic evidence strongly argues for a role of HSPG as the cell surface receptor for IGFBP-5.

A model for the molecular interactions between IGFs, IGF-IR and IGFBPs in SMCs

Based on the findings discussed above, we propose a model to explain the potential mechanism by which various components of the IGF signaling pathway interact with each other to determine the proliferative or migration outcome. In this model (Fig. 7), we incorporate the current knowledge and propose that: (i) the action of IGFs on SMC migration and proliferation is mediated through the IGF-IR; (ii) ligand occupancy of the IGF-IR activates a spectrum of intracellular signaling pathways, including the MAPK pathway, the PI3K pathway, and several PKC isoforms. Activation of MAPK, PI3K and PKC are all involved in IGF-induced proliferation and chemotaxis; (iii) SMCs synthesize and secrete three high-affinity IGFBPs, which in turn regulate IGF bioactivity; (iv) different IGFBPs play distinct roles in determining SMC responses to IGFs – while the soluble, high-affinity forms of IGFBPs in the extracellular fluid such as IGFBP-2 and IGFBP-4 inhibit IGF actions by competing with receptors for ligand binding, others such as IGFBP-5 can be localized to the cell surface after ligand occupancy, thereby ‘bringing’ IGF to the cell surface IGF-IR and promoting IGF actions; and (v) in addition, IGFBP-5 stimulates SMC motility through a novel IGF-independent pathway. IGFBP-5 enters the cell and the nucleus by interacting with HSPG and other yet to be identified molecular partners in the cytoplasm and nucleus. The nuclear IGFBP-5 alters the gene expression directly by interacting with DNA or indirectly by interacting with other DNA-binding proteins. It will be important to determine the molecular events that trigger the cell surface association, internalization and nuclear translocation of IGFBP-5, as well as to identify molecules interacting with IGFBP-5 in the nucleus. Future studies are also needed to elucidate whether nuclear IGFBP-5 may bind DNA and possess transactivating activity. Knowledge gained from these studies should lead to a better understanding of how specific biological action arises in connecting the IGF signal.

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