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Am J Physiol Cell Physiol 296:C954-C976, 2009. First published 11 March 2009;
doi:10.1152/ajpcell.00598.2008

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J Gerontol A Biol Sci Med Sci, August 23, 2011; .

[Abstract] [Full Text] [PDF]

IGFBP-3 Is a Metastasis Suppression Gene in Prostate Cancer

Hemal H. Mehta, Qinglei Gao, Colette Galet, Vladislava Paharkova, Junxiang Wan, Jonathan Said, Joanne J. Sohn, Gregory Lawson, Pinchas Cohen, Laura J. Cobb and Kuk-Wha Lee

Cancer Res, August 1, 2011; 71 (15): 5154-5163.

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Perspectives in mammalian IGFBP-3 biology: local vs. systemic action

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Submitted 17 November 2008; accepted in final form 9 March 2009

Yamada PM, Lee K. Perspectives in mammalian IGFBP-3 biology: local vs. systemic action. *Am J Physiol Cell Physiol* 296: C954–C976, 2009. First published March 11, 2009; doi:10.1152/ajpcell.00598.2008.—Insulin-like growth factor (IGF) binding protein (IGFBP)-3 has traditionally been defined by its role as a binding protein and its association with IGF delivery and availability. Development of non-IGF binding IGFBP-3 analogs and the use of cell lines devoid of type 1 IGF receptors (IGF-R) have led to critical advances in the field of IGFBP-3 biology. These studies show that IGFBP-3 has IGF-independent roles in inhibiting cell proliferation in cancer cell lines. Nuclear transcription factor, retinoid X receptor (RXR)- α , and IGFBP-3 functionally interact to reduce prostate tumor growth and prostate-specific antigen *in vivo*. Moreover, IGFBP-3 inhibits insulin-stimulated glucose uptake into adipocytes independent of IGF. The purpose of this review is to highlight IGFBP-3 as a novel effector molecule and not just another “binding protein” by discussing its IGF-independent actions on metabolism and cell growth. Although this review presents studies that assume the role of IGFBP-3 as either an endocrine or autocrine/paracrine molecule, these systems may not exist as distinct entities, justifying the examination of IGFBP-3 in an integrated model. Also, we provide an overview of factors that regulate IGFBP-3 availability, including its production, methylation, and ubiquitination. We conclude with the role of IGFBP-3 in whole body systems and possible future applications of IGFBP-3 in physiology.

insulin-like growth factor; nonsuppressible insulin-like activity; insulin-like growth factor binding protein-3

INSULIN-LIKE GROWTH FACTOR (IGF) was originally named nonsuppressible insulin-like activity (NSILA) because it stimulated glucose uptake into adipocytes, sharing “insulin-like” activity (81). Whereas anti-insulin antibodies were capable of abrogating the hypoglycemic effects of insulin, they did not abolish the effects of NSILA, hence, the term “nonsuppressible” was incorporated into its name. The term “NSILA” was renamed “somatomedin” in 1972 to describe its hormonal relationship to growth hormone (51).

Soon after the characterization and quantification of NSILA in rat serum (229), tracer technology identified that NSILA-S (serum NSILA) had a carrier protein (132). This was demonstrated by measuring the half-life of NSILA after the infusion of ^{125}I -labeled NSILA-S (IGF) or ^{125}I -labeled NSILA-S plus NSILA-S into rats. NSILA-S of low-molecular weight (~ 7.5 kDa) was cleared 20 min postinjection, whereas the high-molecular weight (~ 100 kDa) persisted for 3 h. Chromatography identified that the ~ 100 -kDa complex contained NSILA-S and its binding protein. The described low- and high-molecular weights (265) presumably corresponded to the molecular weights of IGF-1 and -2 alone (7.5–7.6 kDa) and the IGF-ALS-IGFBP-3 heterotrimeric complex (150 kDa), respectively. Today it is well known that IGF complexes with a leucine-rich acid labile subunit (ALS), and insulin-like growth factor binding protein (IGFBP-3), the most abundant circulat-

ing binding protein. Binding amplifies the half-life of IGF up to 12–15 h (102).

Perfusion of human serum into isolated rat hearts showed that glucose uptake remained unchanged (174), despite reports that free NSILA has insulin-like, hypoglycemic effects (173). This suggested that the insulin-like activity of partially purified NSILA on the rat heart was abolished in the presence of the NSILA-carrier protein complex (174). ^{125}I -labeled NSILA-S action was inhibited by its carrier protein, and the size of the NSILA-S-carrier protein complex prevented IGF efflux from vasculature. In 1976, NSILA was sequenced, revealing that it shared 48% homology with human proinsulin (203) and was renamed IGF-1. In 1989, this transporter of 70–90% of all circulating IGF was officially termed “insulin-like growth factor binding protein-3” (IGFBP-3) [Workshop on IGF Binding Proteins, Vancouver, Canada, 1989 (18)].

Presumably IGFBP-3 is produced and released by hepatic Kupffer and endothelial cells (264) into the systemic circulation to affect auxological growth via endocrine regulation. The majority of *in vitro* studies that have demonstrated IGF-independent actions of IGFBP-3 on cell growth have been performed in culture and therefore simulates autocrine/paracrine regulation. Although this review presents studies that use IGFBP-3 in an endocrine (as an IGF transporter and binding protein) or autocrine/paracrine system (local release of IGFBP-3 from tissues to be reinternalized by the original cell or by neighboring tissues), these systems may not exist as distinct models. This may justify the examination of IGFBP-3 in an integrated model. Figure 1 illustrates a separated view of local and systemic regulation.

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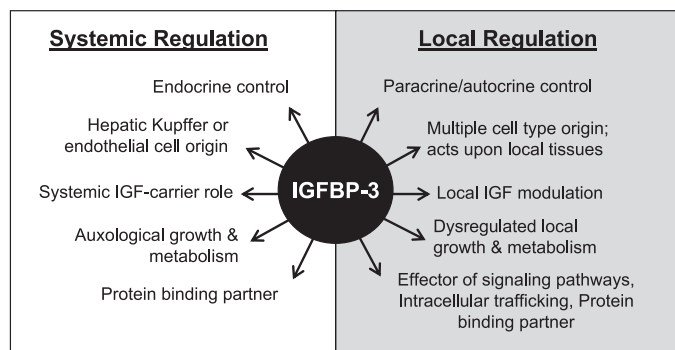


Fig. 1. Local and systemic actions of insulin-like growth factor binding protein-3 (IGFBP-3). The majority of studies employ models that represent either systemic or local regulation and have not incorporated both systems into their model of study. Whether interaction between these systems has physiological significance is unknown.

IGFBP-3 Across Phylogeny

IGFs and IGFBP-3 have been extensively studied in humans, mice (177), and rats (180). The IGF system and its binding proteins have also been examined in a variety of species including cow (88), pig (11), water buffalo (124), cattle (238), sheep (86), horse (75), dog (69), wallaby (33), kangaroo (258), tilapia (190), and zebrafish (157). Of particular interest, a secreted 27-kDa protein that binds to proteins of the insulin superfamily of insect cells (S9) was purified, cloned, and characterized in 2000 (225). This protein was shown to inhibit insulin activation of the insulin receptor and bound to IGF-I, -II, pro-insulin, and mini-proinsulin with high affinity. Eight years later, Imp-L2, the functional equivalent of IGFBP in *Drosophila* was identified in vivo, which is the first documentation of an insect insulin/IGF binding protein (114). Imp-L2 was shown to bind to and antagonize *Drosophila* insulin-like peptide 2 (Dilp2). Imp-L2 overexpression reduced body size, whereas loss of Imp-L2 resulted in increased body size, similar to the phenotypes of the transgenic and knockout IGFBP-3 mice (221, 253). These observations indicated that Imp-L2 is an IGFBP equivalent. In addition, zebrafish have also been used to study the role of IGFBP-3 in growth and development (157). Knockdown of IGFBP-3 delayed pharyngeal skeleton morphogenesis, reduced pharyngeal cartilage differentiation, decreased inner ear size, and disrupted semicircular canal formation in embryos; these effects were rescued with IGFBP-3 expression. IGF binding protein conservation in invertebrates portends a significant physiological role across phylogenies.

Unraveling IGF-Independent Actions

The term IGFBP-3 does not fully describe its roles because it not only functions as a binding partner, but it also has important functions not involving IGF. IGFBP-3 has historically been described as a mediator of endocrine cell growth through its IGF-dependent effects. The function of IGFBP-3 was viewed as a binding protein of IGF-1 to either transport IGF-1 to its receptor, enhancing IGF-1 actions, or sequester IGF-1 from its receptor, inhibiting its action. Advances in the field have enabled researchers to demonstrate evidence for IGF-independent effects of IGFBP-3 using various strategies: 1) IGFBP-3 mutants that do not bind to IGF-1 (26, 115, 257)

or IGF-1 analogs with reduced affinity for IGFBP-3 [long R3-IGF-1, GroPep, Australia, and des-(1-3)-IGF-1] (254, 255), 2) 16- and 22/25-kDa IGFBP-3 fragments with total and partial loss of IGF affinity, respectively (143), 3) transfection of the IGFBP-3 vector into cells lacking IGF-R (197, 236), and 4) use of IGF-1 negative cell lines (breast cancer cells, chondrocytes) (97, 232). Identification of putative IGFBP-3 cell surface receptors and binding to these receptors with specificity and high affinity have suggested IGFBP-3 is capable of IGF-independent actions (184, 186, 256).

Structure

Human IGFBP-3 is composed of 264 amino acids and contains 3 functional domains: a nonconserved central domain and highly conserved cysteine-rich carboxyl (COOH)- and amino (NH₂)-domains. The molecular weight of IGFBP-3 would be 29 kDa based on its amino acid sequence; however, its actual molecular weight ranges from 46 to 53 kDa secondary to potential glycosylation at three different NH₂-linked sites (247). When proteins are separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), IGFBP-3 is detected as a doublet at 42/45 kDa.

Residues 1–87 and 184–264 comprise the NH₂ and COOH domains, respectively. The nonconserved domain spans residues 88–183 or 88–148, with or without the heparin-binding domains (HBD), respectively (256). Another HBD is found in the COOH-terminal, residues 241–259 (90). IGF binding occurs at both NH₂ and COOH terminals (5, 84), and the NH₂-terminal region, including Ile⁵⁶, Leu⁸⁰, Leu⁸¹, contributes to a hydrophobic pocket that is important for IGF-1 binding (26). Many molecular interactions are localized to the COOH-terminal sequence 215–232. For example, residues 228–232 at the COOH terminal are required for ALS, IGF-1, and heparin binding (17, 73). Also contained within the important residues 215–232 in the COOH-terminal domain is a nuclear localization sequence (NLS) responsible for translocation into the nucleus through nuclear transport factor importin-β (210). Residues within the NH₂ terminal and motifs within the COOH terminal are involved with interaction with nuclear partners RXRα and retinoic acid receptor (RARα) (209). A metal-binding domain (MBD) is also contained in the COOH terminal, which spans the NLS and a caveolin-binding sequence (223). Of the six IGFBPs, IGFBP-3 and IGFBP-5 are most similar in structure, where both contain similar functional domains comprising: 1) the IGF binding region of the NH₂ and COOH terminus (23, 84, 129), 2) heparin-binding motifs in the central domain (77, 230), 3) functional transactivation domain in the NH₂ terminal (261), and 4) NLS (210), heparin-binding (21), ALS-binding (73, 235), and cell binding domains (73, 235) in the COOH terminal. Furthermore, since type 1α collagen has been shown to physically interact at a region in close proximity to the NLS domain, binding interaction may ultimately affect cell adhesion and migration (162).

Cell Surface Binding Partners and Internalization

IGFBP-3 contains glycosaminoglycan-binding domains, allowing IGFBP-3 to localize to the extracellular matrix or cellular surfaces. IGFBP-3 attachment to the matrix or cell surfaces decreases the affinity of IGFBP-3 for IGF-1, enhancing IGF action. Glycosaminoglycans, heparin, and heparin

sulfate bind to the COOH terminal of IGFBP-3 (77). Like the COOH terminal domain, the central domain also contains two heparin-binding consensus sequences, which are unmasked only when the COOH terminal domain has been deleted (70, 77). Additional glycosaminoglycans have been shown to bind to IGFBP-3. For example, dermatan sulfate was shown to bind to either domain by inhibiting heparin binding. Dermatan sulfate is found in blood vessels and heart valves and may have a role in coagulation and wound repair (77). Heparin binding was inhibited with lower efficacy by chondroitin sulfate A, a structural component of cartilage, and hyaluronic acid, a major component of extracellular matrix (ECM) (77). IGFBP-3 contains essential glycosaminoglycan binding sequences that not only bind heparin and heparan sulfate, but this critical sequence also binds to the ECM and cell surfaces (21).

IGFBP-3 binds to fibrinogen and fibrin (29). Similar to the IGFBP-3-plasminogen-IGF-I complex, IGF-1 does not compete for binding to IGFBP-3, but instead IGF-1 binds to IGFBP-3-fibrin and binding most likely occurred at the heparin-binding domain. The affinity between the IGFBP-3-fibrin and IGF-1 is similar to the affinity between IGF-1 and type 1 receptor (29). These observations suggest that the fibrin-immobilized IGFBP-3 complex may play a role in concentrating IGF-1 at the wound, aiding in tissue repair. This interaction may be detrimental to the cardiovascular system when tissue repair resides at the vascular level. Also, fibronectin was discovered to bind to both nonglycosylated and glycosylated IGFBP-3. Competitive inhibition of IGFBP-3 was observed in the presence of IGFBP-5 and heparin, whereas IGF-1 and IGFBP-1 had no effect on binding interaction between IGFBP-3 and fibronectin. The binding affinity of the IGFBP-3-fibronectin complex to IGF-1 is similar the affinity of IGF to IGFBP-3 (99).

Although a specific IGFBP-3 cell surface receptor has not been published, there are two putative receptors: one that was cloned via a yeast two-hybrid experiment using the midregion of IGFBP-3 (256) and another termed low-density lipoprotein-related protein-1 (LRP-1)/ α 2M receptor (117, 146). The former has yet to be validated and the latter has been supported with data from a single laboratory; exact receptor structure and signaling mechanisms are yet to be elucidated. IGFBP-3 bound to the surface of human breast cancer cells through putative high-affinity IGFBP-3 membrane receptors (256). Fragments containing the midregion of IGFBP-3 were able to inhibit radiolabeled IGFBP-3 binding, suggesting that the midregion interacts with the breast cancer cell surface receptor. High affinity binding sites were putatively identified in estrogen receptor (ER)-negative (Hs578T) and ER-positive (MCF-7) human breast cancer cells human. Testing all six IGFBPs indicated only IGFBP-5 was a weak competitor, suggesting IGFBP-3 binding to breast cancer cell receptor is specific and not due to nonspecific glycosaminoglycan interactions (256). The interaction between IGFBP-3 and these receptors suggested that IGFBP-3 may have IGF-1 independent actions on cell growth.

Presumed IGFBP-3 binding to a cell surface receptor induced a rapid and transient increase in intracellular free calcium concentrations in breast carcinoma cells (MCF-7) (201). The increase in calcium concentrations were mediated through a pertussis toxin-sensitive pathway, suggesting its receptor may be coupled to a G_i protein to regulate intracellular signal-

ing. Calcium concentrations increased when IGFBP-3 was bound to heparin or IGF-1, suggesting that receptor binding occurs in the least conserved region (201). Although intracellular localization of IGFBP-3 was not observed in this study, diffuse cellular surface and cytoplasmic staining of IGFBP-3 has been visualized in keratinocytes (248), demonstrating that IGFBP-3 is an intracellular molecule. Still, evidence for IGFBP-3 cell surface receptors and coupled intracellular signaling pathways would be strengthened by confirmation from independent laboratories.

IGFBP-3 was shown to bind to transforming growth factor (TGF)- β type V receptor and inhibited cell growth independent of IGF (117, 146). Surprisingly, matrix-assisted laser desorption/ionization (MALDI) analysis revealed that the TGF- β type V receptor is identical to the low-density lipoprotein-related protein-1 (LRP-1)/ α 2M receptor (118). An LRP-1 antagonist, receptor-associated protein, inhibited IGFBP-3 binding to 125 I-labeled TGF- β 1 and 125 I-labeled IGFBP-3 binding TGF- β type V receptor. Furthermore, 125 I-labeled IGFBP-3 affinity labeled TGF- β type V receptor could be immunoprecipitated with antibodies specific to LRP-1 and TGF- β type V receptor, providing additional evidence that the TGF- β type V and LRP-1 receptors are identical (118). Serum IGFBP-3 levels were upregulated in mice with impaired LRP-1 function (Fut8 $^{-/-}$ mice), suggesting that LRP-1 is necessary for IGFBP-3 internalization and impaired LRP-1 function results in increased serum IGFBP-3 levels (151). LRP-deficient cells also had reduced IGFBP-3 internalization compared with LRP-expressing mouse embryonic fibroblasts. Treatment with receptor-associated protein inhibited IGFBP-3 internalization in mouse embryonic fibroblasts, indicating that IGFBP-3 is a ligand of LRP-1, and LRP-1 is crucial for internalization (151). Whether the LRP-1 receptor is the primary IGFBP-3 receptor and if this receptor is identical to the breast cancer cell receptor remains unknown.

Yeast two-hybrid analyses indicated that IGFBP-3 binds to latent TGF- β binding protein-1 (LTBP-1), a component of the latent TGF- β complex (100). LTBP-1 is a part of the structural component of the ECM and is a 160- to 240-glycoprotein containing 8-cysteine and calcium binding EGF-like repeats (166). LTBP-1 is involved in sequestration of latent TGF- β in the ECM and delivery of TGF- β to the plasma membrane (166). Binding occurs when IGFBP-3 is bound to IGF-1; the affinity between IGFBP-3 and recombinant LTBP-1 is estimated to be equal or greater than the affinity of IGFBP-3 for fibronectin (99). Of great importance, IGFBP-3 signaling appears to involve an active TGF- β signaling, where IGFBP-3 stimulates phosphorylation of Smad2 and Smad3 to inhibit breast cancer cell proliferation (65, 66). Although in vitro studies indicate that IGFBP-3 and latent TGF- β complex interact via LTBP-1, in vivo studies are warranted to determine the whether LTBP-1 is functionally important in mediating IGFBP-3 binding to plasma membranes (100) and intracellular signaling.

IGFBP-3 possess basic COOH-terminal nuclear localization signals (195), allowing IGFBP-3 to exert intracellular actions. Transferrin associates with IGFBP-3 in the COOH terminus (149, 244); the caveolin-scaffolding domain consensus sequence also resides near the COOH terminal end and IGFBP-3 bound to caveolin-1 on breast cancer cells (27). These facts lead to the discovery that caveolin-1 mediates IGFBP-3 inter-

nalization via a caveolin-scaffolding docking sequence (149). Caveolae are glycolipid rafts that are involved in endocytosis and signal transduction (226), and caveolin-1 has been implicated to be a principle structural scaffold that organizes intracellular trafficking and cytoplasmic signal transduction (85, 205), providing a mechanism for IGFBP-3 internalization and signaling.

Coimmunoprecipitation assays demonstrated that IGFBP-3 binds to transferrin, which in turn binds to its receptor to form a ternary complex. Cotreatment of anti-transferrin receptor antibody and cholesterol depletion agents completely abolished endogenous and exogenous IGFBP-3 uptake. This suppression also inhibited IGFBP-3-mediated apoptosis in prostate cancer cells to show functional importance of endocytic internalization. Caveolae, transferrin, and the transferrin receptor function together to collectively mediate endocytosis of IGFBP-3 (149). Whereas transferrin is required for full-length IGFBP-3, it is not required for IGFBP-3 fragments lacking the transferrin-binding domain in breast cancer cells (27). A serine phosphorylation domain peptide (SPD) that lacks the region that interacts with transferrin is still able to induce C2-ceramide cell death in Hs578T cells, indicating that transferrin is not essential for IGFBP-3 action. A related study reported that cellular uptake of IGFBP-3 is mediated by metal ion-stimulation of the MBD (223). Full-length IGFBP-3 was shown to bind nickel, iron, and zinc via the MDB; the MDB was reported to have physical interaction with caveolin-1 and transferrin receptor. Thus the MDB may be involved in intracellular translocation of IGFBP-3 (223). IGFBP-3 binding to nickel or iron-charged resin was inhibited in the presence of IGF *in vitro*; however, whether MDB-mediated internalization occurs in an *in vivo* model remains to be determined.

Autocrine motility factor/phosphoglucose isomerase (AMF/PGI) was identified as a binding partner for IGFBP-3 in solubilized T47D and MCF-7 human breast cancer cell membranes (176). AMF/PGI is endocytosed in tumor cells and is utilized as a cell-penetrating peptide to deliver toxins to the intracellular space (136), similar to cell-penetrating peptides derived from IGFBP-3 and IGFBP-5 (90). IGFBP-3 antagonized its induction of migration of these breast cancer cells. Whether a classical receptor will be discovered and characterized for IGFBP-3 or whether the "receptors" described in the current paper are part of larger protein complexes in an endocytic pathway remains an unanswered question.

Nuclear Binding Partners

IGFBP-3 had previously been shown to translocate into the nucleus in opossum kidney cells (156); recombinant IGFBP-3 has been visualized in human breast cancer cells (T47D) (213); endogenous nuclear IGFBP-3 has also been detected in human lung cancer cells (127) and during cell division in keratinocytes (248). IGFBP-3 also has been demonstrated to localize to the nuclei of myoblasts through MBD interaction, where IGFBP-3 bound to RNA polymerase II binding subunit 3 (Rpb3) (188). IGFBP-3 interaction with Rpb3 is dependent on a functional NLS as a NLS mutant failed to associate with Rpb3. Since Rpb3 regulates gene transcription and gene expression through recruitment of the polymerase complex to specific transcription factors, interaction between IGFBP-3 and Rpb3 could provide

a functional role for IGFBP-3 in modulation of gene transcription.

Initial findings in human breast cancer cells (T47D) suggest that NLS of the COOH terminal region is involved in nuclear import of recombinant IGFBP-3 (213). Using fluorescently labeled IGFBP-3, IGFBP-3 was rapidly taken up during rapid cell division. In a follow-up study, wild-type IGFBP-3 underwent translocation, but not a NLS mutant form, through a NLS-dependent pathway into an intact nuclear envelope. When the nuclear envelope was permeabilized, wild-type IGFBP-3, but not the mutant form, accumulated in the nucleus, suggesting that the NLS was involved in binding IGFBP-3 to the nuclear components. IGFBP-3 was recognized by importin- β and α/β -heterodimer, suggesting that IGFBP-3 nuclear importation is dependent on its NLS and importin- β nuclear transport factor (210). When the plasma membrane is compromised (i.e., detergents), nuclear transport of IGFBP-3 occurs in almost all cells (210).

IGFBP-3 interacts with nuclear receptors: retinoid X receptor (RXR)- α (159), RAR (212), and Nur77 (148). Binding to RXR α was identified using multiple methods, including a yeast two-hybrid screen (159). Evidence of IGFBP-3-RXR α functional interaction was further demonstrated by abrogation of IGFBP-3-induced apoptosis in RXR α -knockout cells, indicating that RXR is required for IGFBP-3-induced apoptosis. Combination treatment of IGFBP-3 and RXR ligands synergistically induced apoptosis in prostate cancer cells (161).

IGFBP-3 sequestered RAR and blocked the formation of RAR:RXR heterodimers (212). The presence of IGFBP-3 substantially reduced the number of binding sites for all-trans retinoic acid (atRA), a specific ligand for RAR α , decreasing atRA binding to RAR:RXR heterodimers. To show interaction in a different way, IGFBP-3 depletion from an *in vitro* system increased sensitivity of RA-resistant breast cancer cells to the growth inhibitory effects of atRA (212). Residues within the NH₂ terminal and motifs within the COOH terminal are essential for interaction with RXR α and RAR α and subsequent modulation of RAR/RXR signaling (209).

IGFBP-3 induces RXR α -Nur77 translocation from the nucleus to mitochondria (150). IGFBP-3 treatment was observed to induce a rapid appearance of extra nuclear GFP-Nur77 in RXR α ^{+/+} cells, but not in RXR α ^{-/-} cells, as observed with confocal microscopy. Mitochondrial isolation from cells transfected with IGFBP-3 showed a threefold increase in mitochondrial RXR α , whereas this response was absent in cells transfected with control expression vector. IGFBP-3 trafficking to organelles other than the nucleus has yet to be described. Figure 2 summarizes IGFBP-3 structure and intracellular trafficking. Figure 3 outlines IGFBP-3 binding partners in relation to its potential function.

REGULATION OF IGFBP-3 TRANSCRIPTION, AVAILABILITY, AND ACTION

IGFBP-3 affinity to IGF controls bioavailability and action of IGF (Fig. 4). For example, IGFBP-3 is able to sequester IGF from its receptor because IGFBP-3 has greater affinity for IGF than it does for its receptor, thus reducing IGF bioavailability and action. However, proteolysis and fragmentation of IGFBP-3 (44, 141, 241) and attachment of IGFBP-3 to proteins on the extracellular matrix (172) reduces its affinity to IGF.

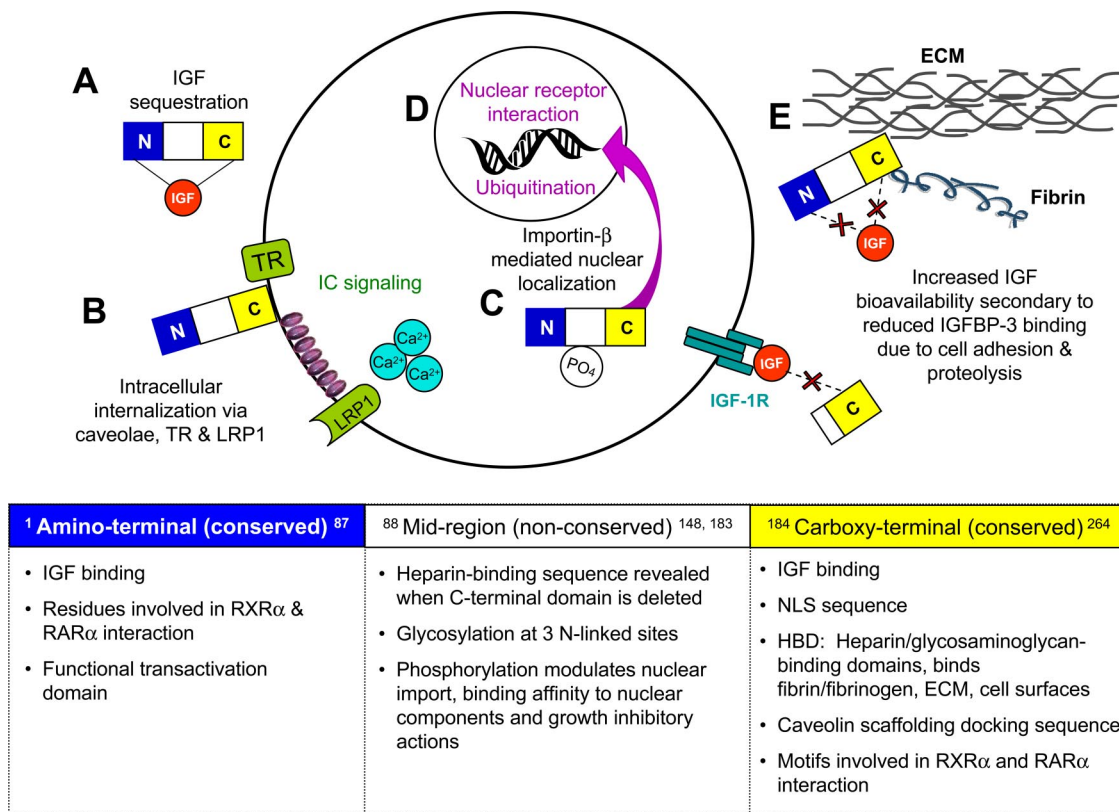


Fig. 2. IGFBP-3 and cellular interaction. **A:** IGF binds at the NH₂ and COOH terminus; although this figure depicts IGFBP-3 as a linear structure, its proposed tertiary structure has been described as a horseshoe, allowing an IGF molecule to bind at both the NH₂ and COOH terminus simultaneously. The IGFBP-3:IGF-1 affinity is greater than IGF-1:IGF-R affinity, resulting in IGF sequestration. **B:** caveolin-1, transferrin, and the transferrin receptor (TR) function together to mediate endocytosis of IGFBP-3 and may be involved in intracellular trafficking and cytoplasmic signaling transduction. IGFBP-3 also may be internalized via lipoprotein-related protein (LRP)-1. Whether LRP-1 and the breast cancer cell receptor are identical or which terminus interacts with LRP-1 is unknown. IGFBP-3 may initiate a rapid increase in calcium concentrations and regulate intracellular signaling. **C:** phosphorylation of IGFBP-3 may increase its nuclear import. Nuclear localization of IGFBP-3 is mediated by nuclear transport protein importin- β , allowing interaction with nuclear transcription factors. DNA-PK can phosphorylate nuclear IGFBP-3 to modulate DNA repair/replication and apoptosis. **D:** ubiquitination of IGFBP-3 occurs in the nucleus, and one of its 19 lysine residues probably serves as an anchor for polyubiquitination. **E:** interaction between the COOH terminal of IGFBP-3 and proteins attached to the extracellular matrix (ECM) [i.e., latent transforming growth factor- β binding protein (LTBP-1)] or type 1 α collagen affects cell adhesion and migration. Binding to ECM or fibrin decreases the affinity of IGFBP-3 to IGF-1, increasing IGF bioavailability. Proteolyzed IGFBP-3:IGF-1 affinity is less than IGF-1-R:IGF-1 affinity, preserving IGF-IR sensitivity secondary to IGF-1 binding. Superscript numbers in IGFBP-3 molecule represent residues within each domain, with varying residues in the nonconserved domain depending on the presence or absence of the heparin binding domain. RXR, retinoid x receptor; RAR α , retinoic acid receptor.

The result is enhanced IGF bioavailability and augmented IGF action at its receptor. In addition to proteolysis, IGFBP-3 activity can be affected by other posttranslational modifications such as phosphorylation, methylation, glycosylation, and ubiquitination. IGFBP-3 levels are also affected by the rate of its transcription. IGFBP-3 gene transcription is affected by numerous agents, such as retinoic acid (RA), estrogen, p53, TGF- β , tumor necrosis factor (TNF)- α , vitamin D, sodium butyrate (NaB), and follicle stimulating hormone (FSH) (187). This section will review different ways in which IGFBP-3 binding affinity, trafficking, and activity can be modulated.

Regulation of Gene Transcription

Vitamin D. 1,25-Dihydroxyvitamin D3 (vitamin D) and its analogs have been shown to decrease cell proliferation in prostate carcinoma cells in vitro and in rat prostate in vivo; this response is associated with increased IGFBP-3 expression (181). This observation is well documented in LNCaP cells, an androgen-dependent cell line (22, 170). An approximate three-fold induction of IGFBP-3 mRNA expression and protein

secretion in LNCaP cells has been reported after a 24-h exposure to vitamin D (170). Moreover, 4 days of exposure to vitamin D plus sense oligodeoxynucleotides (ODN) inhibited cell growth in LNCaP cells, whereas this response was abrogated in antisense ODN and vitamin D treatment (22). Treatment induced an increase in IGFBP-3 mRNA as soon as 6 h posttreatment with a maximal response observed 12 h post-treatment (22).

Vitamin D analogues have been studied as anti-cancer therapies because they do not exert hypercalcemic effects like their naturally bioactive counterparts. A variety of homologues have been used to induce IGFBP-3 expression and/or secretion from a variety of cell lines [i.e., human osteosarcoma cells, PC-3 (121), Hs578T, and MCF-7 (46)]. The anti-proliferative actions of vitamin D are mediated through vitamin D receptor (VDR), a classic nuclear receptor that is usually composed of two direct repeats of 6 bases, separated by a 3-nucleotide spacer (CDR3 motif) (139). A 15-bp sequence containing 2 hexameric core sites were identified as a potential IGFBP-3 vitamin D response element (VDRE) (192). 1,25 OH₂D₃ re-

Heterotrimeric Complex
 IGF-1
 IGF-2
 ALS

Cell Surface Association
 ECM
 LTBP-1
 Type 1 α collagen
 LRP-1 receptor
 Transferrin
 Caveolin
 AMF/PGI

Wound healing/CVD
 Fibrinogen, Fibrin
 Fibronectin
 Plasmin
 Plasminogen
 Dermatan sulfate

Nuclear Import and Interaction
 Importin β
 RXR α
 RAR α
 PPAR γ
 Rpb3

Proteases
 MMP
 hK2, hK3
 PSA
 Aspartic protease cathepsin D
 HPV-16 E7 oncoprotein
 uPA and tPA

Viral Oncoprotein
 HPV-16 E7

Survival Factor
 Humanin

Golgi-associated glycosyltransferase
 GalNAc-T14

Fig. 3. IGFBP-3 binding partners, organized by potential function. See text for more definitions.

cruited the VDR/RXR heterodimer to the VDR site in the IGFBP-3 promoter; mutations of this element abrogated the proper hexameric sequence (192). It has been suggested that vitamin D interacts with RXR since both VDR and RAR act through this common nuclear receptor. Normal action of a specific RAR antagonist AGN193109 antagonized retinoids but failed to antagonize vitamin D in human papillomavirus-immortalized cervical epithelial cells, which suggests vitamin D acts through VDR and not retinoid signaling molecules (4). Thus vitamin D may involve signaling through nuclear receptors VDR/RXR but not RAR/RXR to increase IGFBP-3 gene expression.

Steroid receptor coactivator 3 (SRC-3/AIB1/ACTR/NCOA-3) is a transcriptional coactivator for nuclear receptors including VDR (158). Rapid serum IGF-I degradation in SRC-3^{-/-} mice was associated with a drastic reduction of serum IGFBP-3 levels that cannot be restored by transgenic IGF-I compensation. In addition, disruption of SRC-3 in mice decreased the expression of IGFBP-3 and several other known VDR target genes. SRC-3-deficient cells were unable to support vitamin D-induced IGFBP-3 promoter activity, and IGFBP-3 mRNA was reduced in SRC-3^{-/-} mice. SRC-3 maintained circulating IGF-I by controlling IGFBP-3 expression (158).

p53. The DNA elemental sequence of IGFBP-3 to which p53 interacts in vitro was identified as a consensus binding site containing two copies of a 10-bp motif separated by 0–13 bp (62). Alterations of this motif lead to a loss of p53 affinity. In addition, p53 mutants failed to bind to the consensus dimmer; these mutants represented altered “hot spots” often present in human cancer that results in a loss of tumor suppression. Two p53-responsive elements were identified in the first and second introns of the IGFBP-3 gene, and highly purified wild-type p53 bound to the consensus element, whereas the mutant p53 failed to bind (25). These investigators used a differential cloning approach to demonstrate that the IGFBP-3 gene coding is regulated by p53 binding. Activation of this p53 transgene increased in IGFBP-3 mRNA expression and protein in media conditioned by colon carcinoma cells, cells that have an inducible wild-type p53 transgene and undergo apoptosis upon p53 induction (25). These results are consistent with their previous observation that activation of osteosarcoma cells containing temperature-sensitive p53 element Saos-2-D4H lead to increased IGFBP-3 mRNA expression (24).

Functional p53 domains dictate target gene specificity such that different isoforms differentially regulated target gene expression. Activation domain 2 (AD2) was required for p53-dependent apoptosis (263) and the COOH-terminal basic domain (BD) is a regulatory domain where deletion increases p53-specific binding activity in vitro (106). Interestingly, naturally occurring isoforms of p53 with truncated COOH-terminals induced IGFBP-3 expression, whereas full-length forms could not induce expression. Furthermore, histone deacetylase (HDAC) activity restores the ability of exogenous full-length p53 to induce IGFBP-3. Simultaneous p53 DNA stabilization and inhibition of HDAC restored the ability of endogenous full-length p53 to induce IGFBP-3 (106).

Since p53 modulates IGFBP-3 gene transcription, factors that affect p53 are also capable of regulating IGFBP-3 availability. For example, total body irradiation (and resultant DNA damage) induced p53 in the thymus and small intestine of p53^{+/+} female mice, resulting in increased IGFBP-3 mRNA as early as 6 h postirradiation (95). A human lung carcinoma cell line transfected with a plasmid containing the gene E6 contains less p53 versus the same lung carcinoma cell transfected with an empty plasmid. After exposure to etoposide, a chemotherapeutic agent, both cell types increased IGFBP-3 secretion into conditioned media (95). Treatment with adriamycin, a genotoxic drug, increased IGFBP-3 secretion in p53^{-/-} PC-3 cells showing that IGFBP-3 can also be induced independent of p53-mediated gene transcription (95).

Of note, p53 activity (10) and IGFBP-3 mRNA was greatly increased during cellular senescence in human diploid fibroblasts (91) and human prostate epithelial cells (214). Since IGFBP-3 gene activity was activated by p53, this suggested that p53 activity during cellular senescence may be required for induction of IGFBP-3. Increases in p53 were accompanied with consistent IGFBP-3 mRNA levels in all conditions, indicating that p53 activity does not affect IGFBP-3 mRNA. Thus increased p53 activity is associated with increased IGFBP-3 mRNA; however, p53 did not contribute to IGFBP-3 gene overexpression in this system (165).

Hypoxia. Hypoxia can induce IGFBP-3 mRNA through p53-independent (67, 95) and -dependent mechanisms (39). Induction of IGFBP-3 mRNA was observed in response to

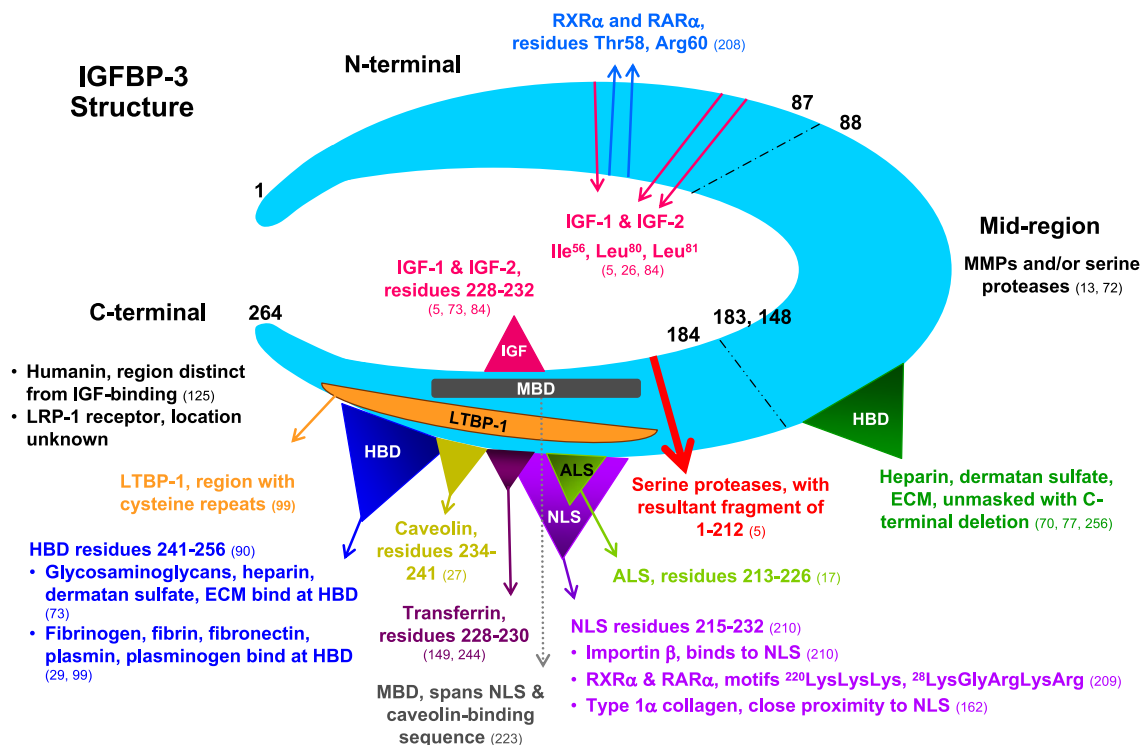


Fig. 4. IGFBP-3 binding partners. Dashed line separates IGFBP-3 into 3 domains; numbers represent residues at the end of each domain. Binding partners are depicted within the region/sequence involved in molecular interactions.

hypoxia in wild-type embryonic stem cells (67). Increasing dosages and hypoxic exposure time both increased IGFBP-3 mRNA in a dose-dependent manner in p53^{+/+} lung carcinoma cells (95). Still, when these methods were repeated using p53^{-/-} PC-3 cells, similar results were observed, suggesting that hypoxia can modulate IGFBP-3 mRNA expression independent of p53. In fact, many cancer cell lines had IGFBP-3 mRNA induction by hypoxia regardless of their p53 load/status (95).

Androgen. Androgen actions on IGFBP-3 transcription are mediated by the androgen receptor (AR) (87), in which the AR binds as a homodimer to the androgen response elements (ARE) in the distal region of the IGFBP-3 promoter (193, 239). Some investigators have shown that androgens have inhibitory effects on IGFBP-3 expression in LNCaP cells, an androgen-dependent human prostate cancer cell line (9, 92, 137). Alternatively, androgen treatment has been shown to stimulate IGFBP-3 expression in the same cell line (170). A possible explanation for the discrepancy is the biphasic response of androgen treatment on LNCaP cells, where low doses of androgen stimulated growth, whereas high concentrations resulted in growth inhibition (147).

Methylation

Aberrant DNA methylation is the most common molecular abnormality in cancer (126). It involves epigenetic modification at the CpG island (cytosine-phosphate-guanine site) in the promoter region, to cause chromatin condensation and gene silencing (245). Hypermethylation of the CpG islands may result in transcriptional silencing by physically blocking the binding of the transcription proteins to the promoter. Alternatively, methylated DNA may be bound by proteins referred to as methyl-CpG-binding domain proteins, which in turn recruit

additional proteins to the locus (i.e., histone deacetylases, chromatin remodeling proteins) that are capable of modifying histones and silencing genes. Since IGFBP-3 is a tumor suppressor gene, silencing the IGFBP-3 gene would presumably increase tumor growth.

Poor prognosis in stage I non-small cell lung cancer was associated with IGFBP-3 methylation (37, 38). This relationship was illustrated in another study that showed aberrant DNA methylation caused a reduction in IGFBP-3 expression by 75% in hepatocellular carcinomas (104). Similarly, hepatitis B virus X-protein (HBx), a transcriptional transactivator of viral and cellular promoters, repressed IGFBP-3 by promoting HBx/HDAC1 complex formation (218). Hepatitis B virus (HBV), which increases the risk of hepatocellular carcinoma, encodes oncogenic HBx. HBx recruited HDAC1 and deacetylated specificity protein (Sp)-1 in a p53-independent fashion, resulting in reduced Sp1 binding onto targeted DNA and suppression of IGFBP-3 (218). Methylation plays a critical role in tumorigenesis through gene silencing and chromatin remodeling.

Growth Inhibitory Agents

Anti-estrogen. Pure anti-estrogen, ICI-182780, and Tamoxifen, a partial ER antagonist, increased IGFBP-3 secretion from MCF-7 cells, whereas cotreatment of anti-estrogens with estradiol reversed this effect (194). The capacity of ICI-182780 to stimulate IGFBP-3 gene transcription was 10 times that of Tamoxifen (120). Nuclear run-off assays showed that IGFBP-3 gene transcription was increased by ICI-182780 and decreased by estradiol in MCF-7 cells (122). IGFBP-3 mRNA was three to four times greater in ICI-182780 treated MCF-7 cells versus controls and more than 20 times greater compared with estradiol-treated cells. To demonstrate that IGFBP-3 is integral in

mediating anti-proliferative actions, recombinant IGFBP-3 was used to inhibit basal and estradiol-stimulated cell proliferation, and IGFBP-3 antisense ODN abrogated the growth inhibitory effects of anti-estrogen (122).

TGF- β . Anti-estrogen treatment stimulated TGF- β secretion 8- to 27-fold in MCF-7 cells, resulting in attenuated ER-negative human breast cancer cell growth. This effect was reversed with anti-TGF- β antibodies, suggesting that TGF- β is hormonally regulated and may have autocrine and paracrine functions in breast cancer cells (135).

An increase in both IGFBP-3 mRNA expression and IGFBP-3 secretion was observed within 8-h and 2-days post-TGF- β 2 treatment of ER-negative human breast cancer cells (Hs578T), respectively; 4 days later cell growth was suppressed (185). Gucev et al. (97) repeated this experiment in a different human breast carcinoma cell line that is IGF-1 negative (MDA-MB-231). Their data illustrated that 3 days of TGF- β 2 treatment resulted in two- to threefold increase in IGFBP-3 protein levels in conditioned media. A 5-day treatment of exogenous recombinant human nonglycosylated IGFBP-3 reduced cell survival in a dose-dependent fashion. Since this cell line is devoid of IGF-1, results suggested that IGFBP-3 inhibits cell proliferation in an IGF-1 independent manner.

TGF- β was observed to be a potent stimulator of IGFBP-3 production in fibroblasts (168). In PC-3 cells, TGF- β treatment induced a large elevation of IGFBP-3, which lead to apoptosis (197). IGFBP-3 sense and TGF- β 1 induced apoptosis, whereas an IGFBP-3 antisense oligomer blocked TGF- β 1-induced apoptosis in PC-3 cells, suggesting that IGFBP-3 is a crucial intermediary step in mediating apoptosis. Apoptosis was attenuated when neutralizing antibodies of IGFBP-3 blocked TGF- β induction of apoptosis, providing additional evidence that increased IGFBP-3 expression is critically involved in TGF- β -mediated programmed cell death.

IL-1. Treatment of interleukin (IL)-1 β caused a time- and dose-dependent induction of IGFBP-3 mRNA in Leydig cells. This treatment caused an increase in IGFBP-3 transcription, contributing to the inhibitory effects of IL-1 β on cell steroidogenesis (243). IL-1 β treatment also caused a dose-dependent increase in IGFBP-3 in conditioned media; IL-1 β treatment increased IGFBP-3 transcription rate and did not change the stability of IGFBP-3 mRNA.

Whereas IL-1 β is produced by activated macrophages and is readily involved in pro-inflammation (59), IL-1 α is constitutively expressed in testes, localized to Sertoli cells, and is involved in differentiation of Leydig cells in rats (128). IL-1 α -stimulated Leydig cells resulted in increased IGFBP-3 secretion (45). However, when Leydig cells were incubated with both GH and IL-1 α , intact IGFBP-3 levels decreased in culture media. Furthermore, cell culture media catalyzed the degradation of IGFBP-3 with simultaneous appearance of fragments, suggesting Leydig cells produce an IGFBP-3 protease (45).

IL-6. Prominent IL-6 production is commonly observed in systemic juvenile idiopathic arthritis and this disease is accompanied by stunted growth and decreased IGFBP-3 and IGF-1 levels (56). While treatment with IL-6 increased biosynthesis of IGFBP-3 protein and total mRNA in a dose-dependent manner in cocultures of hepatocytes and Kupffer cells (152), rhIL-6 treatment in CB6F1 mice resulted in a significant decrease in IGFBP-3 levels compared with saline-treated

mice (56). Elevated pro-inflammatory cytokine IL-6 decreased IGFBP-3 levels and caused a decreased association of IGF-1 in the 150-kDa complex. Thus growth impairment may be attributed to IGFBP-3 reduction and shortened half-life of IGF-1 as decreased IGF-1 levels appeared to be secondary to increased clearance.

Chronic overexpression of pro-inflammatory cytokine IL-6 in transgenic mice resulted in a 50% reduction in circulating IGFBP-3 levels and a marked decrease in growth rate so that adult transgenic mice are 50–70% the size of control littermates (55). The decrease in IGFBP-3 was in part attributed to increased proteolysis as intact IGFBP-3 protein was decreased in transgenic mice. Whereas the mechanism explaining the IL-6-stimulated reduction in IGFBP-3 levels is not clear, IL-6 may directly affect IGFBP-3 production or the effects may be secondary to IGFBP-3 proteolysis.

TNF- α . Pro-inflammatory cytokine TNF- α has been reported to increase IGFBP-3 secretion and mRNA expression in MCF-7, resulting in inhibition of cell proliferation (206). TNF- α abrogated the inhibitory effects of estradiol on IGFBP-3, resulting in IGFBP-3 accumulation. This response has also been observed in cultured rat aortic vascular smooth muscle cells (VSMC), which normally secrete only trace amounts in IGFBP-3 (8). In the absence of TNF- α stimulation, IGFBP-3 could not be detected in conditioned media or with Northern blots. Treatment with increasing dosages of TNF- α over a 24-h period increased both IGFBP-3 secretion and mRNA expression with no change in other IGFBPs. Blocking TNF- α induction with cycloheximide and actinomycin D inhibited induction of mRNA expression and protein secretion of IGFBP-3, providing further evidence that TNF- α regulates IGFBP-3 (8).

Retinoic acid. As an active derivative of vitamin A, retinoic acid (RA) regulates the growth of a variety of cell lines, including human breast cancer cells (2, 97, 169, 215), osteoblast cells (262), and cervical epithelial cells (109). RA antagonizes cellular proliferation of human breast carcinoma cells presumably by increasing secretion of anti-proliferative IGFBP-3 in vitro (74). However, others observed secretion of IGFBP-3 from this cell line, perhaps only in the absence of estradiol stimulation (120, 169). atRA, a FDA-regulated drug, caused a threefold increase in IGFBP-3 expression in a human breast cancer cell line, indicating de novo synthesis of IGFBP-3 (2).

In a separate study, lower atRA concentrations stimulated a twofold increase in IGFBP-3 protein secretion into conditioned media (169). Dose-dependent increases in IGFBP-3 mRNA expression with atRA treatment was observed in MCF-7 cells (215). Increasing treatment durations resulted in a dose-dependent increase in IGFBP-3 mRNA expression with maximal expression achieved after 48 h. RAR β expression was induced with atRA stimulation through an RAR α -dependent pathway in MCF-7 (215) cells and human bronchial epithelial cells (103).

In a human breast carcinoma cell line, RA resulted in a two- and threefold increase in IGFBP-3 mRNA and protein in conditioned media, respectively (97). To show that IGFBP-3 was essential for the anti-proliferative effects of RA, cells were treated with a combination of antisense IGFBP-3 ODN and RA. This treatment (but not sense ODN) failed to increase IGFBP-3 protein in conditioned media by 80%, indicating

IGFBP-3 is directly involved in mediating inhibition of cell proliferation (97). Similar responses to RA have been documented in cervical epithelial cells, human osteoblast cells (262), and human prostate adenocarcinoma cells (123).

Sodium butyrate. As a histone deacetylase inhibitor and 4-carbon fatty acid, sodium butyrate (NaB) exerts different morphological and biochemical effects in cell culture through regulation of nuclear enzymes (191) to ultimately affect gene expression and cell growth (140). For example, it inhibited hyperacetylation of histones H3 and H4 (202) resulting in increased transcriptional activity in HeLa cells (60). Other effects include inhibition of protein methylation (20), reduced RNA polymerase molecules and activity (164), increased ADP-ribose polymerase activity (191), and more recently discovered are its apoptotic effects on prostate cancer cells (32). NaB transcriptionally upregulated IGFBP-3 mRNA and protein levels in PC-3 and LNCaP cells in both dose- and time-dependent manners, with maximal induction after 2-h of treatment (234). NaB involves two distinct regions in the IGFBP-3 promoter in PC-3 and LNCaP prostate cancer cells. PC-3 cells are p53 null, implying NaB acts independently of p53 (234). It has been proposed that NaB increases IGFBP-3 expression in breast cancer cells by activating the IGFBP-3 promoter through a Sp1/Sp3 multiprotein complex (242).

Growth Stimulatory Agents

Growth hormone and IGF-1. In systemic models, IGF-1 and IGFBP-3 are correlated with GH secretion, as patients with GH deficiency (GHD) have reduced serum concentrations for all three proteins, and patients with acromegaly have increased levels (105, 107). Systemic GH administration corrects serum IGF-1 and IGFBP-3 concentrations in GHD (19), whereas IGF-1 treatment yields inconsistent effects. Chronic IGF-1 treatment of GH insensitivity increased serum IGFBP-3 in patients (130), whereas additional studies failed to show increases in IGFBP-3 concentrations (98, 144).

In a different systemic model, hypophysectomized rats have been used as a model to evaluate the relationship among IGFBP-3, GH, and IGF-1. Recombinant human GH (41, 259) and IGF-1 (41, 89) infusion induced a sevenfold increase in IGFBP-3 in hypophysectomized rats. In GH-deficient dwarf rats, GH or IGF-1 infusion induced IGFBP-3 mRNA levels in skin, whereas only GH infusion induced message levels in muscle and liver (153). Since changes in IGFBP-3 and IGF-1 occur in parallel, this suggests GH mediates IGFBP-3 induction in part by IGF-1 (41).

Furthermore, local GH and IGF-1 treatment caused a threefold induction of IGFBP-3 protein in media conditioned by human hepatocarcinoma cells in a dose-dependent manner (96). IGFBP-3 mRNA was increased as early as 6 h post-GH treatment, whereas mRNA levels increased 24 h post-IGF-1 treatment. Northern blots also showed that GH had an earlier transcriptional effect on IGFBP-3 mRNA compared with IGF-1. These observations suggested GH may act independently of IGF-1 when augmenting IGFBP-3 mRNA levels. To show that GH or IGF-1 did not affect IGFBP-3 through alteration of mRNA stability, the half-life of IGFBP-3 was measured to be 14–18 h, and this was unchanged by either treatment. Collectively, these results indicated that IGF-1 and GH are true regulators of IGFBP-3 transcription (96).

Epidermal growth factor. Marked reduction of IGFBP-3 levels were observed post-EGF treatment in ECE16–1 cells (human papillomavirus-immortalized cervical epithelial cells) (7). Also, addition of mitogenic EGF to basal human keratinocyte cells (HaCaT) resulted in a 20-fold reduction in IGFBP-3 mRNA (249). Thus epidermal mitogens such as EGF may increase local IGF-1 availability by inhibiting IGFBP-3 synthesis.

Posttranslational Modifications

Proteolysis. Metabolic demands appear to be a major determinant of IGFBP-3 proteolytic activity. For example, pregnancy, NIDDM, and recuperation after surgery or illness results in increased proteolytic activity. Significant IGFBP-3 proteases have been described in extravascular fluids, such as interstitial (252), synovial (68), follicular (50), and peritoneal fluids (119).

Increased IGFBP-3 proteolytic activity has been described in serum from pregnant women (5, 116). Analyses revealed that IGFBP-3 detected in serum obtained during the second month of pregnancy had reduced affinity for IGF-1 and IGF-2 (116). In addition, immunoreactivity to IGFBP-3 was detected at 30 kDa, while 41.5- and 38.5-kDa bands were not detected. These findings could most likely attributed to protease activity (116). Proteolytic modification of IGFBP-3 resulted in cleavage of the midregion, presumably by MMPs (metalloproteases) and serine proteases as determined in vitro (14, 78).

In addition, Western blotting of human pregnancy serum obtained from healthy volunteers during the third trimester revealed a single 30-kDa band and occasional bands at 16 and 20 kDa, whereas nonpregnancy serum contained IGFBP-3 immunoreactivity at 40–43 kDa and 30 kDa (5). Mass spectrometry and amino acid sequence analysis identified the 30-kDa proteolytic fragment obtained from pregnancy serum by the NH₂- and COOH terminus. The fragment corresponded to residues 1–212 of intact IGFBP-3, indicating cleavage occurred in the COOH terminal. The proteolytic fragment was reported to have an 11-fold decreased affinity for IGF-1 and 4-fold decreased affinity for IGF compared with intact IGFBP-3. Even if proteolytic fragments of IGFBP-3 have reduced affinity for IGF, these fragments may still inhibit both IGF and insulin-induced cell growth (141, 142).

The increased proteolytic activity during pregnancy is attributed to the intense cell growth and metabolism. Similarly, increased IGFBP-3 proteolytic activity was observed in serum from NIDDM patients (12). Proteolytic activity was inhibited by serine protease inhibitors, and the authors believed that the increased proteolytic activity in serum from NIDDM (and pregnant) patients resulted from reduced levels of protease inhibitors and not increased proteolytic activity per se (12, 14). Insulin regulated IGFBP-3 proteolytic activity in NIDDM serum, and proteolysis increases IGF bioavailability (14). In addition, IGFBP-3 proteolysis may counteract the resultant catabolic postsurgery state (52). It is believed that increased proteolysis is a compensatory mechanism that increases IGF bioavailability (53).

Prostate-specific antigen (PSA) and human prostatic kallikreins (hK2, hK3) are also capable of inactivating IGFBP-3 by proteolytic degradation. PSA is a serine protease found in seminal plasma that cleaves IGFBP-3 resulting in a marked reduction in binding affinity of the fragments to IGF1 but not

IGF-2 (43). hK2 and hK3 are IGFBP-3 proteases, with hK2 being more effective at lower concentrations (198). Heparin remarkably enhances the ability of hK3 (but not hK2) to degrade IGFBP-3, thus a combination of these molecules may influence IGFBP-3 and the proliferation of normal and abnormal prostatic cell (198).

Aspartic protease cathepsin D has been shown to cleave IGFBP-3, whereas depletion of cathepsin D attenuates proteolysis (47). Urokinase-plasminogen activator (uPA), tissue-plasminogen activator (tPA), plasmin, and plasminogen are capable of modulating IGFBP-3. uPA and tPA were used to proteolyze IGFBP-3 (13), and this may increase IGF-1 bioactivity. These plasminogen-related molecules have been used to dissociate IGF from IGFBP-3 in osteosarcoma cultures, whereas dissociation was inhibited with plasminogen activator inhibitor (PAI)-1 and uPA antibodies (31). Whereas IGFBP-3 binds plasminogen at the HBD, the IGFBP-3-plasminogen complex can also bind to IGF-1 with high affinity to decrease IGF-1 bioavailability (30). In addition, the human papillomavirus (HPV-16) E7 oncoprotein also enhanced proteolytic degradation of IGFBP-3 while proteolysis is inhibited by proteasome inhibitors (167). In summary, IGFBP-3 proteolytic degradation may be regulated through metabolic demands and results in modulation of its anti-apoptotic actions and bioavailability of IGF.

Phosphorylation. Phosphorylation is an important posttranslational modification of IGFBP-3 not only because IGFBP-3 is secreted as a phosphoprotein, but phosphorylation is a critical regulator of nuclear import of proteins (211). The phosphoprotein IGFBP-3 is constitutively phosphorylated at casein kinase 2 (CK2) sites (49). IGFBP-3 has two phosphorylation sites contained within consensus sites, Ser¹¹¹ and Ser¹¹³ (111). Dephosphorylation of these sites by CK2 appear to enhance its binding to ALS and to cellular surfaces (49, 111).

IGFBP-3 can be phosphorylated in vitro by CK and cAMP-dependent protein kinase (PKA) (49). Double-stranded DNA-dependent protein kinase (DNA-PK) can phosphorylate three potential sites in the nonconserved domain of IGFBP-3: Ser¹⁵⁶, Ser¹⁶⁵, Thr¹⁷⁰ (134). All three sites are highly conserved among human, bovine, pig, rat, and mouse IGFBP-3 (211). DNA-PK is present in both the cytosol and nucleus (251); however, it mainly acts in the nucleus (211). It is activated by DNA damage and may be involved in DNA strand repair, replication, gene transcription, and recombination of 2'-deoxy-sio-guanosine (iG) genes (227).

DNA-PK phosphorylation of IGFBP-3 was shown to reduce its affinity for IGF-1 and -2, whereas CK2, PKA, cdc2 (cyclin-dependent protein kinase), and MAPK (mitogen-activated protein kinase) had no effect on ligand binding in a binding assay (211). Interestingly, in vitro IGFBP-3 phosphorylation by DNA-PK increased IGFBP-3 nuclear import resulting in elevated maximal levels of nuclear accumulation, whereas other kinases failed to increase nuclear import. It is possible that DNA-PK acts in the cytosol to enhance IGFBP-3 nuclear import.

Furthermore, inhibiting IGFBP-3 phosphorylation at two of the three putative DNA-PK phosphorylation sites (Ser¹⁶⁵ and Thr¹⁷⁰) in 22RV1 cells with site-directed mutagenesis resulted in apoptosis identical to the wild-type IGFBP-3 (42). However, mutation of Ser¹⁵⁶ inhibited apoptosis and reduced nuclear accumulation. These results suggest that phosphorylation IGFBP-3 by DNA-PK regulates the growth inhibitory role

of IGFBP-3. Taken together, cumulative data suggest that IGFBP-3 phosphorylation can affect nuclear import, its binding affinity to other nuclear components, and growth inhibitory actions.

Glycosylation. Each of the three potential *N*-glycosylation sites in central region of IGFBP-3, Asn⁸⁹, Asn¹⁰⁹, Asn¹⁷² increases the molecular weight of IGFBP-3 by 4, 4.5, and 0–5 kDa, respectively (71). The absence of *N*-glycosylation at any site does not prevent its secretion from the cell. However, glycosylation may confer IGFBP-3 protease resistance by physically protecting susceptible sites or increasing the rigidity of the protein (72). The interaction of IGFBP-3 with cellular binding elements and IGFs has been speculated to involve negative charges on the targets. Charge reversal of the target significantly reduces binding affinity of the peptides, contributing to alterations in protein-protein, protein-cell, and cell-cell interactions (72). In addition, *N*-acetylgalactosaminyltransferase 14 (GalNAc-T14) was identified as an IGFBP-3 binding partner via yeast two-hybrid screens and glutathione-*S*-transferase (GST) pull-down assays (250). Interaction was further demonstrated with coexpression of IGFBP-3 and GalNAc-T14 in human embryonic kidney cells and co-immunoprecipitation. GalNAc-T14 is probably involved in *O*-glycosylation of IGFBP-3; however, there are no predicted *O*-glycosylation sites on IGFBP-3. Therefore, the function of GalNAc-T14 is unknown and whether the interaction has biological significance remains to be determined.

Of clinical significance, study of children with congenital disorders of glycosylation (CGD) shows incomplete glycosylation of ALS and IGFBP-3 is associated with lower levels of IGF-1, IGF-2, IGFBP-3, and ALS compared with controls. Ternary complex formation of IGF, IGFBP-3, and ALS was impaired in CDG. Linear growth was improved with partial normalization of ALS and IGFBP-3 glycosylation, demonstrating probable functional significance of IGFBP-3 glycosylation (175).

Ubiquitination. The ubiquitin-protease system serves as a proteolytic degradation pathway for IGFBP-3. Ubiquitination is achieved through successive actions of three enzymes: E1, E2, and E3. E1 forms an ubiquitin-adenylate intermediate, acting as a bond between the COOH terminal carboxyl group of ubiquitin and the E1 active site; E2 is an ubiquitin-conjugating enzyme that attaches ubiquitin moieties to the target protein; E3 functions as the substrate recognition module that recruits the target, guides the transfer of the ubiquitin from E2 to the targeted protein's lysine residues allowing for elongation of the polyubiquitin chain (189). During ubiquitination, E3 recruits targeted proteins for degradation, E2 attaches the ubiquitin marker to the target protein, and E1 forms the bond between the ubiquitin and the E1 active site. Once the substrate is ubiquitin-tagged for proteolysis, the substrate is fed into the catalytic core for proteolysis and recycling (110).

IGFBP-3 is targeted to the ubiquitin-protease pathway, where degradation of IGFBP-3 is dependent on E1 as modeled in a cell line that expresses a temperature-sensitive mutant of ubiquitin-activating enzyme (ts20b) (207). Polyubiquitination of IGFBP-3 requires nuclear localization because cytosolic IGFBP-3 has a longer half-life compared with nuclear IGFBP-3, suggesting greater proteolytic degradation of nuclear IGFBP-3 (207). Results of this study demonstrated that nuclear IGFBP-3 is highly polyubiquitinated at multiple lysine residues and ubiquitination can

be inhibited by specific 26S protease inhibitors that stabilize nuclear IGFBP-3. Importantly, the molecular mechanism of IGFBP-3 inactivation by human papilloma virus E7 is inactivation of growth-inhibitory IGFBP-3 (167) is via interaction with the NLS sequence (208). Figure 5 summarizes factors that modulate IGFBP-3 transcription, availability and action.

IGFBP-3 ACTION IN CELLULAR AND WHOLE BODY MODELS

Cancer

The IGF-IGFBP system has been implicated in the pathogenesis and progression of cancer (154). Multiple epidemiological studies have examined the relationship between serum IGF, IGFBP-3, and cancer incidence; however, results are controversial and are attributed to non-standardized laboratory methods (6, 199, 204). The majority of empirical IGFBP-3 research has shown potentiation or inhibition of cell proliferation (61). IGFBP-3 controls IGF availability in which IGFBP-3 maintains IGF-IR sensitization or slowly releases IGF-1 to its receptor, resulting in a growth stimulatory effect. Or, IGFBP-3 may bind IGF to inhibit interaction with its receptor. Another explanation for this discrepant action involves disruption of cholesterol-stabilized membrane complexes or fibronectin receptor antibodies, which consistently reverses IGFBP-3 apoptosis on breast cancer cells in vitro (27). IGF-dependent models show that IGFBP-3 is capable of inhibiting growth through sequestration of IGF-1. Additionally, in vivo animal models suggest that IGFBP-3 suppresses tumor formation and growth inhibition independent of IGF. This section will focus on these pro- and anti-proliferative actions in cellular and animal models.

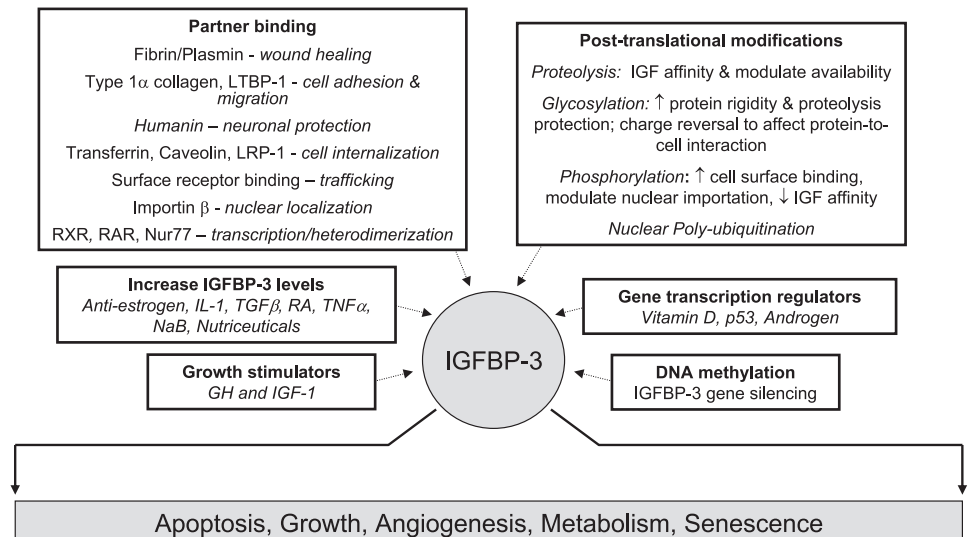
In vitro. IGF-DEPENDENT GROWTH STIMULATION. Although IGFBP-3 is most known for its growth inhibitory effects, IGFBP-3 has the capacity to maintain growth stimulatory effects. Preincubation of bovine fibroblasts with IGF-1 resulted in decreased IGF-R binding and subsequent decreased IGF-1-stimulated DNA synthesis (48). However, the addition of IGFBP-3 to this system during the preincubation blocked the decrement in receptor availability and maintained IGF-R sensitization. IGFBP-3 may enhance IGF-1 activity by slowly releasing IGF-1 to its receptor and inhibit IGF-R downregulation normally

caused by high free IGF-1 exposure (94). Since IGF-1 has a lower affinity for proteolyzed IGFBP-3 than IGF-R, it may act to preserve IGF-IR sensitivity secondary to IGF-1 binding.

IGF-DEPENDENT GROWTH INHIBITION. IGFBP-3 inhibited cell proliferation in human promyelocytic leukemia cells, whereas an IGF-1 analog with reduced affinity for IGFBP-3, [des-(1-3)-IGF-1] failed to attenuate growth (155). Thus IGFBP-3 sequestration of IGF-1 prevented IGF-R binding while des-(1-3)-IGF-1 could bind to IGF-R to preserve growth. Similarly, IGFBP-3 inhibited estradiol production in human granulosa cells when stimulated by FSH and IGF-1, whereas des-(1-3)-IGF-1 failed to produce the same effect in vitro (16).

IGFBP-3 can also be used to inhibit cell growth in cells resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR TKI). EGFR TKI blocked homo- and heterodimerization of phosphatidylinositol 3-kinase (PI3K) and Akt (63) to inhibit survival and cause apoptosis (231). However, some cancers acquire resistance to EGFR TKI (64). Thus IGFBP-3 can be used to inhibit activation of IGF-R and subsequent downstream activation of cell survival pathways. To illustrate this point, a cell line with wild-type EGFR gene amplification, A431, was used to induce acquired resistance to EGFR TKIs (A43-gefitinib resistant). IGFBPs are down-regulated in A43-gefitinib resistant cells and immunoprecipitation of conditioned media, and subsequent incubation with ¹²⁵I-labeled IGF-1 demonstrated substantially more intense IGFBP-3 binding in parental (gefitinib-sensitive) versus gefitinib-resistant cells, indicating lack of IGFBP-3 may contribute to increased activation of IGF-R pathway in gefitinib-resistant cells (101). Reintroduction of IGFBP-3 reversed the resistant phenotype. Only combination treatment of recombinant IGFBP-3 and gefitinib resulted in marked inhibition on cell growth in A431-gefitinib-resistant cells, whereas either treatment alone had no effect on cell growth (101). Clinical trials conducted in patients with non-small cell lung cancer orally ingested EGFR TKI and this prolonged patient survival was prolonged with improvements in disease-related symptoms and tumor regression (82, 138, 216). Furthermore, combination treatment of gefitinib and IGF-R-specific monoclonal antibody treatment in

Fig. 5. An overview of the regulation of IGFBP-3 transcription, availability and action. ↑, increase; ↓, decrease; RA, retinoic acid; NaB, sodium butyrate.



mice with EGFR gene amplification prevented tumor recurrence, whereas either treatment alone failed to inhibit tumor growth. The role of IGFBP-3 has not been investigated *in vivo* and whether combination treatment of IGFBP-3 and gefitinib would produce results of similar amplitude remains unknown.

IGF-INDEPENDENT GROWTH INHIBITION. Evidence suggests that IGFBP-3 affects cell proliferation through IGF-independent mechanisms (183). These independent effects were first manifest by transfection of an IGFBP-3 overexpression vector into cells lacking IGF-R. Overexpression of IGFBP-3 in murine fibroblast cells with disrupted IGF-R gene resulted in growth inhibition, whereas no effect was observed in wild-type cells. These results indicate that IGFBP-3 acts independently from the IGF-R to inhibit cell proliferation (236). In a different study, a 16-kDa IGFBP-3 fragment with total loss of IGF affinity inhibited IGF-1-stimulated DNA synthesis in a chick embryo fibroblast assay to the same extent as intact IGFBP-3. In addition, a 22/25-kDa fragment with 50-fold decreased affinity for IGF-1 compared with intact rhIGFBP-3 also reduced IGF-1-stimulated DNA synthesis by 50%. The fragment with weak affinity for IGF-1 is therefore a weak antagonist of IGF action, whereas the fragment with no affinity for IGF was able to abrogate IGF-stimulated cell proliferation (143).

Recombinant IGFBP-3 treatment resulted in DNA fragmentation and a dose-dependent induction of apoptosis in a p53 negative prostate cancer cell line (PC-3) (197). Furthermore, IGF-R-negative fibroblast cells derived from an IGF-R knockout (KO) mouse were treated with exogenous IGFBP-3 and increased apoptosis. Transfection of IGFBP-3 into IGF-R-negative murine fibroblasts demonstrated that DNA fragmentation was more prevalent in cells transfected with IGFBP-3, and not surprising, a substantial increase in apoptosis occurred (197). Apoptosis was only partially suppressed with exogenous IGF-1, and apoptosis was unaffected by addition of long (L)R3-IGF-1, an IGF analog that does not bind IGFBP-3 (197). Taken together, these data suggest IGFBP-3 is capable of inducing apoptosis in an IGF-I-independent manner.

Similarly, IGFBP-3 treatment on undifferentiated chondrocytes, a cell line that does not express IGF and IGFBP-3, resulted in a 40-fold increase in pro-apoptotic STAT-1 mRNA (232), whereas no increase in STAT-1 was observed with combination treatment of IGF-1 and IGFBP-3 or with IGF-1 alone. Upregulation of STAT-1 was also observed when chondrocytes were treated with a mutant IGFBP-3 incapable of binding IGFs, showing IGF-independent regulation of STAT-1. An antisense STAT-1 ODN abolished IGFBP-3-induced apoptosis independent from IGF action. Moreover, demonstration of STAT-1 nuclear localization and phosphorylation in response to IGFBP-3 treatment provides additional evidence of IGF-independent action (232).

Another mechanism of presumed IGF independence involves IGFBP-3 activation of a phosphotyrosine phosphatase, indicating IGFBP-3 can inhibit IGF-R activation to attenuate mitogenic signaling (200). Normally, tyrosine kinase activity leads to tyrosine phosphorylation of IRS-1 and IRS-2 to initiate PI3K and MAPK. However, IGFBP-3 stimulated phosphotyrosine phosphatase, inhibiting IRS-1 and IRS-2 signaling, and thus suppresses cell proliferation (200). Additionally, GGG-IGFBP-3, a mutant lacking intrinsic IGF binding affinity, suppressed several key players in the apoptotic cascade, including the c-Raf-MEK-ERK pathway and p38 kinase in rat

insulinoma cells in a dose- and time-dependent manner (40). These results suggest that IGFBP-3 inhibits cell growth through an IGF-independent pathway.

Data from a different study that employed a non-IGF binding IGFBP-3 mutant suggest that IGFBP-3 binding to a cell surface receptor or nuclear translocation into human breast cancer cells are not required for growth inhibition (28). Breast cancer cells (T47D) were infected with a mutant IGFBP-3-expressing adenovirus. These cells had reduced cell surface association and did not localize to the nucleus and still induced caspase-dependent apoptosis in T47D cells. Furthermore, incubation of mutant IGFBP-3 with vector-infected T47D cells with LR3-IGF-I had no significant effect on the ability of mutant IGFBP-3 to induce apoptosis. Thus this study showed that the IGFBP-3 mutant inhibited growth even with reduced cell surface binding and non-nuclear localization, suggesting IGFBP-3 may function in multiple pathways to inhibit growth in breast cancer cells (28) or that the mechanism of tumor suppression may be cell-type specific.

Apoptosis may also be mediated by IGFBP-3, RXR α , and Nur77. IGFBP-3 binds nuclear receptor RXR α , which is required for the apoptotic effects of IGFBP-3 (159, 161). RXR α heterodimerizes with orphan nuclear receptor Nur77 to assist translocation of Nur77 out of the nucleus. In the nucleus, Nur77 functions as a transcription factor to mediate cell proliferation, whereas in the mitochondria it mediates apoptosis. In fact, translocation of Nur77 into the mitochondria induced apoptosis in leukemia (260), lung (54), ovary (112), and colon cancer cells (246).

The relationship among IGFBP-3, RXR α , and Nur77 was confirmed utilizing RXR α ^{+/+} and RXR α ^{-/-} embryonic carcinoma cells. IGFBP-3 treatment reduced nuclear Nur77 in RXR α ^{+/+} cells, consistent with Nur77 translocation from the nucleus to mitochondria, whereas nuclear Nur77 was increased in RXR α ^{-/-} cells (150). Rapid mitochondrial translocation of Nur77 by IGFBP-3 was mediated in part by an IGF-R independent mechanism as IGFBP-3 still maintained its ability to induce apoptosis in IGF-R negative embryonic fibroblast cells.

A follow-up study confirmed that Nur77 and IGFBP-3 associate in the cytoplasmic compartment in prostate cancer cells (148). To illustrate that Nur77 translocation occurs *in vivo*, LAPC-4 xenografts were implanted in severe combined immunodeficiency (SCID) mice and after treatment with IGFBP-3 or saline, tumor sections were stained against Nur77. In tumors removed from mice treated with saline, Nur77 staining exhibited predominantly nuclear staining. However, tumors removed from mice treated with IGFBP-3 treatment had primarily cytoplasmic staining of Nur77. Collectively, these data present a role for IGFBP-3 as a multicompartmental signaling molecule.

IGF-INDEPENDENT GROWTH INHIBITION THROUGH OTHER EFFECTORS. IGF-independent mechanisms may also be regulated through other compounds. As reviewed earlier, RA (2, 97, 169, 215), TGF- β (168), TNF- α (196), and 1,25-OH₂D₃ (22, 170) can posttranscriptionally modify IGFBP-3 to potentiate its growth inhibitory effects. 1,25-OH₂D₃ can increase IGFBP-3 protein and mRNA levels through posttranslational gene regulation. Since addition of exogenous IGFBP-3 inhibited cell proliferation in LNCaP cells, it is interesting that antisense IGFBP-3 ODN or neutralizing IGFBP-3 antibodies abrogated the growth inhibitory effects of 1,25-OH₂D₃. IGFBP-3 upregu-

lated expression of p21/WAF1 protein to cause cell arrest, and IGFBP-3 neutralizing antibodies completely blocked 1,25-OH₂D₃ induced upregulation of the cyclin-dependent kinase inhibitory protein p21/WAF1. Thus these data suggest that IGFBP-3 is required for the anti-proliferative effects of 1,25-OH₂D₃ and p21/WAF1 may be responsible for the growth inhibitory effects (22).

IGFBP-3 is also able to modify the actions of these anti-proliferative agents. For example, treatment of ER-negative human breast cancer cells with RA or TGF- β ₂ resulted in significant growth inhibition and a concomitant twofold increase in IGFBP-3 protein and mRNA versus untreated controls (97). Exogenous IGFBP-3 treatment alone inhibited cell growth by 40%. However, antisense IGFBP-3 ODN inhibited RA- and TGF- β ₂-induced increase of IGFBP-3 protein and mRNA expression. As a result the anti-proliferative effects were attenuated with either treatment. Cell proliferation was not attenuated by sense IGFBP-3 ODN, suggesting that IGFBP-3 affects RA and TGF- β ₂-associated growth inhibition (97).

Pro-inflammatory cytokines such as TNF- α and IL-1 stimulate apoptosis via induction of DNA fragmentation (58). Antisense IGFBP-3 ODN blocked TNF- α and IL-1-induced DNA fragmentation in rat insulinoma and hamster insulin-secreting tumor cells, respectively (217). In a different study, antisense IGFBP-3 ODN also inhibited TNF- α apoptosis and DNA fragmentation in mesangial cells (237). IGFBP-3 antisense enhanced Akt phosphorylation (Thr³⁰⁸) while recombinant hIGFBP-3 inhibited Akt phosphorylation; this provides a mechanism by which IGFBP-3 inhibits cell survival.

TNF- α treatment increased IGFBP-3 expression in PC-3 cells in a dose- and time-dependent manner (196). The apoptotic response due to TNF- α was abolished with cotreatment of antisense IGFBP-3 ODN or IGFBP-3 neutralizing antibodies and inactivated survival protein Bcl-2. This indicated that IGFBP-3 and concomitant Bcl-2 inactivation is necessary for TNF- α -induced growth inhibition in PC-3 cells (196). These studies indicated that IGFBP-3 may regulate induction of pro-inflammatory cytokines and apoptosis in tumor cells.

Quercetin, a flavonoid found in grapes, green vegetables, and onions, induced apoptosis of PC-3 cells (240). This was accompanied with a decrease in IGF-1 and -2 and accumulation of IGFBP-3 in conditioned media. Also, anti-apoptotic Bcl-2 and Bcl-xL protein expression was significantly reduced, whereas pro-apoptotic Bax and caspase-3 expression were upregulated (240).

IGF-INDEPENDENT CELL SURVIVAL AND PROLIFERATION. The action of IGFBP-3 can be reversed with fibronectin and depletion of membrane cholesterol (27). IGFBP-3 normally induces apoptosis in breast cancer cells (Hs578T, MCF-7). However, IGFBP-3 action was reversed in non-IGF-responsive Hs578T cells when plated onto fibronectin such that IGFBP-3 enhanced cell survival and proliferation (171). Furthermore, when a specific antibody for the fibronectin receptor, α 5 β 1, was applied used in C2-ceramide induced apoptosis, IGFBP-3 reversed apoptosis to promote cell survival (27). IGFBP-3 also reduced C2-induced apoptosis in the presence of filipin, an agent used to deplete membrane cholesterol. Similar results were observed in the presence of nystatin, an agent used to disrupt cholesterol-stabilized complexes. IGFBP-3 intracellular localization was increased in the presence of nystatin,

indicating the reversal of the action of IGFBP-3 was not due to reduced IGFBP-3 entry into the cell.

In a different breast cancer cell line that has no detectable caveolin-1 (MCF-7), a SPD reversed C2-induced apoptosis in the presence of an agent that depletes membrane cholesterol M β CD (27). This finding was surprising because SPD (which lacks the region that interacts with transferrin) could affect MCF-7 cells even in the absence of caveolin-1. Caveolin-1 may facilitate actions of full-length IGFBP-3 but more interesting is the observation that fragments of IGFBP-3 can still exert similar actions. Furthermore, SPD action on cell proliferation is IGF independent because SPD is non-IGF binding. IGFBP-3 and its fragments such as SPD can either inhibit or promote cell survival; the action is dependent on the status of cholesterol-stabilized integrin receptor complexes (27).

In vivo. GROWTH INHIBITION. Mice bearing human prostate 22RV1 tumor xenografts were fed apigenin, a flavonoid present in parsley and celery (219). Daily oral intake of apigenin resulted in significant increases in tumor IGFBP-3 mRNA and apoptosis, concomitant with decreased tumor volume compared with control mice. An in vitro model was then employed to demonstrate that apigenin induces IGFBP-3. 22RV1 cells were treated with apigenin or an IGFBP-3 antisense ODN alone, or a combination of the two. Apigenin treatment resulted in a 41% decrease in the number of viable cells, increased apoptosis. Moreover, there was evidence of poly (ADP-ribose) polymerase cleavage, indicative of diminished DNA repair capacity. IGFBP-3 antisense ODN treatment resulted in a 9% increase in viable cells, and no evidence of poly (ADP-ribose) polymerase cleavage. The combination treatment reversed the apoptotic effects of apigenin by 31%, suggesting that growth inhibition caused by apigenin is related to IGFBP-3.

Continuous green tea polyphenol infusion for 24 wk substantially reduced IGF-1 and increased IGFBP-3 levels in the prostate of transgenic adenocarcinoma of the mouse prostate mice. Treatment was associated with attenuated protein expression of PI3K, phosphorylation of Akt, and significant inhibition of vascular endothelial growth factor (VEGF), uPA, MMP-2, and MMP-9, genes that promote angiogenesis (3). Similarly, dietary feeding of silibinin, the active component of flavonoid antioxidant silymarin (milk thistle extract) significantly inhibited tumor volume in DU145 tumor xenograft nude mice. Moreover, silibinin feeding before tumors were introduced also resulted in reduced tumor weight. These anticancer effects were attributed to increased plasma IGFBP-3 (224).

To examine effect of IGFBP-3 on angiogenesis, human CaP xenografts (22RV1) were established in SCID mice and mice were treated with either rhIGFBP-3 or saline over a 16-day period (160). There was a sevenfold increase in transferase-mediated nick end-labeling (TUNEL)-positive staining and caspase-3 staining in tumors of mice treated with IGFBP-3 versus mice treated with saline. CD31 staining in microvessels and endothelial cells was reduced by half in tumors treated with IGFBP-3 versus control, indicating IGFBP-3 suppresses intra-tumoral angiogenesis. Furthermore, cotreatment of IGFBP-3 and VEGF, a potent stimulator of vascular formation, inhibited VEGF-induced vessel formation in human endothelial cellular vessel formation in matrigel. Treatment with IGF-1 induced vascular formation, which is consistent with its angiogenic effects. Treatment with IGFBP-3 blocked the an-

giogenic effects of IGF-1 and treatment with LR3-IGF-1 partially blocked vascular formation, suggesting that a fraction of anti-angiogenic effects of IGFBP-3 were independent from IGF-1. In a different experiment, IGFBP-3 inhibited growth of new vascular vessels in chicken embryo chick allantoic membrane, illustrating that IGFBP-3 inhibits angiogenesis *in vivo*. The results of this study indicate that IGFBP-3 increases apoptosis and attenuates tumor growth and angiogenesis (160). These *in vivo* results are consistent with earlier *in vitro* work that showed IGFBP-3 induced rapid apoptosis in endothelial cells in an IGF-independent manner (79).

In contrast to the findings discussed above (160), Chang et al. (36) showed that IGFBP-3 exerts pro-angiogenic effects and promotes endothelial precursor cell migration in CD34⁺ (but not CD14⁺ monocytes), tube formation, and differentiation into endothelial cells. Injection of hematopoietic stem cells transfected with the plasmid-expressing IGFBP-3 into the vitreous humor of mouse pups attenuated vascular regression, increased vessel stabilization, and lead to quicker blood vessel development during hyperoxic insult. These angiogenic changes accounted for the reduced retinal ischemia in pups; these observations were absent in pups injected with an empty control vector. Pups treated with the IGFBP-3 plasmid showed early enhanced pro-angiogenesis and reduced anti-angiogenesis as measured with vascular blood vessel formation and preretinal neovascularization, respectively. Furthermore, pups treated with IGFBP-3-expressing plasmids had increased intraretinal vascular density and reduced preretinal vascular density in the same midperiphery region, perhaps indicating IGFBP-3 does not provide complete protection from hyperoxia-induced vessel regression or specificity of IGFBP-3 action.

Likewise, IGFBP-3 was also shown to stimulate neovessel formation in human endothelial cells (93). In this study, IGFBP-3 upregulated VEGF and matrix MMP-2 and -9. The discrepancy in IGFBP-3 action on cell growth may be model and local environment specific. For example, IGFBP-3 exerts pro-apoptotic effects in carcinoma models, whereas “healthy” cells exposed to hyperoxic conditions and IGFBP-3 have enhanced angiogenesis, neovascularization, and cell survival. Indeed, utilizing IGFBP-3 KO mice, IGFBP-3 suppressed retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth in a noncancer model (163). In addition, IGFBP-3 may have a role in hematopoietic stem cell and endothelial precursor cell function during vascular development (36).

IGF-INDEPENDENT GROWTH INHIBITION. To examine the effect of IGFBP-3 on tumor growth *in vivo*, SCID mice with LAPC4 xenografts were treated with either IGFBP-3, an RXR ligand (VTP 194204), a combination of the two, or saline for 3 wk (161). Although differences in gross tumor growth and apoptosis were absent or modest with either IGFBP-3 or the RXR ligand treatment, respectively, TUNEL assays indicated massive cell death with combination treatment. Combination treatment resulted in a 50% reduction in tumor growth and a 40% reduction in serum PSA levels. IGFBP-3 and RXR ligand in combination was essential to prevent tumor growth through induction of apoptosis (161). Interaction between RXR and IGFBP-3 presumably provides evidence for IGF-independent effects on tumor growth.

In another study, investigators aimed to differentiate whether the pro-apoptotic effects of IGFBP-3 are IGF dependent or independent. They bred the LPB-Tag mice (that have increased susceptibility to prostate cancer) with CMVBP-3 and PGKBP-3 mice (that have overexpression of IGFBP-3) and PGKmBP-3 (which express nonbinding IGF-1 mutant, I56G/L80G/L81G). The LPB-Tag mouse model represents the deletion of the simian virus 40 large T-antigen (Tag) under long probasin promoter (LPB), which is a murine cancer model that is androgen-related, prostate-specific, and of epithelial origin (131). The PGKBP-3 and CMVBP-3 model were developed by cloning human IGFBP-3 cDNA downstream of either the mouse phosphoglycerate (PGK) promoter or the cytomegalovirus (CMV) promoter, respectively, to result in IGFBP-3 overexpression (177). Whereas the I56G/L80G/L81G-mutant IGFBP-3 does not bind IGF-1, it still retains its IGF-independent apoptotic effects *in vitro* (220).

Overexpression of IGFBP-3 attenuated prostate tumor development in both LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice, with the latter having a greater effect (220). The rate of tumor growth was significantly lower in the LPB-Tag/CMVBP-3 mice versus LPB-Tag/PGKBP-3 mice. LPB-Tag/CMVBP-3 mice had greater transgene expression in prostate tissue compared with LPB-Tag/PGKBP-3 mice, but circulating IGFBP-3 levels were similar. Therefore, tumor growth retardation was mainly due to paracrine/autocrine effects of IGFBP-3 because LPB-Tag/CMVBP-3 mice had significantly slower tumor growth, higher transgene expression in prostate tissue, and similar circulating IGFBP-3 levels compared with LPB-Tag/PGKBP-3 mice (222). The LPB-Tag/PGKmBP-3 (non-IGF-binding transgenic) and LPB-Tag/WT had similar tumor growth. However, from 15 to 21 wk of age the PPB-Tag/PGKmBP-3 mice had a significant reduction in tumor growth. This suggests that early prostate tumor development in these mice is IGF-1 dependent and later development may be independent of IGF-1 (222).

Metabolism

In vitro. Chan et al. (35) showed that IGFBP-3 inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The investigators believed it was most likely dependent on GLUT-4 translocation but independent of IGF-1 and IGF-R action. Exposure to insulin alone resulted in a threefold increase of GLUT-4 translocation in adipocytes. However, exposure to insulin in conjunction with IGFBP-3 or IGFBP-3 NLS mutant caused significant reductions in GLUT-4 translocation to the plasma membrane, 39% and 35%, respectively. Whereas IGFBP-3 did not affect tyrosine phosphorylation of the insulin receptor, IGFBP-3 reduced insulin-stimulated Thr³⁰⁸ phosphorylation of Akt (but not Ser⁴⁷³ phosphorylation). These observations suggest Akt may be involved in IGFBP-3 inhibition of GLUT-4 translocation and insulin-stimulated glucose uptake (35).

In a different study, IGFBP-3 was also shown to inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes (133). However, in contrast to the previously mentioned study, IGFBP-3 inhibited insulin-receptor phosphorylation. In addition, TNF- α treatment induced endogenous production of IGFBP-3 in 3T3-L1 adipocytes, and IGFBP-3 inhibited adiponectin expression in mature adipocytes. This suggests that

during times of chronic inflammation and increased circulating TNF- α , endogenous production of IGFBP-3 would increase in adipocytes, resulting in a subsequent reduction in adiponectin production.

Treatment and cellular expression of IGFBP-3 inhibited adipocyte differentiation of 3T3-L1 fibroblasts, whereas an IGFBP-3 mutant unable to bind to nuclear receptors failed to elicit the same response (34). IGFBP-3 interfered with PPAR γ -dependent adipocyte differentiation while the mutant that does not bind RXR α or PPAR γ (a nuclear receptor known to dimerize with RXR α) did not block the ligand-induced transactivation of the PPAR response element. Using a His6 pull-down assay, IGFBP-3 bound strongly to His6-PPAR γ , whereas the mutant failed to bind PPAR γ . IGFBP-3 interaction with PPAR γ was further demonstrated with coimmunoprecipitation using 3T3-L1 lysates, demonstrating that PPAR γ is an *in vitro* binding partner of IGFBP-3 in adipocytes. IGFBP-3 was also shown to inhibit PPAR γ heterodimerization with RXR α *in vitro* (34).

Whereas TNF- α increased IGFBP-3 production in 3T3-L1 adipocytes (133), TNF- α blocked IGFBP-3 secretion from primary human muscle myoblasts to reduce myoblast differentiation (76). In addition, IGFBP-3 secretion into conditioned media increased during differentiation of myoblasts to myotubes. IGFBP-3 antisense treatment reduced differentiation and IGFBP-3 secretion into conditioned media, suggesting IGFBP-3 is necessary for differentiation of myoblasts.

In vivo. Increased IGFBP-3 proteolysis has been implicated in noninsulin-dependent diabetes mellitus patients, indicating that IGFBP-3 may be a key modulator in metabolic disease (12). Although initial reports suggested that IGFBP-3 overexpression in transgenic mice resulted in selective organomegaly and had little effect on metabolism (178, 179), use of a different promoter to overexpress IGFBP-3 resulted in dramatic metabolic effects in mice (177). Human IGFBP-3 cDNA was subcloned downstream of the mouse phosphoglycerate (PGK) promoter or the cytomegalovirus (CMV) promoter and transgenic (Tg) mice overexpressing IGFBP-3 were generated by injecting transgenes into pronuclei of fertilized CD-1 zygotes (177). Tg mice exhibited ubiquitous hIGFBP-3 mRNA expression, whereas wild-type (WT) controls had no hIGFBP-3 mRNA expression (221).

Silha et al. (221) reported that both transgenic mouse strains were lighter at birth and at 12 wk when compared WT, which corresponded to decreased body length. Liver and epididymal fat weights were heavier in CMVBP-3 Tg mice (but not PGKBP-3) mice while brain and kidney weights were reduced. Although the opposite findings would be expected in IGFBP-3 knockout (KO) mice, heavier liver (and quadriceps) weights were reported in 8-wk-old IGFBP-3 KO mice while body weights and body lengths were greater in KO mice (253). In a follow-up study, Silha et al. (221) reported that fasting blood glucose levels were significantly elevated in CMBVP-3 and PGKBP-3 Tg versus control mice, with CMVBP-3 Tg mice having the highest fasting glucose levels. CMVBP-3 mice (but not PGKBP-3) also had higher basal insulin concentrations when compared with WT. Similarly, in response to intraperitoneal glucose stimulation, glucose tolerance tests demonstrated that Tg mice had greater glucose concentrations over 180 min when comparing the areas under the glucose concentration-time curves. Tritiated 2-deoxyglucose uptake was mea-

sured under basal conditions, and CMVBP-3 Tg mice had reduced clearance from the circulation versus WT mice, whereas uptake into skeletal muscle and adipose tissue were also reduced (221). This indicates that overexpression of IGFBP-3 decreases glucose/insulin sensitivity at the periphery; however whether central (hepatic) insulin resistance existed in this model is unknown. In a recent report, IGFBP-3 KO mice had no observed differences in glucose concentrations during a glucose tolerance test compared with WT mice (253). This may indicate that IGFBP-3 contribution to whole body metabolism is complex and may reflect a scenario where genetic deletion of IGFBP-3 along with overexpression of IGFBP-3 can contribute to a similar metabolic phenotype.

To show that IGFBP-3 affects peripheral insulin action through central mechanisms, IGFBP-3 was infused into the third ventricle of the hypothalamus in male rats (180). Intracerebroventricular (ICV) infusion of IGFBP-3 significantly blunted insulin action at the liver during a hyperinsulinemic clamp. Under hyperinsulinemic conditions, IGF-1 infusion decreased the contribution of glycogenolysis to hepatic glucose production, whereas IGFBP-3 infusion resulted in a significant increase in glycogenolysis. In a healthy liver, insulin stimulation should reduce hepatic glucose production and glycogenolysis. Since IGFBP-3 infusion caused an increase in glycogenolysis, this indicates IGFBP-3 is associated with hepatic insulin resistance. Furthermore, IGFBP-3 infusion significantly attenuated peripheral glucose uptake while IGF-1 infusion marginally increased peripheral glucose uptake under hyperinsulinemic clamp conditions *in vivo*. This is in accordance with Silha et al. (221) who showed that IGFBP-3 overexpression reduced glucose uptake in skeletal muscle and adipose tissue. Collectively, these data indicate IGFBP-3 is associated with hepatic insulin resistance and decreased peripheral glucose sensitivity. Balancing IGF-1 and IGFBP-3 actions are paramount in maintaining glucose homeostasis (180). Importantly, this publication demonstrated the presence of IGF-1, IGF-R, and IGFBP-3 transcripts in the rat hypothalamus utilizing RT-PCR. A more recent publication also localized IGFBP-3 in murine and human neurons that secrete hypocretin, a neuropeptide associated with narcolepsy (113).

Ning et al. (182) reported that the generation of an IGFBP-3 null mouse had no obvious metabolic phenotype. Therefore, they generated a triple KO mouse (IGFBP-3, -4, -5) to study the role of IGFBPs in metabolism. The triple KO mouse had reduced body weights at birth and at 9 wk of age and reduced gonadal fat pad and quadriceps muscle masses when expressed relative to their body weight compared with their WT counterparts. Since MAPK stimulates cell growth, differentiation, and survival (80), decreased muscle weight may be attributed to decreased MAPK activation. Basal glucose levels were reduced by about ~30% in triple KO mice; blood IGF-1 bioactivity and levels were reduced in triple KO mice.

The triple KO mice also had higher insulin levels after challenged with glucose, and the increased insulin levels persisted for 30 min postglucose injection. The mice had increased beta cell area, accounting for increased insulin secretion postglucose challenge, which the authors believed enhanced glucose clearance postglucose challenge. The triple KO mice had reduced IGF-1 levels and since IGF-1 can inhibit insulin secretion *in vivo* (145), this may explain the increased insulin secretion in the KO. The KO mice most likely had increased

insulin activation of its receptor, which results in increased PI3K activity and formation of phosphatidylinositol-3 phosphate, leading to increased glucose uptake (83). In addition, the triple KO mice tended to have higher levels of phosphorylated AKT levels compared with WT, which may have resulted from increased insulin binding activity to its receptor. Although insulin kinetics in the triple KO mice were different (greater insulin secretion) from WT, interestingly the insulin tolerance test revealed that insulin sensitivity was not different between the triple KO and WT mice. The authors concluded that the triple KO mouse had postnatal growth retardation and increased insulin levels secondary to increased beta cell area.

Other Aspects of IGFBP-3 Action

Neuronal protection. Humanin was identified as a binding partner of IGFBP-3, with binding involving the COOH-terminal domain of IGFBP-3 in a region distinct from IGF binding (125). The 24-amino acid peptide is involved in protecting neurons from death associated with Alzheimer's disease and amyloid- β cytotoxicity (108, 233). IGFBP-3 is associated with humanin and antagonized its prosurvival effects in A172 glioblastoma cells. Interestingly, addition of IGFBP-3 potentiated humanin's rescue ability in primary murine cortical neurons from amyloid beta cytotoxicity and cell death.

Erectile dysfunction. Systemic infusion of IGF-1 or insulin results in vasodilation (15) and since IGF-1 is produced locally (57), it may be more functionally important in regulating local blood flow. Streptozotocin-induced diabetes in rats resulted in increased IGFBP-3 gene expression in major pelvic ganglia (MPG) by twofold; this response was absent in controls (1). Insulin alone or insulin plus free oxygen scavenger treatments were used to inhibit gene expression in diabetic MPG. Furthermore, a 10-fold increase in IGFBP-3 mRNA and IGFBP-3 transcripts of ~3, 2.6, and 1.5 kb were detected in penile tissue of diabetic rats but were absent in controls. This increase in IGFBP-3 could be corrected by insulin alone or insulin plus free oxygen scavenger treatments. Also, intracavernous pressure and IGFBP-3 mRNA levels in MPG and penile tissues were inversely correlated, indicating IGFBP-3 may be reducing local IGF bioavailability and inhibiting vasodilation. Similar findings were reproduced by a different group who also used streptozotocin-induced diabetes to increase IGFBP-3 expression in rats (228). Moreover, increased IGFBP-3 protein was reported to be localized to the epithelium of the urethra, penile endothelium, and smooth muscle in the corpus cavernosum. Thus hyperglycemia in combination with increased IGFBP-3 expression may have a role in the development of erectile dysfunction.

CONCLUSIONS

It seems that the context in which IGFBP-3 was first described to inhibit IGF-1 action (132) has focused its function to a carrier protein. Since this initial observation, IGFBP physiological actions of IGFBP-3 have been extensively studied, including 1) its endocrine and local regulation of IGF-1 availability and action on cell growth and metabolism, and 2) promotion of apoptosis in cellular and animal models through both IGF-1-independent and -dependent pathways. The latter has led to the development of anti-cancer therapies that promote cellular apoptosis through regulation of gene

transcriptional activity or co-administration of IGFBP-3 with preexisting therapies. IGFBP-3 binds to nuclear proteins including transcriptional factors. Because transcription factors have been localized to other intracellular organelles, this suggests that IGFBP-3 may also localize to other intracellular compartments and organelles. Studies showing cytosolic and nuclear localization of IGFBP-3 suggest that it has IGF-independent functions. Current research demonstrating IGFBP-3's IGF-independent roles in suppressing tumor formation and carcinoma cell growth have used autocrine/paracrine models. Although this in itself is not a novel concept, where local IGFBP-3 is able to modulate cell growth, the interplay between IGFBP-3 systemic and local regulation is a concept that may bear physiological significance. As IGFBP-3 has been shown to have a combination of IGF-dependent and -independent roles, this could perhaps reflect the interaction between the endocrine and autocrine/paracrine systems. To understand the role of IGFBP-3 in whole body physiology, the juxtaposition of systemic and local regulation of IGFBP-3 should be further investigated in both healthy and diseased models. Further exploration of IGFBP-3 employing this perspective may provide insight into the pathophysiology and therapy across a wide continuum of disease.

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