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Insulin-Like Growth Factor-Binding Proteins in Serum and Other Biological Fluids: Regulation and Functions*

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I. Introduction

THE insulin-like growth factors (IGFs) are growth-promoting peptides that share significant structural homology with insulin. However, unlike insulin, IGFs circulate in plasma complexed to a family of structurally related binding proteins. These are called IGF-binding proteins (IGFBPs). Although the existence of IGFBPs in circulation was suspected more than three decades ago, it was not until the mid 1980s to early 1990s that the six known IGFBPs[1] were cloned and sequenced (1–7). Early studies (8–12) in which human plasma was fractionated according to molecular size by gel filtration chromatography indicated that the IGFs migrated in high molecular weight fractions. Subsequently, Burgi *et al.* (12) and Hintz and Liu (13) demonstrated that the high molecular weight fractions containing the nonsuppressible insulin-like activity (NSILA) could be dissociated into smaller molecular mass fractions (5–10 kDa) under acidic conditions. These data suggested that the NSILA or somatomedin peptides were originally complexed with larger carrier proteins in plasma.

In subsequent studies, Zapf *et al.* (14) incubated plasma with radiolabeled IGF and detected the somatomedin activity at 40 kDa. This binding was highly specific for the IGFs with no competition from insulin, suggesting the presence of specific high-affinity binding proteins for the IGFs. Furthermore, Kaufmann *et al.* (15) showed that the half-life of ^{125}I-labeled NSILA was reduced when excess unlabeled NSILA was injected into normal rats, suggesting that NSILA is bound to carrier proteins. The binding of NSILAs to the carrier proteins in serum provided a possible explanation for the absence of insulin-like effects of endogenous NSILA *in vivo*.

The discovery of the six different binding proteins currently known to exist did not occur all at the same time (1–7, 16). The first three IGFBPs were purified in the mid-1980s. The association of [^{125}I]IGF in rat serum initially with a small molecular binding protein complex and a shift to a larger complex after a few minutes confirmed the presence of two binding proteins (14). The first was a major binding protein in serum identified to be a GH-regulated acid-labile 150- to 200-kDa complex eventually designated as IGF+IGFBP-

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[1] Oh *et al.* (349) have recently shown that purified recombinant human mac25 protein (termed IGFBP-7), which shares 20–25% identity with IGFBPs, binds IGFs but with several fold lower affinity than that of IGFBP-3.

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3+acid labile subunit (ALS) complex. The second was a GH-independent 50 kDa acid-stable protein (2, 17). Later it was shown that this 50-kDa small molecular binding protein consisted of IGFBP-1 and IGFBP-2. By the late 1980s and early 1990s, three additional binding proteins, IGFBPs 4 through 6, were isolated and characterized. All six binding proteins share ~35% sequence identity with each other. Several excellent reviews on the structure, molecular and cellular aspects, and biological actions of these binding proteins are available (1–7) and therefore will not be the focus of this review. The recent development of specific assays for measurement of various IGFBPs in circulation and in other biological fluids has led to significant new information on serum regulation of IGFBPs and their functions. The main focus of this review is to present the data regarding the characterization of the IGF-IGFBP complexes in serum and other biological fluids and to evaluate their regulation and functions.

II. Characteristics of the IGFBPs

First, a brief review of the general characteristics of the IGFBPs will provide the background information necessary to evaluate the functions of IGFBPs in serum and other biological fluids. For detailed information on this topic, several excellent reviews are available (1–7). IGFBPs are produced by a variety of biological tissues and found in various biological fluids (18–25). Although all six known IGFBPs belong to the same gene family, several features distinguish these IGFBPs from each other. The general characteristics of the six known IGFBPs are summarized in Table 1. IGFBP-1, a nonglycosylated protein of 30 kDa, was first isolated from mid-term amniotic fluid (20). This binding protein shares sequence identity with the placental protein 12 (19, 26), which is synthesized by endometrium and decidua. It is present in the amniotic fluid in concentrations 100–500 times higher than in serum. IGFBP-2, originally isolated from rat liver (BRL)-3A cell line (27), is a nonglycosylated protein of 31–36 kDa and is found in significant amounts both in serum and cerebrospinal fluid (1). The major form of binding protein present in human circulation is IGFBP-3; its molecular mass ranges from 38 kDa to 43 kDa depending on the number of sites glycosylated (28). In circulation, this glycoprotein is associated with an IGF molecule and an 80-kDa acid-labile subunit (ALS) to form a 150- to 200-kDa complex (2, 29, 30). This complex consists of IGFBP-3 and IGF-I + IGF-II in an equimolar ratio, suggesting that most of the IGFBP-3 in serum is likely to be saturated.

IGFBP-4, a nonglycosylated protein of 25 kDa and 32–36 kDa, was first isolated from medium conditioned by human osteosarcoma TE-89 cells (31) and from adult rat serum (32). Subsequently this nonglycosylated protein was isolated from a variety of cell types from different animal species and from human adult serum (1, 3). IGFBP-5 was first purified from adult rat serum, from human bone extract, and from medium conditioned by the U-2OS human osteosarcoma cell line as 29- and 23-kDa fragments (33–35). This binding protein was later purified as a 31-kDa fragment from the conditioned medium of T98G human glioblastoma cells (36) and a 22-kDa fragment from human cerebrospinal fluid (18). IGFBP-6 was purified as a 34-kDa fragment (37) from human cerebrospinal fluid and from transformed human fibroblast cell cultures (38).

Although the IGFBPs differ in their structure and binding specificity, it is not clear whether these differences contribute to functional differences among the various IGFBPs. For example, it is not known whether there is any functional significance for glycosylation of the IGFBPs and why some

Table 1. General characteristics of the human IGFBPs

	No. of amino acids	Core molecular mass (kDa)	Special feature	IGF affinity	Modulation of IGF action	Source in biological fluids
IGFBP-1	234	25.3	RGD	I = II	Inhibition and/or potentiation	Amniotic fluid, serum, placenta, endometrium, milk, urine, synovial fluid, interstitial fluid, and seminal fluid
IGFBP-2	289	31.4	RGD	II > I	Inhibition	CSF, serum, milk, urine, synovial fluid, interstitial fluid, lymph follicular fluid, seminal fluid, and amniotic fluid
IGFBP-3	264	28.7	N glycosylation	I = II	Inhibition and/or potentiation	Serum, follicular fluid, milk, urine, CSF, amniotic fluid, synovial fluid, interstitial fluid, and seminal fluid
IGFBP-4	237	25.9	2 extra cysteine	I = II	Inhibition	Serum follicular fluid, seminal fluid, interstitial fluid and synovial fluid
IGFBP-5	252	28.5	Extracellular matrix, hydroxyapatite binding	II > I	Potentiation	Serum and CSF
IGFBP-6	216	22.8	2 less cysteine O-glycosylation	II > I	Inhibition	CSF, serum and amniotic fluid

RGD, Arg-Gly-Asp.

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of the IGFBPs bind IGF-II with preferential affinity (39, 40) compared with IGF-I. Interestingly, none of the IGFBPs bind IGF-I with preferential affinity. In any case, the differences in structure (glycosylation, number of cysteine, RGD sequence), binding affinity, and tissue-specific expression are consistent with the general idea that different IGFBPs have discrete functions. Based on the findings that extracellular fluids of certain tissues (described in *Section IV*) are enriched with specific IGFBPs and that tissues surrounding the body fluid express the same IGFBPs in high abundance (41, 42), it is speculated that the IGFBPs may function locally to regulate IGF actions. However, there is no experimental data to demonstrate that this is in fact true.

III. Target Cell Actions of the IGFBPs

The main focus of this review is to present the data on the characterization of the IGF/IGFBP complexes in serum. However, at this time, we felt that a brief description of the target cell actions of IGFBPs would be beneficial to the reader to get a perspective as to the overall functions of these binding proteins in serum and other biological fluids. An earlier review by Jones and Clemmons (4) describes in detail the biological functions of individual binding proteins in various target cells. A summary of our current understanding of the biological actions of various IGFBPs is discussed below.

A. To modulate IGF actions

Studies in a number of laboratories including ours have shown that IGFBPs are capable of modulating IGF-induced cell proliferation both in a positive and negative manner (3, 4, 43–45). Several IGFBPs, including IGFBP-1, IGFBP-2, IGFBP-4, and IGFBP-6, inhibit IGF action by binding to IGFs and preventing the binding of IGFs to IGF receptors (3, 4). In contrast to the phosphorylated IGFBP-1 that inhibits IGF actions, the nonphosphorylated form of IGFBP-1 potentiates the effect of IGF-I on DNA synthesis in porcine smooth muscle cells (4). Coincubation of human fibroblasts with IGF and IGFBP-3 showed an inhibitory effect while preincubation with IGF had growth-potentiating effect (43). It was suggested that binding of IGFBP-3 to the cell surface reduces its affinity for IGF-I and results in a potentiating effect (44). In contrast to other IGFBPs, IGFBP-5 is stimulatory for a variety of cell types (45–48).

Thus, the different binding proteins may modulate IGF action differently, and the same binding protein can have an IGF-inhibiting or potentiating role under different conditions. The factors that determine these differences include IGFBP phosphorylation, IGFBP proteolysis, and IGFBP cell surface association, among others. These variables may modulate IGF action in target tissues by altering the binding affinity of the IGFBPs to IGFs.

B. To facilitate storage of IGFs in extracellular matrices

Another important role of IGFBPs may be to help in the storage of IGFs in the extracellular matrices of certain tissues. In this regard, Jones *et al.* (45) provided evidence for fixation of IGFs via IGFBP-5 binding to extracellular matrix proteins. We found evidence that IGFBP-5 may help fix IGFs in bone since the complex of IGFBP-5 and IGFs, but not IGFs alone, bind to hydroxyapatite (34, 49). In terms of the significance of fixation of IGFs in extracellular matrices such as bone, it is speculated that the stored IGFs may be released during the osteoclastic bone resorption phase of bone remodeling to stimulate nearby osteoblasts during the bone formation phase of remodeling (50). Similarly, IGFs stored in extracellular matrices of soft tissues may have a role in wound healing.

C. To exert IGF-independent effects

Recent evidence suggests that some of the IGFBPs may mediate their effects on target cells by an IGF-independent pathway. This concept has evolved from a number of experimental studies, including the study by Jones *et al.*, which found that IGFBP-1 stimulated smooth muscle cell migration by an IGF-independent mechanism involving integrin receptors (51). IGFBP-3 has been shown to inhibit proliferation of breast and prostate cancer cells by a cellular signaling pathway independent of IGFs (52, 53). In addition, Rajah *et al.* (54) have recently shown that IGFBP-3 induces apoptosis of the p53-negative prostate cancer cell line, PC3, through a novel pathway independent of either p53 or the IGF-IGF receptor-mediated cell survival pathway. Consistent with the idea that IGFBP-3 may have IGF-independent effects on certain types of cells, two recent reports have provided evidence for nuclear localization of IGFBP-3 (55, 56). The significance of this finding is not clear. However, this exciting finding may clarify the direct intrinsic actions of some of the IGFBPs on cells. We and others have found evidence that IGFBP-5 may promote cell proliferation in osteoblasts, possibly through putative cell surface-binding sites (46, 47). Although studies from a number of laboratories support the possibility that IGFBPs may have IGF-independent effects in certain cell types, further experimental evidence is needed to verify this mode of IGFBP action.

Thus, the explosion of IGFBP research during the past several years has provided evidence that IGFBPs may have both IGF-dependent and IGF-independent actions. Based on the complexity of IGFBP functions, it is clear that we cannot fully appreciate the significance of changes in IGFBP levels in serum and local body fluids until we know more about the functions of these IGFBPs.

IV. IGF-IGFBP Complexes in Biological Fluids

A. Serum

As mentioned above, the major pool of IGFs circulate in human serum as 150- to 200-kDa complexes (28, 57–59). In addition to the large molecular mass complex, two other pools of IGFs exist in serum, the free and the 50-kDa IGF pool. Hardouin *et al.* (60) were the first to characterize the different IGFBPs present in adult human serum. These authors found evidence for the presence of five different molecular forms of IGFBPs in human serum and showed that the various IGFBPs were distributed in two complexes in the serum, the 150- to

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200-kDa complex primarily containing the GH-dependent IGFBP-3 (61, 62) and the 50-kDa complex consisting of other forms of IGFBPs (1).

Figure 1 shows the relative distribution of various IGF pools in human serum. In circulation, about 75–80% of the IGFs are complexed to IGFBP-3 and the acid labile α-subunit to form the 150- to 200-kDa complex (57, 59, 63). This is possible because under normal conditions, the total IGFs and IGFBP-3 in serum are in equimolar concentrations (63). A smaller percentage (20–25%) of the IGFs are associated with low molecular mass IGFBPs (57). Less than 1% are found in the free form in circulation (64, 65).

Studies by Baxter, Martin, and colleagues (28, 58) and Guler *et al.* (57) to identify the constituents of the 150- to 200-kDa complex led to the purification of two components of the complex from human serum: an acid-stable glycoprotein of 38–43 kDa (IGFBP-3) with IGF-binding activity and an acid-labile glycoprotein of 85 kDa that does not bind to the IGFs (Fig. 2). Subsequently, the reconstitution of the 150- to 200-kDa complex was achieved by using purified acid-labile subunit (ALS or α-subunit), IGFBP-3 (β-subunit), and IGF-I or IGF-II (δ-subunit). The role of ALS appears to be to increase the molecular mass of the IGF+IGFBP-3 complex so that the access of the circulating IGF to the extracellular fluid and thus to the various tissues is limited (57, 58).

Baxter and co-workers (66–69) have shown that the affinity constant for the formation of IGF-IGFBP-3 complex ($2–3 \times 10^{-10}$ M) is considerably higher than the affinity of the ALS for the IGF-IGFBP-3 heterodimer (5×10^{-8} M). They have demonstrated that human IGFBP-3 injected into normal rats is distributed within a few minutes between a 50-kDa and a 150- to 200-kDa complex. ALS circulates in its free form 2- to 3-fold molar excess (30) with respect to its ternary complex and hence is readily available for complex formation. However, since very little unbound IGF-I is present, some other source for IGF must be available for ternary complex formation to occur. It is suggested that there may be a rapid exchange between IGF-I associated with the small molecular mass binding protein complex (50 kDa) and the 150- to 200-kDa complex. There is also the possibility that the IGF-I may be associated with the cell surface or lining capillaries and dissociated by the high-affinity IGFBP-3 (59). The stabilization of IGFs occurs when it binds to the IGFBP-3 and then forms a complex with ALS (59). In IGF-deficient animals, either with diabetes (66) or GH deficiency (GHD) (70), ternary complex formation is impaired, but restored to normal after IGF is injected along with IGFBP-3. Based on these data, Baxter (58) proposed that ALS association with IGFBP-3 requires the presence of IGFs.

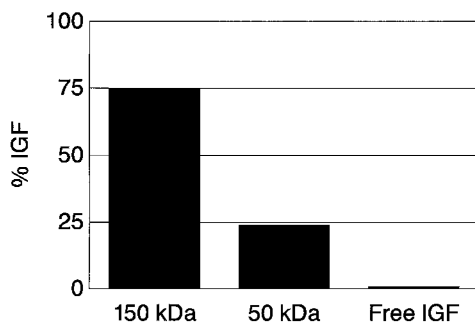

FIG. 1. Relative distribution of various IGF pools in human serum. The distribution of IGFs between the 50-kDa, 150-kDa, and the free pool, as determined before and during continuous subcutaneous infusion of 30 mg/day of rhIGF-I in healthy men (82).

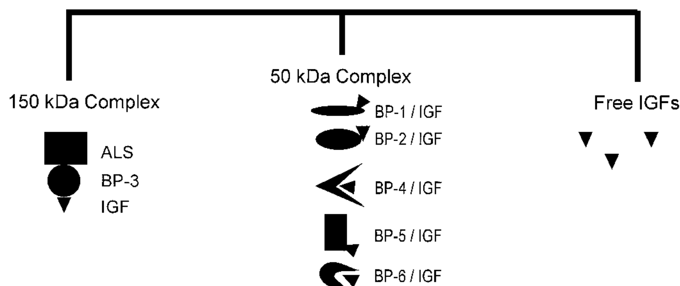

FIG. 2. Proposed model of the forms in which IGFs circulate in human serum. The 150-kDa complex consists of 7.5 kDa IGF-I or IGF-II plus 38–43 kDa IGFBP-3 and a 80- to 90-kDa non-IGF-binding acid-labile component called ALS. The 50-kDa complex consists of IGF-I or IGF-II bound to one of the remaining five IGFBPs.

The question of whether ALS can form a binary complex with IGFBP-3 in the absence of the IGF ligand is controversial at this time based on recent reports by Barreca and colleagues (71–73) and Lee *et al.* (74). Barreca *et al.* (71) demonstrated that incubation of recombinant human IGFBP-3 and ALS resulted in the appearance of a 150- to 200-kDa complex in the absence as well as in the presence of IGF. They also showed that ALS binding to IGFBP-3 increased the affinity of IGFBP-3 to IGF-I, possibly by inducing conformational changes in IGFBP-3. Based on these results, the authors speculate that ALS may play an important role in regulating the affinity of IGFBP-3 to IGF-I, thus regulating the levels of free IGFs. In addition, Yang *et al.* (75) observed that [^{125}I]IGF-II readily bound to the 150-kDa fraction of adult rat serum to sites with a higher affinity for IGF-II than IGF-I. In subsequent studies, Lee and Rechler (76) demonstrated two different IGFBP-3 complexes in the 150- to 200-kDa fraction of the adult rat serum, one with similar affinity for IGF-I and -II and the other with greater affinity for IGF-II (77). The latter complex is formed from proteolytically nicked IGFBP-3 that is present in the native serum before acidification. The proteolytic cleavage in IGFBP-3 decreases the affinity of the IGFBP-3-ALS for IGF-I and increases the binding of IGF-II. Similar proteolytic nicking of IGFBP-3 occurs during human pregnancy, changing the binding specificity for IGFs (78). In contrast to these results, Baxter and co-workers (79) demonstrated that proteolyzed IGFBP-3 from maternal serum can bind to IGFs and form a ternary complex with ALS with normal affinity. Thus, they speculated that the altered binding affinity of the IGFBP-3 fragment during Western ligand blotting is an artifact resulting from breakage of a labile peptide bond after prolonged acidification or exposure to SDS.

If human IGFBP-3 must first bind to IGF-I before it can form the ternary complex, then the amount of IGF-I associated with the 150- to 200-kDa complex in rats injected with hIGFBP-3 should be twice that of normal rats. However, Lee *et al.* (74) did not observe an increase in the mobilized IGF and thus concluded that IGFBP-3 and ALS can form a binary complex independent of IGF both *in vivo* (80) and *in vitro* (71, 76). Thus the question of whether IGF is required for the

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formation of a complex between IGFBP-3 and ALS remains controversial. One possibility is that there may be two IGFBP-3 pools, one with a higher binding affinity for ALS after first binding to IGF, and the second, which binds ALS even in the absence of IGF but with lower affinity. With differences in binding affinities, the functional roles of ternary complexes of ALS+IGFBP-3+IGF and binary complex of ALS+IGFBP-3 may also be different. Future studies are required to elucidate the extent to which IGFBP-3 forms a binary complex with ALS and if so, whether these binary complexes play a physiological role in modulating the actions of IGF.

One of the proposed functions of plasma IGFBPs is to increase the half-life of IGFs in circulation. When IGF-I and IGF-II are injected into normal rats, they bind to IGFBP-3, increasing their stability and half-life to 4 h compared to 20 min in hypophysectomized rats (81). Guler *et al.* (57) determined the half-lives of free and IGFBP-bound [^{125}I]IGF-I and -II after bolus injection of the tracers in two normal adults. Apparent half-lives of [^{125}I]IGF-I and -II in each of the three IGF serum pools (150 to 200 kDa, 50 kDa, and free IGF), calculated from the respective disappearance rates of the tracer, are shown in Fig. 3. These results demonstrate that the 150- to 200-kDa complex is responsible for the relatively long half-life of IGFs and that the 50-kDa and the free IGF pool have a rapid turnover and account for most of the daily IGF production (82). Another important role of IGFBP binding to IGF is in modulating IGF action. The ternary complex does not permeate the capillary endothelial barrier, but the smaller IGF-IGFBP complexes can easily do so and facilitate tissue-specific IGF action (3, 4, 57, 59). On the other hand, the endocrine actions of IGFs bound to IGFBP-3 may be achieved by specific proteolytic enzymes that dissociate the ALS+IGFBP-3+IGF complex, and it is suggested that this may increase the bioavailability of IGFs. An additional mechanism altering IGF bioavailability has been proposed by Yamamoto and Murphy (83). They identified the presence of a protease in rat serum that cleaves IGF-I into des(1–3)IGF-I. Since this form dissociates from the binding proteins easily, it may serve to increase IGF bioavailablity. The role of proteases in modulating IGF bioavailability is discussed in detail in *Sections VIII* and *IX*.

Different forms of IGFBPs have been identified in a variety of biological tissues other than serum. Although their exact source and role are not clearly known in many of these systems, if the production of these binding proteins is tissue-specific, it could imply a local paracrine or autocrine role for IGFBPs in regulating IGF-I and -II actions. The binding proteins identified in the other biological fluids are discussed in the section below.

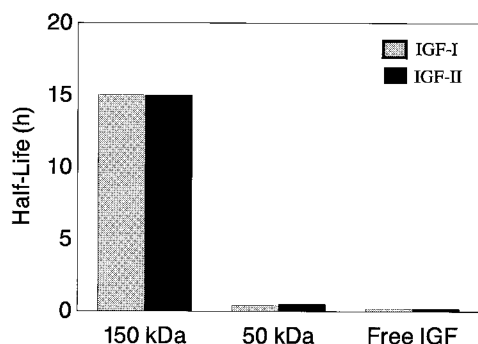

FIG. 3. Apparent half-lives of various IGF pools in human serum. Apparent half-lives of [^{125}I]IGF-I and [^{125}I]IGF-II in each of the three IGF serum pools (free, 50-kDa, and 150-kDa) were calculated from the respective disappearance rates of the tracer after intravenous bolus injection (82). Values are mean estimations from two healthy men.

B. Milk

Human milk contains IGFBP-1, -2, and -3 (84), the functions of which remain unclear. In addition, IGFBP-4 has been identified in rat, porcine, and bovine milk (85, 86). As with serum, IGFBP-3 is the major binding protein of IGFs in milk. Although maternal serum is the source of rat milk IGFBP-3 (21), the 150- to 200-kDa complex is not translocated from serum into milk. It is suggested that IGFBP-3 may enter milk from circulation in the free form or complexed to IGF-I. On the other hand, IGFBP-2 and -4 are produced locally by the mammary gland as shown by expression of their respective mRNA in the mammary tissue (21). IGFBP-1 in human milk (87) parallels the level of IGF-I in that immediately after birth, both milk IGF-I and IGFBP-1 decline. The exact role of IGFBPs in milk remains to be explored, but it is possible that they protect against the degradation of milk IGF-I or that they modulate the local mitogenic activity of the IGFs.

C. Urine

IGFBP-1, -2, and -3 have been detected in healthy adult urine by Western ligand blot analysis (25), and the concentration is approximately 3 orders of magnitude less than that in serum (88). Previously, IGFBP-2 was shown to be the predominant form in dialyzed adult urine, but when urine is not dialyzed, IGFBP-3 is the major binding protein (88). The reason for this discrepancy is not known. It appears that urinary IGFBP-3 originates mainly from the kidney and/or the urinary tract and (89), unlike the serum, is not found as a 150- to 200-kDa complex in the urine. Quantification by RIA show that urinary IGFBP-3 is age dependent, with an increase at approximately age 9–11 yr that corresponds to the pubertal rise in serum IGFBP-3 and is followed by a decline until it plateaus at approximately age 26 yr (89). Although the antiserum used in the RIA recognizes both intact and proteolyzed fragments of IGFBP-3, there is no evidence for the presence of urinary protease in normal individuals from age 4–45 yr (25, 89). It is suggested that urinary IGFBPs may have diagnostic utility, but this has not been established.

D. Cerebrospinal fluid (CSF)

Although several different binding proteins have been identified in human CSF, IGFBP-2 is the major form present (22). The CSF contains high concentrations of IGF-II (90), which binds IGFBP-2 with 10- to- 20-fold greater affinity than IGF-I (91). Also purified from the CSF is IGFBP-6, which is present at slightly lower concentrations in the CSF than serum (92), but also has a preferential affinity for IGF-II over IGF-I. Both IGFBP-3 and -5 have been identified in the CSF at lower concentrations than IGFBP-2 and -6. It is suggested that the IGFBPs found in the CSF may be synthesized locally

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by glial cells and neurons and not derived from plasma by crossing the blood-brain barrier (93). A 30-kDa IGFBP, corresponding to IGFBP-2, was demonstrated in rat CSF (94). By analogy with other transport proteins synthesized by the choroid plexus, it is suggested that this IGFBP may facilitate the secretion of IGF-II to the CSF and modulate its biological action at distant sites within the brain (94). In a number of disease states related to the central nervous system (CNS) (95, 96), changes in IGFBP concentrations have been documented, suggesting a possible diagnostic utility for the measurement of these binding proteins. Thus IGFBPs are thought to modulate the biological actions of IGFs (39, 94, 97) including a possible regulatory role in growth and differentiation of the CNS. However, the mechanisms remain to be investigated.

E. Follicular fluid

IGFBPs 1–4 (41, 98–104) were identified by Western ligand blotting in human follicular fluid, suggesting that the regulation of IGF action in the ovary is probably under the control of regulatory binding proteins (98). In women with normal menstrual cycles, after ovulation, progesterone stimulates the endometrium to release IGFBP-1 (105), which mediates the cell differentiation effects of IGF-I on the endometrium. IGFBP-1 is synthesized by granulosa cells and is secreted into the follicular fluid (101, 103, 106–109), where the concentration is 4- to 5 times higher than that found in the serum (110). The preovulatory rise in serum IGFBP-1 is not regulated by insulin or ingestion of a meal, nor is it associated with diurnal variation (111). This implies that during the preovulatory phase, IGFBP-1 detected in serum is primarily of follicular origin. IGFBP-1 is thought to inhibit the biological activity of free IGF on androgen-producing theca cells, since that might lead to atresia and anovulation (112).

Changes in the various IGFBPs during atresia and follicular growth have been reported. The levels of IGFBP-2 and -4 are higher in atretic follicles (99, 113) compared with healthy developing follicles of serum, suggesting a role for them in inducing atresia. A decrease in proteolytic activity degrading IGFBP-3 and an increase in IGFBP-2, -4, and -5 protease were observed during follicular growth in ovine follicular fluid, and an increase in IGFBP-3 protease and a decrease in IGFBP-4 and -5 protease were observed during atresia (100, 101, 114, 115). These observations suggest that changes in intrafollicular IGFBP proteolytic activity could be responsible in part for the changes in IGFBP levels seen during growth and atresia (114). Since the expression of various IGFBPs is altered during follicular development and atresia, it is speculated that the changes in IGFBP levels may regulate follicular growth by modulating the local IGF bioavailability.

F. Amniotic fluid

The binding protein isolated from amniotic fluid (AFBP) is a small molecular mass binding protein that is both heat and acid stable (42, 116) and was later identified to be the same as IGFBP-1. It is the major IGFBP in the amniotic fluid and is present in concentrations 100–500 times higher than that found in the serum (117). During pregnancy, a surge in the concentration of amniotic fluid IGFBP-1 reflects its local production in decidual tissues (42, 118). The amniotic fluid IGFBP-1 contributes to the increase in serum IGFBP-1 levels during the second trimester of pregnancy (117). IGFBP-1 is twice as high in preterm amniotic fluid as in term amniotic fluid, suggesting a role for this binding protein in growth and development.

Immunoreactive IGFBP-3 is present in amniotic fluid but at a much lower concentration than that in serum. Western ligand blot analysis of amniotic fluid failed to reveal evidence for the presence of IGFBP-3 in amniotic fluid. This could be due to the presence of IGFBP-3 protease capable of degrading intact IGFBP-3 into fragments that do not bind [^{125}I]IGF tracer. Consistent with this interpretation, incubation of amniotic fluid with radiolabeled IGFBP revealed the presence of a protease(s) specific for IGFBP-3, -4, and -5 (119). These proteases alter the binding affinity of the IGFs for their binding proteins and thereby could modulate the bioactivity of IGFs (119). The role of IGFBP-6 in the amniotic fluid is not yet known, although the levels present are similar to those seen in serum (92).

G. Lymph

The concentration of both IGFs and IGFBPs in lymph are lower than that found in serum (120, 121). Using gel filtration chromatography, it was shown that the IGFBPs present in lymph eluted in the 40- to 50-kDa size range. The finding that little IGF activity eluted as a 150- to 200-kDa complex from lymph is consistent with the fact that the IGF+IGFBP-3 complex does not cross the capillary endothelial barrier. IGFBP-2 is believed to be one of the major binding proteins in the lymph tissue and may originate from both the serum and surrounding local tissues (3).

H. Seminal fluid

IGFBP-1-like immunoreactivity was detected in human seminal plasma (122), with levels similar to those found in human adult serum. Intact IGFBP-3 could not be detected in seminal fluid by Western ligand blot analysis, but Western immunoblot analysis using IGFBP-3 antiserum revealed the presence of immunoreactive IGFBP-3 fragments (122). Since the amounts and ratio of these binding proteins do not correlate with those present in the serum, it is likely that the source of these proteins are specific to the cell population within the local tissues, such as Sertoli cells. Human seminal plasma also contains intact IGFBP-2 and IGFBP-4, while IGFBP-3 is present in the fragmented form (123–126). The prostate-specific antigen (PSA) has proteolytic activity for not only IGFBP-3, but also for IGFBPs -4 and -5. In addition to the PSA protease, an IGFBP-5-specific protease has been identified in seminal plasma (125–127). Since IGFBP proteolytic activities in seminal fluid from normal volunteers, vasectomized patients, or patients with idiopathic azoospermia were not significantly different, the role of IGFBPs and IGFBP proteases in the male reproductive system and male infertility remains to be established.

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I. Other biological fluids

The presence of IGFBPs 1 through 4 has been detected in interstitial fluid obtained from human skin blisters caused by high negative pressure in healthy volunteers (128). The IGFBP-3 concentration was lower than that present in the circulation and was due to increased IGFBP-3 protease activity. Several IGFBPs have been identified in vitreous and aqueous humors (129), but the predominant serum carrier protein IGFBP-3 was not detected in these fluids. This may be due to the presence of increased amounts of IGFBP-3 protease activity in vitreous and aqueous humors (129). Vitreous humor from diabetics had a higher amount of IGFBP-3 proteolytic fragment compared with healthy controls, suggesting that the rate of IGFBP-3 proteolysis is different in vitreous humor of normal and diabetic individuals. Western ligand blotting and immunoprecipitation of normal synovial fluid revealed the presence of IGFBPs 1 through 4, with levels higher in synovial fluid of patients with rheumatoid arthritis (130) compared with controls. These findings suggest that understanding the normal IGF/IGFBP axis in physiological states and the alterations that occur in pathological conditions may provide clues to our understanding of the pathophysiology of different disease states.

Although the enrichment of certain biological fluids with one or more IGFBPs, together with the increased expression of the same IGFBP in the local tissues surrounding the body fluid (Table 2), suggests that these IGFBPs may function locally to regulate IGF actions, more work is required to understand the specific role of these binding proteins in modulating IGF action in various biological fluids.

V. Assays for Circulating Levels of IGFBP

A. Western ligand blotting

Western ligand blotting, originally developed by Hossenlopp *et al.* (131), has been used extensively in detecting the different IGFBPs in biological samples. This technique involves transfer of proteins to nitrocellulose after separation by SDS-PAGE, hybridization with radioligand tracer, and, ultimately, autoradiography. Although not very quantitative or specific, this technique allows for identification of the IGFBPs differing in molecular mass by as little as 1 kDa. Western ligand blotting gives a measurement of the intact IGFBP (5, 25) since the IGFBP proteolytic fragments do not bind IGFs or bind with reduced affinity. Although Western ligand blotting can be used to quantitate certain IGFBPs with more accuracy than others, this assay is not highly quantitative for many of the IGFBPs for the following reasons: First, the transfer of the IGFBPs during electroblotting technique is not always complete and may vary for different IGFBPs (132). Second, the sensitivity of detection is different for the various IGFBPs. For example, IGFBP-6 at lower concentrations could not be detected by Western ligand blotting (133, 134). The reason for the poor detection of IGFBP-6 by ligand blotting may be due to either incorrect folding of IGFBP-6 after transfer to nitrocellulose or the close proximity of the epitopes for the IGF ligand and nitrocellulose in IGFBP-6, which could hinder nitrocellulose-bound IGFBP-6 when binding to IGF tracer (134). Third, it is difficult to accurately quantify certain IGFBPs (*e.g.*, IGFBP-5) by ligand blotting since the molecular masses of glycosylated and nonglycosylated forms of several IGFBPs are too similar for proper separation (3). Despite the above problems, Western ligand blotting has been widely used by researchers in the IGF field and has proved to be an invaluable tool for detection and characterization of the molecular size of the various IGFBPs present in biological fluids.

TABLE 2. Distribution of IGFBPs in biological fluids

Fluid	Major IGFBP
Serum	IGFBP-3
Amniotic fluid	IGFBP-1
Follicular fluid	IGFBP-3
CSF	IGFBP-2, IGFBP-6

B. Western immunoblotting

Antibodies specific for a IGFBP can be used to quantitate IGFBPs in a conventional Western immunoblotting, after size separation by SDS-PAGE. Western immunoblotting can be improved by optimizing protein transfer, antibody binding, and detection systems (*e.g.*, chemiluminescence). In general, immunoblot analysis using polyclonal antiserum usually detects both intact and fragmented forms of the IGFBPs (25). For example, when pregnancy sera were analyzed using the Western ligand blot technique (25, 135), there was no evidence of IGFBP-3, while both immunoblot and RIA detected the presence of fragmented IGFBP-3 that arose from protease activity. In subsequent studies, Baxter and co-workers (79) demonstrated that the proteolyzed IGFBP-3 fragment from maternal serum can bind IGF with normal affinity and that the lack of detection by Western ligand blot analysis is an analytical artifact resulting from using [^{125}I]IGF-I for binding. Another example of the usefulness of the immunoblot assay was shown in diabetics. Patients with untreated insulin-dependent diabetes mellitus (IDDM) showed lower levels of IGFBP-3 compared with healthy controls (136, 137). While the Western ligand blot could only detect the intact fragment, immunoblot assay was able to show a decrease in intact IGFBP-3 and also an increase in fragmented IGFBP-3 compared with controls. This finding led to subsequent investigation of proteolytic activity in these patients. IGFBP-3 protease activity was found to be higher in the serum of untreated IDDM patients compared with age-matched controls (137). The usefulness of Western immunoblotting in the identification of IGFBP fragments has fostered studies on characterization of IGFBP proteases in a variety of biological fluids.

C. RIA

One of the major problems with Western ligand blot and Western immunoblot analysis was the lack of precision, which was overcome by the development of RIA (92, 138–144). At present RIAs for IGFBP-1, -2, and -3 are commercially available. Recent success in purifying IGFBPs from a variety of sources to homogeneity and recombinant expression of various IGFBPs has led to the development of specific

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antibodies suitable for establishment of RIAs for accurate measurement of the various IGFBPs. These RIAs have already provided important new information on the physiological and hormonal regulation of the various IGFBPs. The RIAs for the various IGFBPs do not require an extraction procedure as in the case of the IGFs since endogenous IGFs do not interfere with the assay (138–144). Thus, the various biological fluids can be assayed directly for IGFBPs. The majority of IGFBP RIAs thus far developed utilize polyclonal antiserum, which reacts with both intact and fragment forms of IGFBPs. Although measurement of both intact and fragmented forms of IGFBPs may provide useful information in various clinical settings, one of the disadvantages with the use of polyclonal antisera is that the antisera developed in various laboratories may recognize different fragments. This could lead to inconsistent quantitative results using antisera that recognize dissimilar epitopes for similar biological samples. For example, it is known that serum from children with end-stage renal disease contains increased amounts of fragmented forms of various IGFBPs (145, 146). The quantitative measurements of various IGFBPs in serum from children with end-stage renal disease may depend on whether a particular antiserum used for measurement of a given IGFBP recognizes only selected fragments or all of the forms of that particular IGFBP.

D. Immunoradiometric assay

The immunoradiometric assay (IRMA) is a noncompetitive assay in which the IGFBP to be measured is "sandwiched" between two antibodies. The first antibody, which needs to be specific, is immobilized to the inside wall of the tubes. The second antibody is used as a capture antibody (radiolabeled or enzyme conjugated). Since the two antibodies used for IRMA are typically developed against amino-terminal and carboxy-terminal ends of the molecule, the advantage of this assay is that it is often more specific than RIA (147, 148) and more likely to measure the intact molecule. The disadvantage with IRMA is that it may not reflect production rate as well as RIA since the analyte measured may be degraded during storage or during experimental conditions, resulting in artifactually lower values than that actually present. At present, IRMA is commercially available only for IGFBP-3.

VI. Relative Distribution of IGFBPs in Serum

With the development of improved RIAs and validation techniques for the various IGFBPs, it is now possible to measure the concentrations of IGF-I, IGF-II, and their binding proteins in the circulation (Fig. 4). IGFBP-3 is the predominant form present in serum with levels more than 10-fold higher than the other IGFBPs (59, 149). The concentration of the small molecular mass binding proteins are found in increasing order (IGFBP-4 > IGFBP-5 > IGFBP-2 > IGFBP-6 > IGFBP-1) in human serum (92, 138–144, 149, 150). There is a 50% molar excess of IGFBPs over IGFs in serum, which implies that a very small percentage of the IGFs remain in the free form. Since the antisera used for measurement of various IGFBPs recognize both intact and fragment

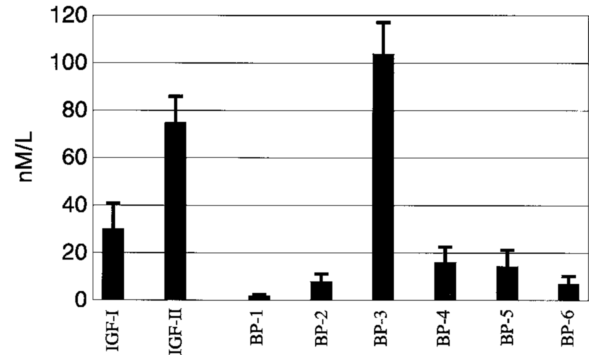

FIG. 4. Concentrations of IGFs and IGFBPs in adult human serum. IGF-I, IGF-II, IGFBP-3, IGFBP-4, and IGFBP-5 values were determined in the author's laboratory. Data for IGFBP-1, IGFBP-2, and IGFBP-6 were compiled from published literature. Values are mean ± SD.[Reproduced with permission from S. Mohan and D. J. Baylink: *J Clin Endocrinol Metab* 81:3817–3820, 1996 (149). © The Endocrine Society.]

TABLE 3. Correlation between IGFs and IGFBPs in normal human serum

IGFBPs	IGF-I	IGF-II
IGFBP-1	Negative	Negative
IGFBP-2	Negative	Negative
IGFBP-3	Positive	Positive
IGFBP-4	Not significant	Not significant
IGFBP-5	Positive	Positive
IGFBP-6	Not known	Not known

forms of IGFBPs, the relative abundance of intact forms of various IGFBPs in adult human serum is not known at this time.

Although approximately 75% of IGFs are bound to GH-dependent IGFBP-3, the relative contribution of non-GH-dependent small molecular mass IGFBPs to the IGF binding capacity (the remaining 25% of IGFs) in serum is not known. If each of the five non-GH-dependent IGFBPs contribute equally to the IGF-binding capacity of serum, one would expect all of the IGFBPs to correlate positively with serum IGF-I and IGF-II levels. Examination of correlation (Table 3) between IGFs and various IGFBPs reveal that only IGFBP-5, in addition to IGFBP-3, showed positive correlation with IGF concentration in normal adult human serum (142). In contrast, serum IGFBP-1 and IGFBP-2 levels showed negative correlation while IGFBP-4 levels did not correlate with IGF concentration. These data suggest that different mechanisms may regulate the amounts of various IGFBPs in serum.

Of the two IGFs, the concentration of IGF-II is about 3-fold greater than that of IGF-I in adult human serum (151, 152). The mechanisms that could contribute to the observed differences in the serum level of IGF-II *vs.* IGF-I include differences in production rate as well as MCR. The daily production rate of IGF-II may be higher than that of IGF-I since the amount of IGF-II produced by several cell types in culture exceeds IGF-I production by 1 order of magnitude (153–156). It appears, however, that the production rate could only contribute to a small extent since Guler *et al.* (82) showed that the IGF-II production rate (13 mg/day) was only slightly higher than that of IGF-I (10 mg/day) in healthy men.

Regarding the MCR, it is known that IGFBPs play a major

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role in extending the half-life of IGFs in the circulation (see *Section IX*). In this regard, the half-life of IGF-II in serum may be longer than that of IGF-I since human serum contains IGFBPs with selective affinity for IGF-II over IGF-I (1, 3). For example, human IGFBP-6 has 50- to 100-fold higher affinity for IGF-II over IGF-I. IGFBP-2 and IGFBP-5 have slightly higher affinity for IGF-II than IGF-I. In addition, Lee and Rechler (76) showed that the 150- to 200-kDa protein complexes in the rat serum have higher affinity for IGF-II than IGF-I. They propose that these 150- to 200-kDa complexes in the adult rat serum contain proteolytically nicked IGFBP-3 and ALS that bind to IGF-II preferentially. Based on these data, it is speculated that the presence of IGFBPs with higher affinity for IGF-II over IGF-I could contribute to a greater half-life of IGF-II over IGF-I. This explains some of the observed differences in the greater abundance of IGF-II *vs.* IGF-I in human serum. However, it appears likely that the differences in IGF-binding affinity of IGFBPs is not the only mechanism that contributes to the greater abundance of IGF-II over IGF-I, since 75% of IGF-II is bound to IGFBP-3 in the form of ternary complex and the intact IGFBP-3 binds IGF-I and IGF-II with similar affinity (3).

Indeed, inasmuch as the differences in production rate and IGFBP affinities to IGFs cannot account for the observed differences in the serum levels of IGF-I and IGF-II, it would seem that some aspect of metabolic clearance, such as the degradation rate, is higher for IGF-I than for IGF-II, thereby contributing to the lower level of serum IGF-I compared with IGF-II in adults. Based on the above analysis, it seems reasonable to conclude that three mechanisms may contribute to the greater serum level of IGF-II than IGF-I in humans: 1) greater production rate of IGF-II than IGF-I; 2) the preferential binding of minor IGFBPs for IGF-II as compared with IGF-I; and 3) a lower degradation rate of IGF-II than IGF-I (the latter two mechanisms would lead to a greater MCR for IGF-I than IGF-II).

If we assume that the actions of IGF-I and IGF-II are similar (*i.e.*, both act via the Type I IGF receptor), then the structural differences between IGF-I and IGF-II would serve some other mechanism than functional activity. This raises the possibility that the differential structure of the two IGFs could lead to differences in MCR. Accordingly, we can speculate that serum contains two pools of reserve IGFs — a smaller IGF-I pool, which is rapidly turning over, and a larger IGF-II pool, which is slowly turning over. If so, the differential structure of the IGFs may produce differential three-dimensional structures with the IGFBPs and, therefore, could lead to a lower proteolysis rate of IGF-II than IGF-I. In this regard, recent studies have shown that exogenous addition of IGF-II to cell-free conditioned medium derived from a number of cell types, including human osteoblasts and fibroblasts, increases the rate of IGFBP-4 proteolysis (157–160). Since IGFBP-4 proteolysis is not induced by the addition of insulin, des(1–3)IGF-I, or des(1–6)IGF-II, all of which bind IGFBP-4 with extremely low affinity, it is speculated that the binding of IGF-II to IGFBP-4 may alter the conformation of the protein and enhance the susceptibility of IGFBP-4 to proteolytic degradation (159). Although these data are consistent with the possibility that the binding of ligand to binding protein may result in altered proteolysis of the ligand and/or the binding protein due to conformation changes, further studies are needed to establish whether or not there are, in fact, different MCRs for the serum IGF-I pool and the serum IGF-II pool. If this proved to be the case, this would open the possibility that the two reserve serum IGF pools provide a metabolic advantage to maintain overall body economy in the face of dramatic changes in functional demands, such as during growth, pregnancy, and starvation. Regardless of whether or not this concept has merit, the findings that IGF-II circulates in greater abundance than IGF-I in human serum, and that IGF-II is produced by several adult tissues in large amounts, are consistent with an important role for IGF-II in human physiology.

The finding that IGFBP-3 is the most abundant IGFBP present in adult human serum does not necessarily mean that the production rate of IGFBP-3 is more than that of other IGFBPs. In this regard, the higher abundance of IGFBP-3 in serum may be due to the fact that the half-life of IGFBP-3 is considerably longer (15–20 h) since it is bound to the 80- to 85-kDa ALS. In contrast, the half-lives of IGFBP-1 and IGFBP-2 have been estimated to be on the order of 1–2 h (161), which suggests that these binding proteins must be produced at a higher rate than that of IGFBP-3 to achieve similar serum levels based on the differences in their half-lives. Thus, it is essential to understand not only the regulation of IGFBP-3 and other IGFBPs in serum, but it is also necessary to know the regulation of different IGFBPs in various extracellular body fluids since the relative levels of these IGFBPs and their corresponding proteases in local body fluids may play a role in regulating the local actions of IGFs depending on the needs of local tissues.

VII. Regulation of Serum IGFBPs

If IGFBPs in serum play an important role in regulating the actions of IGFs (see *Section IX*), then the levels of various IGFBPs should be regulated during various physiological and pathological conditions. Recent studies demonstrate that IGFBPs are regulated during exercise, surgery, pregnancy, and aging and that hormones modulate the levels of one or more IGFBPs in serum and other biological fluids (see below).

A. Physiological conditions

1. Diurnal variation. Plasma IGFBP-1 values are subject to diurnal variation with the levels reaching the lowest during the afternoon and midnight, and highest in the morning (61). In circulation, IGFBP-1 has a free IGF-binding site, suggesting that it is unsaturated in contrast to GH-dependent IGFBP-3, which is normally saturated. It appears that the increase in IGFBP-1 during the morning hours coincides with an increase in IGF-I level, thus reducing insulin-like activity (20). However, this is independent of both IGF and GH (61). In contrast to IGFBP-1, IGFBP-2 and -3 are more stable and do not exhibit diurnal variation nor are they subject to postprandial changes (143, 144). Diurnal variations have not yet been studied for IGFBPs 4–6.

2. Nutrition. Nutritional regulation of IGFBP-1, -2, and -3 has been discussed briefly in a recent review by Thissen *et al.*

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(162), but little is known regarding the nutritional regulation of IGFBP-4, -5, and -6 (163, 164). The metabolic state of an individual is reflected by insulin level, which influences the circulating concentration of IGFBP-1. Insulin-dependent diabetic patients have higher serum IGFBP-1 levels than nondiabetic controls (136, 165). Further, acute steady state hyperinsulinemia reduces the serum IGFBP-1 concentration to values that are 40–70% lower than baseline values in normal individuals and also in diabetic and insulinoma patients (166), suggesting that insulin is involved in the regulation of serum IGFBP-1 levels (167). Serum IGFBP-1 levels fluctuate acutely in response to dietary food intake, with a marked increase (3- to 4-fold higher than baseline) after an overnight fast (168) or long-term dietary restriction (169), and a decline immediately after a meal. This decline in serum IGFBP-1 may be attributed to the direct effect of insulin or insulin-induced changes in glucose transport.

The effect of calorie and protein restriction on the concentrations of the serum IGFBPs is different for adults and children. A 50% calorie reduction for 6 days increased IGFBP-1 levels in healthy adults but not in children. Levels returned to normal after refeeding (170, 171). The differences in these responses were not due to differences in insulin secretion, since both adults and children had a significant decline in fasting C peptide levels. Although insulin is the major regulator of IGFBP-1 concentration (165, 172, 173), this study showed that IGFBP-1 changes in children may not be linked to changes in insulin secretion.

Long-term dietary deprivation decreases plasma IGF-I and IGFBP-1 and may modify the tissue response to IGF by increasing IGF receptor synthesis (174). Another interesting finding is the role of glucagon as a stimulator of plasma IGFBP-1 independent of insulin levels (175). This is evident in healthy subjects, patients with GHD, and IDDM patients who have increased levels of IGFBP-1 when glucagon is administered in spite of an increase in plasma glucose and insulin levels. Based on these data, it is speculated that the nutritional regulation of serum IGFBP-1 level is complex and may be dependent on changes in the level of hormones such as insulin and glucagon, in addition to metabolic changes.

The serum IGFBP-2 level is more stable than IGFBP-1 level and is not influenced by postprandial changes (141). However, serum IGFBP-2 increased markedly in both adults and children on protein restriction (170). Similar observations have been made in patients with anorexia nervosa (171), chronic protein-calorie malnutrition (170), and in prolonged fasting that lasted more than 1 week (172). This increase in serum IGFBP-2 follows a cellular increase in the expression of the IGFBP-2 mRNA in rat liver (174, 176). Although protein refeeding normalized the serum IGFBP-2 levels of undernourished children, high-protein intake is required to achieve complete normalization (177). Thus, nutrition-induced changes in serum IGFBP-2 level appear to be the direct effect of dietary protein on IGFBP-2 expression in liver.

Serum IGFBP-3 levels declined slightly but significantly with calorie restriction in both children and adults (170), but protein restriction caused a decrease in IGFBP-3 only in adults. However, this was normalized after protein refeeding (177). Although serum IGFBP-3 levels are regulated by IGF-I and GH under normal conditions (140), the decrease seen in undernourished children is more likely due to the presence of IGFBP-3-specific protease levels (177). The presence of similar proteolytic activity accompanying a low IGFBP-3 level is seen in other catabolic states (135, 178), pregnancy (135, 179), and in postsurgical patients (180). Age appears to influence changes observed in IGFBPs as a result of dietary modification. The response to calorie restriction among children and adults differs more so than during protein restriction, which may be due, in part, to an overestimation of the energy requirements for children (169).

Weight loss or long-term moderate energy restriction does not alter IGFBP-3 (181). Serum IGFBP-3 was not influenced by a very low calorie diet (VLCD) consumed by normal and obese subjects, while IGFBP-1 increased markedly in controls on VLCD and not in obese subjects (182). The increase in IGFBP-1 is suggested to inhibit the IGF-I feedback regulation of GH secretion, while a similar response is absent in obese individuals. Thus, GH secretion is increased in normal subjects on VLCD, but this response is abolished in obese individuals. The impaired GH secretion in obese subjects resulted in a lowered IGF/IGFBP-3 molar ratio, but this was reversed after weight loss by these subjects (183). Thus, changes in serum levels of IGFBPs induced by VLCD appear to be different between normal and obese subjects.

Based on the observations that serum levels of IGFBPs change depending on the nutritional status, two general conclusions can be made: 1) serum IGFBP-1 and IGFBP-2 levels are regulated differently than IGFBP-3 by nutrition, and 2) the decrease in serum IGFBP-3 with corresponding increases in serum IGFBP-1 and IGFBP-2 levels during malnutrition would decrease the half-lives of IGFs but tend to increase the transport of IGFs across the vascular endothelium and thereby could modulate the bioavailability of IGFs to target tissues (see *Section IX*). Further studies are needed to determine whether nutrition regulates IGF action by altering the ratio of IGFs bound to the 150- to 200-kDa and 50-kDa complexes.

3. Exercise. Exercise increased IGF-I and IGF-II in human adults (184, 185) with the degree of response influenced by the intensity of exercise (186). This increase in IGF level in serum appears to be GH independent, since the increase in serum IGF-I occurred earlier than the increase in serum GH. In addition, GH secretion increased only in high-intensity exercise while IGF increased under both low and high intensities. Circulating IGF-I levels are also influenced differently by different types of exercise. Weight-bearing exercise caused no change in the IGF system (186, 187), while endurance-type exercise induced significant increases in serum IGF-I level (185). Exercise is also accompanied by changes in some of the IGFBPs (increase in IGFBP-1 and IGFBP-3) with or without changes in IGFs, with an overall change in the IGF to IGFBP ratio.

Prolonged exercise increased the need for plasma glucose because of depleted muscle glycogen or increased hepatic glucose output. Prolonged exercise increased serum IGFBP-1 (188, 189), and this was inversely related to serum insulin and IGF-I levels (188). This raised the possibility that serum insulin was the main regulator of IGFBP-1 in circulation during exercise. However, another study (189) showed that serum

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IGFBP-1 increased in response to prolonged exercise even when normal plasma glucose and insulin levels were maintained, suggesting that factors other than insulin levels, *e.g.*, muscle glycogen, may be involved in the regulation of serum IGFBP-1 during exercise.

Increased serum IGFBP-3 levels have been reported in adults after exercise (184, 190). This increase paralleled an increase in IGFBP-3 proteolytic activity. It is speculated that the proteolysis was induced by activation of calcium-dependent protease (191) and might be responsible for the IGF-induced anabolic effect of exercise on muscle tissue. These changes in the IGF system components are observed immediately after exercise and are of short duration (<1 h). It is not known at this time whether the alterations in the IGF system components in circulation play a role in mediating the anabolic effects of physical activity or whether the exercise-associated changes in circulating levels of IGFs and IGFBPs reflect processes that occur in the exercising tissue itself (191).

4. Glucocorticoid. Glucocorticoids inhibit somatic growth in humans in part by suppressing GH secretion and IGF activity. When dexamethasone was administered to healthy male volunteers, it suppressed IGFBP-1 and IGFBP-2 levels while increasing IGF-I and IGFBP-3 levels (192, 193). The mean IGF bioactivity was reduced by 60% over the sampling period (192). This decrease in bioactivity could be due to the induction of serum inhibitors, alteration in IGFBP activity, and/or alteration in secretory profiles of GH. Recently it was shown that the negative effects exerted by glucocorticoid on bone formation may be mediated, in part, via changes in endocrine and local action of IGFs (194). In this study, the reduction in bone formation after glucocorticoid therapy of chronic obstructive pulmonary disease patients was accompanied by a decrease in stimulatory IGF system components including IGFBP-3. Whether or not other stimulatory and inhibitory IGFBPs are also affected remains to be determined.

B. Development and aging

1. Fetal and neonatal development. It is possible that IGFBPs play a major role in regulating the mitogenic and differentiation-promoting effects of IGFs in fetal tissues. IGFBPs 1 to 6 are expressed in the different organ systems of the developing fetus (12–16 weeks) as shown by Northern blot analyses (195, 196). Of the IGFBPs seen in the serum of a human fetus, IGFBPs 1, -2, and -3 originate predominantly from the liver, while only small amounts of IGFBPs 4, -5, and -6 are expressed in the liver. IGFBP-5 mRNA was detected in several cell types during early postimplantation stages of the developing rat, suggesting that IGFBP-5 has a role in the development of different organ systems (197). These binding proteins either cause inhibitory or stimulatory effects on IGF action, depending on the amount of IGFs bound to each of the IGFBPs and the pattern of distribution of these binding proteins in the various fetal tissues.

In order for optimal fetal growth, a constant interaction between the maternal host and the developing embryo/fetus is required. The presence of IGFs, IGFBPs, and fragments of IGFBP-3 in human extraembryonic cavities provide support for maternal-fetal exchange of IGF system components (115). It is suggested that the altered affinities of the proteolyzed IGFBP-3 for IGF-II in extraembryonic cavities may play a role in regulating the bioavailability of IGF-II in the chorion and/or the amnion (115). However, the role of IGFBP-3 protease in modulating IGF bioavailability remains controversial.

There is also a developmental switch during transition from fetal to neonatal life in the IGFBPs present in circulation. In a fetus of less than 27 weeks of gestation, serum contains IGFBP-1, while cord serum contains mainly IGFBP-3 (198). The level of IGFBP-1 is higher in the fetal and cord blood than in adult plasma. In normal weight fetuses, the IGFBP-3 and IGFBP-1 concentration in serum is 15% and 50% of maternal serum levels, respectively (199). Low cord serum IGFBP-1 and elevated IGFBP-3 concentrations were reported in large-for-gestational age fetuses at term birth (200). Since these infants also had elevated cord serum insulin levels, it was suggested that the changes in IGFBP-1 were mediated by insulin, mainly by directing greater delivery of the IGF/IGFBP complex to the target tissue, resulting in the accelerated growth as seen in large-for-gestational age fetuses. IGFBP-3 levels increase significantly during the last trimester of intrauterine life. This is supported by a study (201) that showed an increase in serum IGFBP-3 in preterm infants from birth (3 months preterm) to 2 months past appropriate term age. Thus IGFBP-1 and IGFBP-2 are the predominant binding proteins during fetal life, but they decline during the early neonatal period, with IGFBP-3 becoming the predominant binding protein.

In intrauterine growth-retarded (IUGR) fetuses, there is a marked elevation in cord serum IGFBP-1 and -2 compared with normal fetuses (199, 200, 202). Giudice *et al.* (200) showed that serum IGFBP-3 levels were decreased in IUGR fetuses, but this is in contrast to the results of Lassarre *et al.* (203), who showed that fetal cord serum had higher IGFBP-3 than normal cord serum. Typically, serum IGFBP-3 declines in cord serum due to a specific protease that degrades this protein to increase the amount of IGFs available for stimulation of growth in the target tissues. Thus, in fetal circulation, the increased availability of IGF is due to a molar excess of IGF-I and -II, an increase in IGFBP-2, and a decrease in the ternary IGFBP-3 complex formation. It is therefore speculated that IGFBP-3 protease is less likely to play a significant role in fetal serum in contrast to maternal or neonatal serum (204).

During fetal life, not only is the total plasma IGFBP-3 lower than in the adult circulation, but the amounts of IGF-I and IGF-II bound to this binding protein are also low compared with amounts of IGFs bound to IGFBP-3 in adults. IGF-I levels are depressed in infants with IUGR, suggesting that IGFs play a significant role in promoting growth. However, the majority of IGFs are bound to IGFBP-3 as a 50-kDa ALS-independent complex in infant serum, which is capable of crossing the endothelial barrier, thus increasing the bioavailability of the IGFs (203). The role of IGFBP-4 in neonatal development has not yet been explored, but given the inhibitory effect of IGFBP-4 on IGF action, it is possible that serum IGFBP-4 levels are higher in children with slow growth compared with normally growing children (205). However, future studies need to confirm these speculations.

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2. Puberty. Although low at birth, the serum IGFBP-3 concentration rapidly increases during the first years of infancy (206–208), reaches a peak at puberty (207), and declines during adulthood (208). Girls have higher serum IGFBP-3 levels than boys of comparable age throughout childhood, and levels peak a year earlier than boys during puberty (207, 208). In addition, IGFBP-3 increases with the increasing stage of pubertal maturation. Both the height and body mass index of an individual correlates positively with IGFBP-3 levels independent of age, sex, and pubertal stage. The molar ratio between IGF-I and IGFBP-3 is increased during puberty, suggesting that more biologically active IGF is available in the free form during the pubertal growth spurt. Serum levels of IGFBP-2 show marked age-dependence with high levels at birth and senescence and low levels during puberty (144). Serum IGFBP-1 declines progressively with age, with the lowest concentration observed during puberty (207, 208). The height variability seen among pubertal children correlates with the concentration of IGF-I and IGFBP-3, with lower values in short stature children and higher values in tall children (209). Also, in patients with acromegaly and those with high serum GH levels, circulating IGFBP-3 and IGF-I are increased. Under normal physiological conditions, most of the serum IGFs are bound to IGFBP-3, with an approximate 1:1 molar ratio of total IGF (IGF-I + IGF-II) and IGFBP-3 (60, 63). However, this ratio seems to vary with developmental age, with a greater increase in circulating IGF-I than IGFBP-3 during puberty (208). Changes in serum concentrations of the various IGFBPs during puberty (Table 4) have been proposed to play a role in inducing the growth spurt during puberty.

Changes in the IGF system components may also mediate the increase in bone size associated with puberty (210). An up-regulation in the IGF system as measured by an increase in serum IGF-I, IGFBP-3, and IGFBP-5 was reported in 65 girls belonging to Tanner stages (TS) II-IV. The increase in skeletal width occurred between TS II and III, the same time when the IGF system was up-regulated, suggesting a role for the IGFs and their binding proteins in skeletal growth. The role of GH *vs.* sex steroid hormones in mediating these changes in the IGF system components needs to be explored.

3. Pregnancy. Several changes in the IGF system occur in maternal and fetal circulation during pregnancy, suggesting an important role for the IGF system components in producing the anabolic effects on maternal and fetal tissues. Both serum IGFBP-1 and -2 are found in the maternal serum during the initial weeks of gestation. However, serum IGFBP-2 levels are lower toward the latter part of gestation due to the presence of IGFBP-2-specific protease (179). Serum IGFBP-1 levels are higher during early gestation and plateaus from week 12 through the end of gestation (118).

TABLE 4. Changes in IGF system component levels in serum during puberty and aging

IGF system components	Puberty	Aging
IGF-I	Increase	Decrease
IGF-II	Increase	Decrease
IGFBP-1	Decrease	Increase
IGFBP-2	No change	Increase
IGFBP-3	Increase	Decrease
IGFBP-4	Not known	Increase
IGFBP-5	Increase	Decrease
IGFBP-6	Not known	Not known

Serum IGFBP-3 is the major binding protein for the circulating IGFs (211, 212) in pregnant and nonpregnant women. Binoux *et al.* (213), using competitive binding studies, showed that IGFBP-3 derived from maternal serum had 10 times lower affinity for IGF-I than IGFBP-3 derived from normal human serum and half the affinity for IGF-II. An apparent decline in the acid-stable IGF-binding subunit of IGFBP-3 in the latter part of gestation and the presence of smaller molecular forms of IGFBP-3 in term maternal serum provide strong evidence that specific proteases that cleave the IGF-IGFBP-3 complex (78, 135, 179) are present. The protease detected in the serum of pregnant women is suggested to cause a functional change (78, 214) resulting in a redistribution of IGF-I. This would increase the proportion of free IGF-I (215) at the expense of IGF-I bound to the 150 to 200 kDa complex. Both the intact and fragmented forms of IGFBP-3 have greater affinity for IGF-II than IGF-I (3), a role that could be related to the established importance of IGF-II in fetal development.

On the other hand, Baxter and co-workers (67, 216, 217) demonstrated that IGFBP-3 derived from human maternal serum is not functionally altered. Baxter *et al.* (67) reported that this proteolyzed IGFBP-3 circulates in a ternary complex that is normal in molecular mass and IGF-carrying capacity. IGFBP-3 derived from maternal serum appears similar in size to the IGFBP-3 from serum of nonpregnant women dissociated from ternary complex by acid treatment with normal binding affinity for ALS (217). Ternary complex formation measured by radiolabeled α-subunit binding was identical in serum from pregnant and nonpregnant women after acid treatment of serum that destroys endogenous α-subunit. Based on these data, Baxter and co-workers (79) concluded that IGFBP-3 in native maternal serum is functionally normal. Thus, to date, the functional significance of IGFBP-3 protease in maternal serum remains controversial.

IGF biological activity is modified in situations in which fetal growth is abnormal, such as in multiple or abnormal pregnancies. Maternal IGFBP-3 increases with gestational age and is higher in multiple pregnancy compared with gestational age-matched twin or single pregnancies (218). IGFBP-3 protease activity also increases with increasing fetal number, but is not reversed by embryo reduction. This implies that the protease activity is activated early on in pregnancy and remains irreversible. A similar increase in IGFBP-3 protease activity has been observed in both appropriate-for and small-for-gestational age fetuses with utero placental insufficiency. Based on this evidence, the concept has been advanced that in both multiple pregnancy and under utero placental insufficiency, the increase in protease activity may be an adaptive mechanism to counteract the threat to fetal growth (218).

4. Aging. An age-related decline in serum IGF-I level has been reported by several investigators (219–223). The decrease in serum IGF-I with advancing age could be explained in part by the decrease in serum GH levels (221). Recent studies also demonstrated that serum IGF-II levels declined with age but to a lesser extent compared with IGF-I (222). In addition to

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the IGFs, the serum IGFBP levels were also altered with age. Both the magnitude and direction of change with age were different for the various IGFBPs. Serum levels of IGFBP-1, IGFBP-2, and IGFBP-4 increased with age while those of IGFBP-3 and IGFBP-5 declined with age (138–144, 190). Serum IGFBP-1 showed an age-related increase (138, 224) in both men and women. Fasting IGFBP-1 levels increased with aging (224), but there was no correlation between insulin and IGFBP-1 with advancing age. Although insulin correlates inversely with IGFBP-1 (165), this relationship ceases to exist in aged individuals. This might be due to the development of age-related insulin resistance among these subjects (225). Replacing adrenal steroids suppressed the IGFBP-1 levels while increasing the IGF-I levels and restoring normal mitogenic activities in serum of the elderly (226). The age-dependent pattern of serum IGFBP-2 resembles that of IGFBP-1 in that it increased with aging (143, 144). After puberty there is a steady increase in IGFBP-2 in circulation, and this increase is accelerated at age 60 and above. However, the mechanism that causes the increase in IGFBP-2 is unknown at present.

Serum IGFBP-3 declines with age during adulthood, and this corresponds to an age-related decline in GH secretion (140, 150). In contrast, serum IGFBP-4 levels increase with age (139, 222), indicating that different binding proteins are differentially regulated. The increase in serum IGFBP-4 in elderly women was in part due to the age-related increase in PTH, the production of which is stimulated in calcium deficiency states (227). A 30% reduction in the serum IGFBP-5 levels in women aged 23–85 yr was observed when compared with IGFBP-5 in the serum of prepubertal girls (142). Since serum IGFBP-5 levels show significant positive correlation with age and the treatment of various cell types including osteoblasts with IGFs increased production of IGFBP-5, it is suggested that the decline in IGF production may contribute to the age-related decrease in IGFBP-5 production. Consistent with this interpretation, Nicholas *et al.* (49) reported that the decline in skeletal content of IGFBP-5 showed significant positive correlation with skeletal content of both IGF-I and IGF-II. Whether the age-related decline in skeletal and serum IGFBP-5 reflects an age-related decline in synthesis of this protein by bone and other tissues needs further investigation.

The data in Table 4 demonstrate that multiple deficits in the IGF system components occur as a consequence of aging. The underproduction of stimulatory IGF system components and the overproduction of inhibitory IGF system components could lead to an age-related decrease in the hormonal as well as local actions of the IGFs, all of which could contribute to an impairment in the function of various organs during aging. Further studies are needed to establish the cause and effect relationship between changes in IGF system components and impairment in various body functions with age.

C. Hormonal effects: mechanisms

If IGFBPs play an important role in regulating IGF actions, then the regulation of IGFBPs in various physiological situations would depend on the hormones involved and the target tissue. Thus, the changes in serum levels of IGFBPs during normal physiological conditions such as malnutrition, puberty, pregnancy, and aging may be influenced in part by corresponding changes in the levels of various hormones including insulin, GH, sex steroid hormones, glucocorticoids, and thyroid hormone.

The extent to which changes in the level of a given hormone influences the serum level of a given IGFBP depends on two main factors: 1) the magnitude of change in the production of the corresponding IGFBP in the target tissue(s) and 2) the extent to which the target tissue(s) contribute to the circulating level of the IGFBP in serum. It is thus possible that the changes in the production of one or more IGFBPs in the target tissues in response to hormonal stimuli may be reflected by corresponding changes in serum levels of these IGFBPs. Alternatively, the levels of one or more IGFBPs may change in the local body fluid in response to a hormonal stimuli, and these local changes may not be reflected by changes in corresponding levels of the IGFBPs in serum.

Studies on serum regulation of IGFBPs in response to hormonal stimuli suggest that some hormones may primarily influence the serum level of one IGFBP while other hormones may influence the serum levels of multiple IGFBPs. For example, the level of insulin changes in response to alterations in nutritional status. Accordingly, the serum level of IGFBP-1 changes in response to alterations in insulin level (59). The finding that insulin is the major regulator of IGFBP-1 production in liver, one of the target organs for insulin action, suggests a role for insulin in regulating IGFBP-1 function depending on the nutritional status. In contrast to the specific effect of insulin on IGFBP-1, GH appears to influence the serum level of a number of IGFBPs (59, 228). Since GH mediates somatic growth in a variety of organs and some IGFBPs are more abundantly expressed in certain tissues than in others, it is not surprising that GH influences multiple IGFBPs.

Studies on the mechanisms by which various hormones influence the serum level of known IGFBPs provide evidence for both direct effect of a given hormone on the IGFBP gene in the target tissues and an indirect effect of the hormone secondary to changes in the production of local growth factors. For example, the effect of GH to increase the serum level of IGFBP-3 is thought to be mediated via the following two mechanisms: 1) Based on the findings that GH treatment increases IGFBP-3 expression by a pathway independent of IGFs in liver cells *in vitro* (229), it is proposed that GH may mediate its effects by directly influencing the IGFBP-3 gene in the target tissues; 2) Based on the findings that many of the effects of GH on target tissues are mediated indirectly via IGFs and that changes in the serum levels of IGFBP-3 correlate significantly with changes in serum level of IGFs, it is proposed that GH effects on IGFBP-3 may be secondary to an increase in the production of IGFs (59, 228, 230, 231). In addition to these two mechanisms, GH induces ALS production, thus contributing to the prolonged half-life of IGFBP-3 (66). The extent to which each of these mechanisms contributes to the GH-induced increase in serum level of IGFBP-3 is not known at this time.

Similar to GH effects, a number of other hormones, including sex steroid hormones and glucocorticoids, may also

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influence serum level of IGFBPs by both direct and indirect mechanisms. In this regard, studies on regulation of production of IGFBPs using serum-free monolayer cell cultures have provided evidence for the direct effect of estradiol, testosterone, progesterone, and dexamethasone on the expression of various IGFBPs (153, 232). In addition, it is also known that these hormones influence actions of a variety of locally produced growth factors including IGFs (153, 159, 232). Because many of these locally produced growth factors also influence production of IGFBPs in a variety of target cell types, the changes in the serum level of IGFBPs may be a reflection of not only the direct effect of the given hormone on IGFBP production in the target tissues but also the indirect effect of a given hormone on local growth factor milieu.

Studies on the molecular mechanisms by which hormones influence expression of IGFBPs provide evidence for complex regulation involving both transcriptional and posttranscriptional mechanisms (159). Some hormones may regulate IGFBP level by primarily regulating one control mechanism while other hormones may regulate IGFBP level by regulating multiple control mechanisms. In this regard, it is known that the insulin effect on IGFBP-1 production is regulated primarily at the transcriptional level in liver cells (233). In contrast, the IGFBP-3 levels in the conditioned medium of various cell types in response to various effectors are regulated by mechanisms involving alterations in both synthesis and degradation (191). In addition, there is evidence of the release of cell surface-bound IGFBP-3 by certain growth factors (234). Similarly, IGFs and bone morphogenetic proteins influence IGFBP-5 levels in human osteoblasts by both decreased proteolysis and increased gene transcription (235). Thus, some hormones appear to modulate the IGFBP level by regulating one control mechanism (*e.g.*, proteolysis) while other hormones appear to modulate IGFBP levels by regulating multiple control mechanisms (*e.g.*, gene transcription, mRNA processing, mRNA stability, proteolysis, release of cell surface-bound IGFBP). It is speculated that the complexity of IGFBP regulation may provide the required flexibility for modulating IGF actions by a multitude of systemic and local effectors in various tissues.

D. Pathological conditions

The following section describes the changes in the IGFBP axis in serum during various pathological states and how these changes could be interpreted to explain the corresponding disease states.

1. Diabetes. Serum IGFBP-1 is the only component of the IGF system that is directly regulated by insulin. Several studies (229, 236–238) have shown that in adult and adolescent IDDM patients there is a significant increase in serum IGFBP-1 level. In normal subjects, serum IGFBP-1 levels progressively declined throughout childhood and puberty, but this age dependency did not hold true in diabetic individuals (239). Although levels of serum IGFBP-1 in IDDM patients was 7 times higher than in healthy controls before initiation of insulin treatment (240), the levels were normalized immediately after insulin infusion. Glucagon is known to be a stimulator of IGFBP-1 production in IDDM patients and is independent of insulin action (175). The serum IGFBP-1 in patients with IDDM and severe ketoacidosis was in the phosphorylated form (241). This form has 6-fold higher affinity for IGF-I than the nonphosphorylated form *in vitro* (242). Based on these findings, it is speculated that the increase in phosphorylated IGFBP-1 leads to a decrease in free IGF available for stimulating growth in IDDM patients, which may contribute in part to the low lean body mass observed in these patients (241, 242).

In contrast, in non-insulin-dependent diabetes mellitus (NIDDM) that results in hyperinsulinemia and insulin resistance, conflicting data (165, 173) have been obtained in regard to serum IGFBP-1 levels (*i.e.*, both increases and decreases in serum IGFBP-1 levels). This discrepancy could be due to the type of treatment provided to the patients and how this in turn affects β-cell function and insulin resistance. For example, sulfonylurea increases endogenous insulin secretion and thereby decreased IGFBP-1 secretion, while multiple insulin injections inhibit endogenous insulin secretion, resulting in an increase in serum IGFBP-1 (243). An increase in fasting IGFBP-1 and -2 levels and a decrease in IGFBP-3 levels were observed in normal and NIDDM patients after IGF-I and insulin infusion (244). These changes in the levels of various IGFBPs are likely to alter distribution of IGFs among these binding proteins which, in turn, could modify the bioavailability of IGFs in the target tissues depending on the tissue-specific proteolysis of IGFBPs (see *Section VIII*).

Serum IGFBP-2 is also increased in diabetes; however, insulin therapy normalized the levels (239). This is surprising since it is known that free IGF-I and not insulin is the major regulator of IGFBP-2. Although serum IGFBP-3 is not involved in glucose metabolism, a 30–40% reduction was reported in diabetic patients (237) before starting insulin treatment. After treatment, there was an increase in both serum IGF-I and IGFBP-3, with the former showing a slightly greater increase than the latter. This makes more IGF bioavailable and possibly accounts for the improved linear growth seen in children with IDDM receiving insulin treatment (240).

In several pathological states, IGFBP-specific protease(s) is increased in the serum, which may lead to an increase in the amount of free IGF-I available for growth stimulation. Intact IGFBP-3 measured by ligand blot analysis of children with untreated IDDM was 50% lower than that of age-matched controls (243) while it was 70% lower when measured by immunoblot assay that detected both intact and fragmented IGFBP-3. IGF-I, a known regulator of serum IGFBP-3, was reduced in IDDM patients (245) and may be responsible for the decline in IGFBP-3 levels. After insulin treatment, IGFBP-3 levels increased significantly, with an associated decline in serum IGFBP-3 protease (137). It is postulated that an increase in IGFBP-3 protease activity during insulin deficiency, as seen in IDDM, helps to partly overcome the catabolic state by increasing the bioavailability of IGF for growth stimulation (137).

An increase in protease activity is also observed in gestational diabetes. During the latter part of gestation, there is an alteration in IGFBP-3 levels due to a specific IGFBP-3 protease that increases availability of free IGF-I. Although women with gestational diabetes had a greater increase in

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free IGF-I compared with nondiabetic pregnant women, this change did not seem to be due to a decrease in serum IGFBP-3 level, but rather to an increase in this protease activity (246).

2. Tumors. Extrahepatic non-insulinoma tumor-induced hypoglycemia is accompanied by metabolic abnormalities such as an inhibition of hepatic glucose production, decrease in lipolysis in adipose tissue, and increased consumption of glucose by peripheral tissues (247). Although these effects are characteristic of insulin action, plasma insulin levels are low in these patients (248). It was evident that the tumors in these patients secreted a bioactive big IGF-II (15–25 kDa) as a result of incomplete processing of pro-IGF-II peptide (249). Big IGF-II has an insulin-like action and binds insulin receptor (250). The presence of high levels of IGF-II by itself does not address the issue of tumor-related hypoglycemia. However, it was shown that there is a shift in the distribution of IGF-II from the 150- to 200-kDa to the 50-kDa complex in the circulation (251, 252), since IGFBP-3 and tumor IGF-II failed to complex with ALS (253). The increase in 50 kDa complex is mainly due to an increase in the level of IGFBP-2 (251, 252). Since the 50-kDa IGF complex can cross the vascular endothelial barrier, it seems likely that the bioavailability of IGF-II is increased, resulting in insulin-like actions in target organs.

A proposed mechanism by which big IGF-II secreted by tumors may lead to hypoglycemia is shown in Fig. 5. Consistent with this model, Baxter *et al.* (253) showed that prednisone treatment of a 87-yr-old women with a non-islet cell tumor suppressed pro-IGF-II formation and corrected the hypoglycemia. Prednisone treatment caused a redistribution of serum IGFBP-3 from binary to ternary complex forms. These studies demonstrate the role of ALS and the ternary ALS+IGFBP-3+IGF complex in glucose regulation.

a. Prostate tumors. IGFBP-2 is found in significant amounts in prostate secretions and is elevated in serum of patients with prostate cancer (254, 255). A dramatic increase in serum IGFBP-2 is observed in prostate cancer patients and correlates with PSA levels (255). This is supported by the finding that both normal and cancerous prostate cells in culture secreted IGFBP-2 as the major binding protein (256). Serum IGFBP-3 levels, on the other hand, decreased significantly in patients with prostate cancer. A decrease in IGF-I or the activation of a protease specific for IGFBP-3 may be responsible for the decrease in IGFBP-3. Recent evidence suggests a IGFBP-3 proteolytic activity for PSA (125), as seen by an inverse correlation between PSA and serum IGFBP-3 levels. However, the exact role of these alterations in binding proteins in the pathophysiology of cancer is unclear. It is possible that the decrease in IGFBP-3 may lead to an increase in the amount of free IGF-I in the target tissues, which promotes growth. On the other hand, the decreased IGFBP-3 levels may reduce the half-lives of IGFs and decrease the endocrine IGF actions. However, with respect to the diagnostic utility of IGFBPs as tumor markers for prostate cancer, serum IGFBP-2 may not be as sensitive and accurate an indicator as serum PSA, but may add some information in clinical situations where serum PSA levels are low (254).

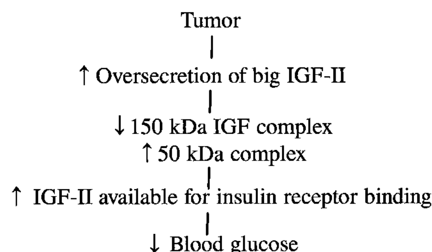

FIG. 5. Proposed mechanism of extrahepatic tumor-induced hypoglycemia. The hypoglycemic state in patients with-non islet cell tumors is associated with an increase in serum level of pro-IGF-II, a decrease in the circulating level of the 150-kDa complex, and a corresponding increase in the circulating level of the 50-kDa complex. The altered distribution of IGFs between the 150-kDa and 50-kDa complexes is likely to be due to the failure of tumor-secreted pro-IGF-II to form a complex with ALS and IGFBP-3. The increase in 50-kDa IGF pool increases the bioavailability of IGFs (because 50-kDa and not 150-kDa IGF complex can cross the vascular endothelium) to produce insulin-like effects in the target tissues. The association between the decreased 150-kDa complex and glucose level in the serum of tumor-induced hypoglycemia emphasizes the central role of the 150-kDa IGF complex in glucoregulation (248–251, 253).

b. Endometrial tumors. IGFs have been shown to stimulate breast cancer cell proliferation *in vitro* (257). With respect to breast cancer, tamoxifen therapy suppressed plasma IGF-I (258) and increased serum levels of IGFBP-1 (259). This increase in IGFBP-1 could either result in a decrease in the biologically available form of IGFs and reduce growth of the tumor or could enhance the delivery of IGFs to the tissues because of the ability of the IGF/IGFBP-1 complex to cross the endothelial barrier, making tamoxifen therapy ineffective in these patients. In women with endometrial cancer, fasting insulin was found to be higher, and serum IGF-I and IGFBP-3 levels were lower than in normal controls (260). It is not known at this time whether the changes in the serum levels of various IGF system components in women with endometrial cancer play a role in modulating the amount of IGFs available for cancer cell growth.

c. Other tumors. Several other tumors are also associated with alterations in IGF and their binding proteins. For example, in one type of tumor affecting the kidney in children (Wilm's tumor), IGF-II levels in serum were lower than in normal controls, but IGFBP-2 was significantly higher in those with the tumor (261). Although it is known that IGFBP-2 sequesters IGF-II and prevents it from binding to the IGF receptors, the exact role of IGFBP-2 in tumor development is not known. Based on the findings that the serum level of IGFBP-2 is increased in various types of tumors, Zumkeller *et al.* (261) concluded that the serum IGFBP-2 measurements may prove to be useful as markers for various malignancies.

Elevation of IGFBP-2 in CSF was observed in patients with malignant CNS tumors (96). The source of CSF IGFBP-2 was not the serum as there was no correlation between these two measurements. Increased expression of IGFBP-2 mRNA in CNS tumors from one patient suggests the possibility that the tumor itself may be the source of the CSF IGFBP-2. Although it is speculated that the IGFBP-2 in the CSF could be a useful marker for CNS tumors, further studies are required to understand the mechanism that causes the increase in IGFBP-2 and the clinical utility of IGFBP-2 measurement as a diagnostic marker for CNS tumors. Children with highly malignant CNS tumors or CNS leukemia (95) have elevated IGFBP-3 in the CSF possibly due to the disruption of the blood-brain barrier and entry of serum IGFBP-3 or to an

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increase in local production of IGFBP-3 by tumor tissues. Chemotherapy for cranial tumors decreased serum IGF-I and IGFBP-3, although GH levels remained within normal limits (262). However, after 12 months, the levels returned to pretreatment values. It is speculated that GH insensitivity seen during chemotherapy might be responsible for the early growth retardation observed in these patients.

3. Laron type dwarfism. Laron type dwarfism (LTD) is an autosomal recessive condition involving a defect in the GH receptor gene. These patients have high circulating levels of GH and low serum IGF-I with impaired receptor function causing growth retardation. In this GH-resistant condition, it appears that the IGFBPs play an important role in modulating the activity of IGFs. Elevated serum IGFBP-1 concentrations were observed in LTD patients, and this may be partly due to low IGF-I levels. After IGF-I administration, the IGFBP-1 levels were down-regulated in LTD patients (263). Low levels of serum IGFBP-3 were seen in LTD subjects compared with normal controls, and most of the IGF-II in circulation appeared bound to IGFBP-2 instead of IGFBP-3. Furthermore, an IGFBP-3-specific protease was identified in the serum of LTD patients but not in controls. Based on these data, it is speculated that the decrease in IGF bioactivity in LTD is largely due to variations in IGFBPs and not in IGF-II levels.

Long-term treatment of LTD with IGF-I resulted in a strikingly progressive increase in serum IGFBP-3 levels with continuous treatment (264–266). This increase in serum IGFBP-3 levels, despite the absence of GH action in LTD, suggests the possibility that IGFBP-3 in humans is regulated directly by IGF-I, independent of GH. An increase in serum levels of other IGFBPs was also detected after IGF therapy. The increase in serum IGFBP-1 levels paralleled the decrease in insulin levels observed with the therapy. Since insulin is the major regulator of IGFBP-1 production, the rise in the latter is most probably due to this observed decrease in insulin (267). The serum IGFBP-2 level was also increased after long-term IGF-I therapy of LTD. Similar increases in the serum IGFBP-2 level were also observed in normal human subjects receiving infusions of IGF-I (268). The functional significance of the increase in serum levels of various IGFBPs as a result of IGF-I administration in modulating the growth-promoting effects of exogenous IGF-I in LTD is not known at this time.

Two forms of IGF- and ALS-associated IGFBP-3 (44-kDa doublet and 28-kDa band) that are capable of forming the ternary complex have been identified in healthy adult serum (28). In GH receptor deficiency (GHRD), the distribution of these two forms differs from that of control serum and explains the alterations in IGF distribution in these patients. Although in adults with GHRD, recombinant IGF-I treatment increases serum IGF-I and decreases serum IGF-II without any change in ALS (269), the IGF distribution between the two molecular forms of IGFBP-3 is not corrected (270). Thus IGF-I therapy in GHRD patients is expected to have a minimal role in prolonging the half-lives of IGFs since IGF-I therapy in these patients has failed to increase the formation of the 150- to 200-kDa IGF complex. However, a recent study demonstrated the potential beneficial effects of short-term IGF-I therapy in LTD (271) although it is not known whether long-term IGF-I treatment would result in continuous improvement in growth velocity in these patients.

4. GHD. In GHD the patients have a low circulating level of GH but normal receptor function. The serum IGFBP-1 levels in GH-deficient patients were higher than in healthy controls (138, 272), and this could be due to low endogenous insulin production in these subjects (273, 274). In addition to insulin, the role of other known stimulators of IGFBP-1, such as glucagon and catecholamines, in altering serum IGFBP-1 needs to be explored. As for serum IGFBP-3, both in congenital and idiopathic GHD, the levels were diminished (60). Similarly, in patients with hypopituitarism secondary to certain tumors, the low levels of IGFs were accompanied by low serum IGFBP-3.

It is known that the serum levels of IGFBP-3 are dependent on GH and/or IGF-I production. GH and/or IGF effects on IGFBP-3 levels are mediated via an increased synthesis of IGFBP-3 or an alteration in IGFBP-3 breakdown (275–277). IGF-I can also induce IGFBP-3 level independent of GH activity as shown by an increase in the serum IGFBP-3 level after treatment with recombinant IGF-I (278). However, the changes in serum IGFBP-3 level in IGF-I-treated children are much smaller compared with those changes induced by GH treatment, probably due to lack of increase in ALS production during IGF-I treatment (278, 279). In terms of whether or not changes in IGFBP-3 production play a role in mediating GH-induced growth, it is speculated that the absence or presence of IGFBP-3 plays a role in the growth of pygmy children. It is proposed that the absence of IGFBP-3 in these children accounted for their normal growth during childhood, in spite of the low levels of IGF-I, while during puberty the increase in IGFBP-3 level resulted in the failure of the normal growth spurt (63).

In human serum, levels of IGFBP-3 were shown to be low in hypopituitary patients and increased after administration of GH as assessed by immunoanalysis and Western ligand blot analysis. Similarly, circulating levels of human IGF-I and ALS are considered to be directly regulated by GH. Administration of GH in the absence of IGF-I to GH-deficient rats showed that continuous administration of GH, as opposed to twice daily GH injections, is superior in restoring the IGF-IGFBP-3-ALS complex (279, 280). These data are consistent with the idea that ALS and IGF-I are directly regulated by GH while IGFBP-3 is directly regulated by IGF-I (280). Also, the IGF/IGFBP-3 ratio and the induction of all the components of the IGFBP-3 complex were observed with continuous infusion of GH. It is suggested that continuous infusion of GH would up-regulate GH receptors and stimulate production of IGF-I and provide a synergistic effect on growth promotion.

Prepubertal GH-deficient children treated with GH showed an increase in serum IGF-I and IGFBP-2, suggesting that they are the major correlates of pubertal growth (231). GH-deficient children treated with GH sustained an increase in circulating levels of IGFBP-5 and IGF-II, suggesting that IGFBP-5 and IGF-II may also play a role in mediating some of the anabolic actions of GH (228). However, whether or not this effect is due to a direct influence of GH on IGFBP-5 or due to a GH-induced increase in IGF-I and II on IGFBP-5

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(281–283) is unclear. GHD not only impairs longitudinal growth, but also decreases bone mineral content in humans. GH has been shown to have important effects on skeletal metabolism *in vitro* and *in vivo* (123, 184, 283–286). Thus, in patients with GHD of childhood onset, GH administration is often used to increase the bone mineral content (287, 288).

In contrast to GH, administration of IGF-I in adults with GHD for 7 consecutive days increased serum levels of IGF-I but decreased serum levels of IGF-II (231). Daily injections of IGF-I had no significant effect on serum levels of IGFBP-1, IGFBP-2, and IGFBP-3. In contrast, long-term treatment of 20 GH-deficient patients, aged 22–57 yr, with GH increased serum levels of IGF-I, IGFBP-3, and ALS (289). However, the percentage increase in IGF-I was higher than the other two peptides (*i.e.*, IGFBP-3 and ALS) resulting in a higher ratio of IGF-I/ALS and IGF-I/IGFBP-3 after treatment of GH-deficient adults with GH. In addition, the increase in lean body mass during GH treatment showed significant positive correlation with age, thus suggesting that IGF-I can be used as a marker in monitoring changes in lean body mass during GH-replacement therapy.

Determination of spontaneous GH secretion in detecting GHD is a time-consuming procedure, and alternate markers are often evaluated to determine their usefulness as a diagnostic measure in detecting GHD. Blum *et al.* (140) in their earlier work showed that IGFBP-3 was subnormal in GH-deficient patients and that it could serve as an excellent screening parameter for GHD. In a recent study (290), a 17.7-kDa form of IGFBP-3 that is GH dependent was detected in the serum and urine of healthy children but was absent in healthy adults and GH-deficient children. Since IGFBP-3 increases in response to GH treatment secondary to an increase in IGF-I, quantification of urinary IGFBP-3 could provide a simple and noninvasive test to detect GHD and monitor the response to GH therapy. The IGFBP-3 in urine is degraded and, therefore, the usefulness of urinary IGFBP-3 as a diagnostic tool may be partly dependent on patient-to-patient variations in this proteolytic cleavage. Further, monitoring the levels of this molecular form of IGFBP-3 as a screening tool for GHD may be considered only after further studies support these findings. Serum levels of IGF-I, IGFBP-3, and ALS were measured after GH treatment in GHD adults (291). It was observed that the sensitivity of IGFBP-3 and ALS to the GH dose was lower than that of IGF-I. Thus IGF-I levels in serum are much more useful as a diagnostic tool than serum IGFBP-3 levels for detecting GH excess in adults or in those receiving GH replacement therapy. This is because it is more sensitive than IGFBP-3 and ALS to GH doses in the high range (291). Although the value of additional measurement of either IGFBP-3 or ALS levels is limited, these measurements could improve the reliability of diagnostic assessment.

5. Acromegaly. Acromegaly is a disorder caused by hyperfunction of the pituitary gland with normal to high production of GH. Plasma levels of IGF-I and IGFBP-3 were elevated markedly in untreated acromegalic patients compared with age-matched controls (292–294). Although regulated by GH, the increase in IGFBP-3 was mediated in part via an increase in IGF-I (292). Insulin, glucose, and gut peptides have a role in altering the levels of IGFBP-3 (293–295), but further studies are needed to completely understand the physiological regulation of IGFBP-3 in acromegaly. Acromegalic patients typically demonstrate elevated serum IGFBP-1 and IGFBP-2 (294) and an elevated ratio of IGF-I/IGFBP-3, suggesting that the increase in GH level in acromegalic subjects may result in increased availability of free IGF-I (294). This ratio, however, does not provide additional diagnostic value over IGF-I measurement in clinical settings, since like IGF-I, the ratio of IGF-I to IGFBP-3 is higher for acromegalic patients compared with healthy controls. In a recent case report, Yoshida *et al.* (296) showed that a nonpulsatile GH secretion and enhanced tissue sensitivity to GH may induce hypersecretion of IGF-I and IGFBP-3 resulting in clinical acromegalic gigantism. The findings that the levels of IGF-I and IGFBP-3 were increased in acromegalic subjects while they were decreased in GH-deficient subjects are consistent with an important role for GH in regulating IGF-I and IGFBP-3 levels *in vivo*.

6. Osteoporosis. All of the IGFBPs identified thus far (IGFBP-1 through -6) are also produced by human bone cells (31, 34, 46, 153, 297–299). IGFBPs exhibit significant biological effects on bone cells, and the production of the IGFBPs is regulated by a variety of osteoregulatory agents, emphasizing a key role for IGFBPs in regulating osteoblast cell proliferation and differentiation (34, 153, 297, 300–302). A number of studies demonstrate that circulating levels of IGF-I and bone mass are positively correlated in healthy subjects and that subjects with osteoporosis have low circulating levels of IGF-I (284, 303, 304). Recent studies also showed changes in the serum levels of various IGFBPs in osteoporotic subjects compared with normal individuals (304–306). Johansson *et al.* (284) reported that serum levels of IGFBP-3 showed significant positive correlation with bone mineral density at multiple sites. Serum IGFBP-3 levels were found to be considerably less in osteoporotic subjects compared with age-matched controls (305) and was shown to increase in response to treatment with recombinant human GH (306). In addition, serum IGFBP-5 levels, measured by RIA using polyclonal antiserum, were found to be low in osteoporotic subjects (142). However, further studies are needed to evaluate whether the low levels of IGFBP-5 in the serum of osteoporotic patients represent that of intact IGFBP-5 or fragments or both.

Using ligand blot analysis, it was shown that serum IGFBP-4 levels are higher in elderly women with hip fractures with elevated PTH levels compared with age-matched controls (227). However, this needs to be confirmed using RIA in a larger population. It was also shown that the increased serum IGFBP-4 levels in those who are advanced in age show significant positive correlation with serum PTH levels (139). PTH treatment increased IGFBP-4 production in human osteoblasts *in vitro* (301). In addition, serum IGFBP-4 levels are increased during oral 1,25-dihydroxyvitamin D_3 therapy in psoriasis patients (307). These findings suggest that during calcium deficiency, the increase in serum PTH and 1,25 dihydroxyvitamin D_3 may, in addition to stimulating bone resorption, inhibit bone formation by stimulating bone cell production of IGFBP-4 (307–309). These data demonstrate that patients with senile osteoporosis who have hip

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fracture and secondary hyperparathyroidism also have a highly significant increase in the ratio of inhibitory to stimulatory IGF system components (222, 241). These two changes could lead to a decrease in bone formation and a decrease in hip bone density that may increase their propensity toward hip fractures.

7. Renal failure. Patients with chronic renal failure (CRF) demonstrated a higher capacity to bind IGFs compared with age-matched controls (310, 311). This is contributed by a significant increase in circulating IGFBP-2 and -3 (312), with a moderate increase in IGFBP-1 (145, 313). Serum IGFBP-2 and -3 levels of CRF patients are high, unlike that of serum IGFBP-1, which is reduced due in part to the high insulin levels seen in these patients. The increase in IGFBP-2 seems to be due to an increase in hepatic production and/or lowered rate of renal filtration. This excess IGFBP in CRF patients is believed to act as an inhibitor of IGF action by decreasing the free biologically active IGF (314, 315). This could explain the growth deficit seen in CRF subjects and the improvement seen in longitudinal growth after GH treatment (146, 314).

In children with chronic renal insufficiency (CRI), the growth deficit observed is reversed after renal transplants. Growth is not always sustained in these patients because a number of factors, including the treatments they receive, impair growth (316). Prednisone treatment of patients with CRI increased serum IGFBP-3 levels (311, 312, 315, 317, 318) and decreased growth rate. In contrast to IGFBP-3, serum IGFBP-1 levels were higher before prednisone treatment, but returned to normal after treatment, probably as a result of an increase in insulin concentration. It is not known whether the increase in IGFBP-3 and/or decrease in IGFBP-1 after prednisone therapy in CRI patients has any role in mediating the impairment of growth in CRI patients.

Nephrotic syndrome results in damages of the capillary wall of the arteries that supply the glomerulus and clinically leads to the loss of large amounts of protein in the urine, resulting in hypoalbuminemia. It is therefore likely that IGF-I/IGFBP complex is also filtered, affecting the circulating levels of these binding proteins. Serum IGF-I concentration and the binding to the 150-to 200-kDa complex were lower in nephrotic rats than pair-fed controls (317), while the serum level of IGFBP-2 was increased due to an increase in liver synthesis. It was shown in this study that the decrease in serum IGF-I was compensated for by an increase in a IGFBP-3 protease, which shifted the binding of IGF-I from the 150- to 200-kDa complex to the low molecular mass complex so that it permeates the vascular compartment and maintains tissue IGF-I availability.

8. Other pathological conditions. Serum IGFBP-1 and GH were elevated in cirrhotic patients compared with control subjects (319, 320). The authors suggested that this might be due to the insulin resistance seen in cirrhosis as insulin regulates IGFBP-1 production in the healthy state (319). On the other hand, a decreased level of IGFBP-3 was observed due to decreases in hepatic production. While the concentration of IGFBP-3 correlated with the severity of the hepatic disease, IGFBP-1 did not show a similar relationship. It is clear that IGFBP-1 and -3 are regulated differently and serve different functions.

Growth and wasting accompany end-stage liver disease in children and accelerated growth is feasible only after liver transplant. Before the transplant, the circulating levels of IGF-I and IGFBP-3 are low (321), similar to those observed in acute and chronic renal failure (319). The low levels of IGF-I could arise from the state of malnutrition or the presence of liver disease itself. After receiving the liver transplant, these patients demonstrate an increase in both IGF-I and IGFBP-3, with the latter increasing relatively higher than control values. It is believed that the higher IGF-I level in circulation after liver transplantation compared with the pretransplantation level would increase the endocrine action of IGFs for growth stimulation.

Serum IGFBP-1 levels were markedly increased in patients before transplant, and this persisted after transplant. IGFBP-1 thus exerts an inhibitory effect on IGF action. However, unlike in healthy subjects, insulin does not regulate IGFBP-1 levels, as high insulin secretion in these patients does not suppress IGFBP-1 production. A rise in serum IGFBP-2 as a result of dietary restriction in these patients was also observed, but the level continued to be elevated for reasons not understood. A combined increase in serum IGFBP-1 and -3 post-transplant is speculated to play a role in the growth failure or slow catch-up observed in patients after liver transplant surgery.

In severely ill patients, catabolism leads to major changes in IGFBP production and circulation in the serum, which in turn can influence IGF bioavailability. The major binding protein in the serum, IGFBP-3, was greatly decreased in severely ill patients due to the release of substrate-specific IGFBP-3 protease. Similarly, after surgery such as cholecystectomy (180) and major heart surgery (322), there is an increase in proteolysis of IGFBP-3. Based on these findings, it is speculated that the release of IGFBP-3 protease during surgery is an adaptive mechanism to ensure an adequate supply of IGF to the tissue for counteracting the catabolic state (178).

Serum IGFBP-3 decreased in hypothyroid patients and was within normal levels in hyperthyroid patients. In contrast, serum IGFBP-1 was elevated in the hyperthyroid group (323) or increased in hypothyroid patients receiving T_4 treatment (324). The patient is typically in a catabolic state in hyperthyroidism, and this may be due, in part, to elevated IGFBP-1, which suggests a decrease in bioactivity of IGFs in plasma. These conclusions have been questioned since the collection of the plasma from hyperthyroid subjects and the reference pool was not done at the same time of day. This suggests that the changes in IGFBP-1 levels between the healthy controls and hyperparathyroids may have been partly due to diurnal variation.

Based on the above mentioned discussion, it is obvious that the serum IGFBP levels are altered under various physiological and pathological conditions. Table 5 summarizes the key regulators of various IGFBP levels in circulation. Multiple mechanisms are known to be involved in regulating serum levels of various IGFBPs, including alterations in the production of IGFBPs in target cell types and degradation of IGFBPs by specific proteases. The presence of multiple binding proteins and the complexity of regulation are consistent

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TABLE 5. Regulators of serum IGFBP levels

IGFBP	IGF carrier function in serum	Daily variation	Major regulators	Alterations during physiological situation	Alterations during pathological situation
1	Minor	Yes	Insulin Glucagon	Aging	Diabetes
2	Minor	No	IGFs, GH, nutrition	Aging	Undernutrition, Tumor
3	Major	No	IGFs, GH, glucocorticoids	Puberty Aging	GH deficiency Acromegaly
4	Minor	Not known	PTH	Aging	Osteoporosis
5	Minor	Not known	IGFs, glucocorticoids	Puberty Aging	Osteoporosis
6	Minor	Not known	Retinoic acid	Not known	Not known

with a key role for IGFBPs in regulating the actions of IGFs in response to a multitude of systemic and local effectors.

VIII. IGFBP Proteases in Circulation

In a variety of physiological and pathological conditions, the relative ratio of IGFs to IGFBPs may vary to facilitate tissue-specific growth-promoting effects of the IGFs. One of the mechanisms by which this can be accomplished is by the activity of a specific IGFBP protease that degrades the IGF+IGFBP-3 complex to increase the free IGF concentration. IGFBP-3-specific protease is triggered under a variety of stress-related conditions and enables the cleavage of the IGF+IGFBP complex, making more free IGF available for growth-stimulating activity.

A. Proteases under normal conditions

IGFBP-3 proteolysis is known to occur under normal conditions (25, 191, 325–327) in a variety of biological fluids such as peritoneal, follicular, amniotic, seminal, and cerebrospinal fluids. In addition, amniotic fluid contains a IGFBP-1-specific protease and cerebrospinal fluid contains a IGFBP-2 protease (91). In addition to tissue fluids, a variety of cell types in culture secrete proteases capable of degrading IGFBPs. Some of these proteases are relatively specific to a given IGFBP while others are relatively nonspecific. For example, bone cells in culture produce matrix metalloproteases and plasmin, which degrade multiple IGFBPs as well as other proteins (328). In addition, human bone cells as well as fibroblasts in culture produce proteases that are relatively specific to IGFBP-5 (281, 282, 329). Endogenous IGF-II and tumor necrosis factor-β stimulate IGFBP-4 protease (282, 330, 331), and IGF-II inhibits IGFBP-5 protease (282). The finding that IGFs regulate IGFBP levels by regulating proteolysis in a variety of cell types raises the interesting possibility that IGFs function to regulate IGFBPs, as well as *vice versa*. IGFBP-2-, -4-, and -5-specific proteases have been detected in smooth muscle cells (332) with IGFBP-4 protease capable of increasing the bioavailable IGF-I. IGFBPs 1–4 were found in human skin interstitial fluid (128) with IGFBP-3 in the modified form due to an increased IGFBP-3 protease activity (128, 333). Although IGF concentration in the interstitial fluid is lower than in serum, it is more available due to proteolytic activity for IGFBPs (128). Synovial fluid from both normal individuals and patients with rheumatoid arthritis (130) also contain IGFBP-3 protease activity, which acts to regulate IGF bioavailability. IGFBP-4 levels in estrogen-dominant follicular fluid were lower than levels found in androgen-dominant follicular fluid because of the presence of a metalloserine IGFBP-4 protease activity in the former. It is speculated that the presence of IGFBP-4 protease in estrogen-dominant follicular fluid would decrease the inhibitory IGFBP-4 level and increase bioavailability of IGF. It is also speculated that the available IGFs stimulate estradiol production along with gonadotropins in the growing follicular tissues (41). Significant proteolysis of IGFBP-3 was shown in the reproductive tract of men, and the intact IGFBP-3 levels were inversely related to the PSA concentration (334). Thus, proteases have been postulated to aid in modulating IGF action in a tissue-specific manner.

B. Pregnancy-associated proteases

One of the first identified IGFBP proteases was a pregnancy-associated IGFBP-3-specific protease that is active from the second trimester of pregnancy in humans (135, 179). This is specific to the reproductive tissues and helps to meet the *in utero* fetal growth demand. The potential role of IGFBP-3 protease in regulating the bioavailability of IGFs has been discussed earlier. In addition to IGFBP-3 protease, a IGFBP-5 protease that degrades IGFBP-3, -4, and -5 was detected in maternal serum and amniotic fluid (119). The protease activity is also increased in relation to fetal demands as seen by a higher serum protease activity in mothers with multiple fetuses or with fetuses affected by *in utero* placental insufficiency (203) and in GH-deficient pregnant rats (275). The regulation of these proteases is now being investigated.

C. Proteases under catabolic and disease states

Another common condition that increases serum protease activity is a general catabolic state. For example, IGFBP-3 specific protease was detected in patients with severe illnesses and in a catabolic negative nitrogen balance (178, 180). Protease activity is more significant during fasting and declines after parenteral nutrition (322, 325), suggesting that there is a specific response to catabolism. Another example of a catabolic state occurs in patients with acute and chronic renal failure. These patients showed an increase in urinary IGFBP-3-specific protease, which was responsible for the complete absence of intact IGFBP-3 in the urine (276). Significant proteolytic activity of IGFBP-3 protease was observed in acute diseases of childhood such as Kawasaki disease, bacterial meningitis, and mycoplasma pneumonia (277). A high amount of IGFBP-3 protease activity was de-

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tected in the CSF of children with CNS tumors (95). It is speculated that IGFBP-3 proteolysis plays a role in overcoming catabolic conditions by increasing the availability of IGF-I.

Insulin has an important role in regulating the activity of IGFBP-3-specific protease as shown in studies that demonstrated an elevated serum IGFBP-3 protease activity in untreated NIDDM (335) and IDDM (137) patients. This appears to be regulated by insulin levels, as shown by a decline in the protease activity after insulin therapy. Therefore, the catabolic state induced by insulin deficiency is counteracted to some extent by an increase in the IGFBP protease activity.

The observations that IGFBP protease activity was detected in various biological fluids during pathological and physiological situations suggest a role for protease in conditions of increased growth such as in pregnancy, and also when a person is in a catabolic state indicated by an illness, infection, cancer, or diabetes. The increase in protease activity during these situations helps to increase the dissociation of IGF from the 150- to 200-kDa complex so that more free IGF is available for promoting growth. The finding that a number of IGFBP proteases have been identified in a variety of biological fluids (Table 6) and the finding that the rate of IGFBP proteolysis can be regulated by a variety of systemic and local effectors (327, 336–342) raise interesting possibilities for the involvement of IGFBP protease in regulating the endocrine and local actions of IGFs (see Figs. 6 and 7).

IX. Endocrine Functions of IGFBPs in Serum

IGFBPs have been proposed to play a role in modulating the actions of IGFs by regulating their availability to target tissues. In this regard, the serum levels of IGFBPs may play an important role in regulating the endocrine actions of IGFs. The proposed functions of IGFBPs that aid in regulating the endocrine actions of IGFs are: 1) inhibition of insulin-like activity; 2) a prolongation of the plasma half-lives of IGFs; and 3) regulation of the rate of transport of IGFs from the vascular compartment to enhance the growth-potentiating effects of IGFs. In addition to the endocrine effects, IGFs are also involved in local regulation, where IGFs produced by one cell type act in an autocrine or paracrine manner. The finding that certain extracellular body fluids are enriched with one or more IGFBPs and that local body fluids contain protease(s) capable of degrading IGFBPs into forms that do not bind IGFs or bind IGFs with very low affinity are consistent with a role for IGFBPs in modulating the actions of IGFs either positively or negatively in the local milieu.

A. To prevent insulin-like effects

In adult humans, the mean IGF concentration is about 800 μg/liter, with IGF-I and -II contributing about 200 μg/liter and 600 μg/liter, respectively. This amount is 1000-fold higher than mean insulin concentration. Despite the fact that insulin-like activity of IGFs is only 5% of insulin, the IGFs could in theory contribute 50 times more insulin-like activity than insulin alone due to their abundance (6, 58, 59, 149, 343). However, this does not occur because the activity of IGFs is largely neutralized as a consequence of IGFBP binding. Thus, without the IGFBPs, the effect of IGFs would overwhelm the effect of insulin alone, and because IGF expression is not tightly controlled by blood glucose levels, blood glucose homeostasis would not be possible.

B. To increase the half-lives of IGFs

IGFBPs are known to facilitate IGF endocrine action by increasing the half-life of the IGFs in circulation. Both IGF-I and IGF-II, when injected into hypophysectomized rats, disappeared in approximately 20–30 min, while in normal rats the half-life was extended to about 15–20 h (81). This difference is due to the fact that the majority of IGFs circulate in serum as a 150- to 200-kDa ALS+IGFBP-3+IGF ternary complex as described previously. The stabilization of IGFs thus depends mainly on IGFBP-3, with other IGFBPs having minimal roles in this function. IGFBP-1 and -2, for example, had shorter half-lives in serum (161) and thus seem unlikely to play any role in stabilizing the IGFs in the circulation. Thus, by increasing the half-life of IGFs and protecting them from rapid degradation, IGFBP-3 alters the ratio of free IGF to bound IGF, which in turn regulates the metabolic actions of the IGFs. Consistent with the idea that IGFBPs may increase the endocrine actions of IGFs by increasing their half-lives, several reports document that systemic administration of an equimolar dose of IGFBP-3+IGF-I complex is more effective than IGF-I alone in stimulating bone formation in ovariectomized rats (344, 345).

C. To control the transport of IGFs from the vascular space

The transportation of serum IGFs from the vasculature into the extracellular fluid is necessary in order for IGFs to elicit a growth-stimulating response. For this to be accomplished, the IGFBPs bound to the IGFs in circulation must be either degraded or should be able to cross the endothelial barrier (Fig. 6). The majority of IGFs exist as a ALS-IGFBP-3-IGF complex and, as such, other IGFBPs, including IGFBP-1 and IGFBP-2, remain unsaturated in plasma since they are not the predominant binding proteins (59) for IGFs.

TABLE 6. Characterization of IGFBP proteases in biological fluids

Protease	Class	IGFBP specificity	Broad protein specificity	Biological fluid
BP-3 protease	Serine protease	BP-3	Not known	Maternal serum
Matrix metalloprotease	Metalloprotease	BP-3	Yes	Maternal serum
Plasmin	Serine protease	BP-3, BP-1, BP-4, BP-5	Yes	Serum
Cathepsin	Acid protease	BP-3	Yes	Serum
PSA	Serine protease	BP-3, BP-5	Yes	Seminal plasma
7S NGF	Serine protease	BP-3, BP-4, BP-5, BP-6	Yes	Serum

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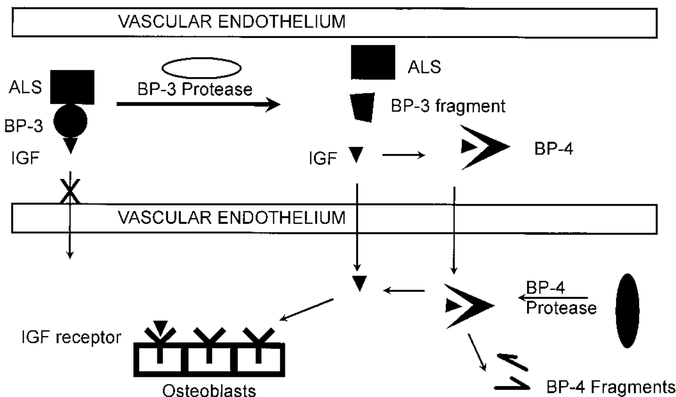

FIG. 6. Modulation of IGF bioavailability by IGFBP proteolysis. IGF proteases may regulate the availability of IGFs by controlling the transport of IGFs from the vascular space into tissue space. The majority of IGFs exist as ALS-IGFBP-3-IGF complex in the serum, which does not cross the vascular barrier. Since transportation of serum IGFs from the vasculature into the tissue space is necessary in order for IGFs to elicit hormonal growth-stimulating responses, the IGFs bound to IGFBP-3-ALS complex must be released first. This can be accomplished by the IGFBP-3 protease produced by vascular endothelial cells or by IGFBP-3 protease present in serum. Proteolysis of IGFBP-3 by IGFBP-3 protease results in disruption of this complex and release of IGFs. IGFs, thus released, may get transported into the tissue space or may bind to other IGFBPs such as IGFBP-4 and cross the endothelium. Because the small molecular mass IGFBPs are present in excess, this is likely to occur. The binding of IGFs to these small molecular mass IGFBPs may protect the IGFs from degradation and may also increase half-life in the circulation. Upon transport into the tissue space, IGFBP-4 protease produced by target cells may release the IGFs to bind to IGF receptor and exhibit a growth-promoting response. Thus IGFBP protease may play a role in controlling the transport of IGFs into the tissue space and regulating the availability of free IGFs in the tissue space.

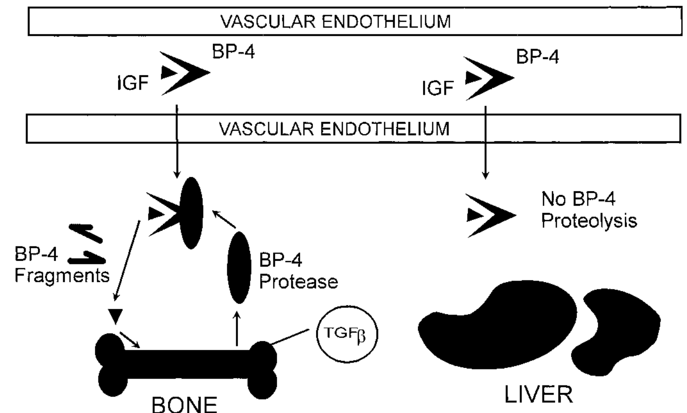

FIG. 7. Tissue-specific regulation of IGF bioavailability by IGFBP proteases. IGFBP proteases may also regulate bioavailability of IGFs in a tissue-specific manner. Based on the finding that proteolysis of IGFBP4 can increase the amount of local IGF available for receptor interaction and based on the findings that a number of local growth factors (*e.g.*, TGFβ) can regulate IGFBP 4 proteolysis (330), it can be speculated that some effectors may increase the local production of IGFBP proteases in specific tissues and, in turn, may degrade the inhibitory IGFBPs in extracellular fluid and thus increase the free level of IGFs for receptor interaction. Thus, tissue-specific regulation of IGFBP proteolysis may provide a mechanism to increase site-specific bioavailability of serum IGFs depending on the local needs.

However, their ability to cross the endothelial barriers intact (346) makes them important IGF transporters to target tissues. Unlike the complex with IGFBP-1 and -2, the IGFBP-3-IGF-ALS complex, which contains more than 75% of the circulating IGFs, does not migrate from the vascular compartment. The ALS+IGFBP-3+IGF complex serves as a reservoir for IGF release, which is determined by 1) a drop in free IGF or 2) the action of certain proteases. Proteolytic cleavage can reduce the affinity of the IGFBP-3 for IGFs by 20–30 times (135) and facilitates equilibration with the smaller molecular mass binding protein complex in serum (Fig. 6). Because the affinity of IGFs to IGFBP-3 is reduced upon binding to glycosaminoglycans, it is speculated that glycosaminoglycans present on the surface of endothelial cells may control the efflux of the IGFs out of the vascular compartment (346). Although this is an attractive theory, further experimental data are required to support this function for extracellular matrix proteins produced by vascular endothelial cells.

The shifting of IGF from the 150- to 200-kDa complex to a 50-kDa IGF complex does not necessarily increase IGF bioavailability in target tissues since many of the IGFBPs (*e.g.*, IGFBP-1, IGFBP-2, IGFBP-4, and IGFBP-6) inhibit IGF actions by preventing the binding of IGFs to their receptors. Thus, the inhibitory IGFBP in the 50-kDa IGFBP+IGF complex must be degraded in the local tissue to release the IGF from this circulating complex in a bioavailable form (Fig. 7). Based on the above mentioned discussion, two sequential mechanisms are essential to increase the availability of free IGFs to local tissues: First, release of IGFs from the 150- to 200-kDa complex by disruption of ALS+IGFBP-3+IGF complex (*e.g.*, proteolysis of IGFBP-3); second, release of IGFs from a 50-kDa inhibitory IGFBP+IGF complex by disruption of this complex (*e.g.*, proteolysis of inhibitory IGFBP-4). Consistent with the idea that locally produced IGFBP proteases may play a role in regulating IGF action, a number of studies have shown that the activity of the IGFBP proteases in biological fluids may be regulated based on the physiological and/or pathological conditions. In addition, *in vitro* studies have shown evidence that a number of cell types and cultures produce proteases capable of degrading one or more IGFBPs, which are regulated by both local and hormonal effectors (100, 124, 157, 282, 329, 331, 347, 348).

Thus the 150- to 200-kDa and 50-kDa IGFBP complexes may have a specific role either to increase half-life, prevent hypoglycemia, or to facilitate transport of IGF out of the vascular compartment. These functions ultimately are responsible for increasing the bioavailability of free IGF for mitogenic/differentiation activity in the target tissues. Several mechanisms have been suggested to cause an increase in the bioavailability of free IGFs in the target tissues, such as 1) increasing the ratio of IGFs/inhibitory IGFBPs; 2) increasing the ratio of stimulatory IGFBPs/inhibitory IGFBPs; 3) increasing or decreasing the rate of proteolysis of inhibitory or stimulatory IGFBPs, respectively; and 4) increasing IGF receptor abundance in the target tissue (149). Essentially, factors that can alter the effective concentration of IGFBPs can, in turn, regulate the availability of free IGF.

Since IGFs present in the 50-kDa complex can exist as complexes of both stimulatory and inhibitory binding proteins, it is essential that the regulation of these two types of

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binding proteins occur in a reciprocal manner to produce a significant biological effect. Several physiological and pathological conditions, including age, development, nutrition, exercise, surgery, and pregnancy, have been shown to affect the ratio of IGF to IGFBP (78, 162, 178–180, 214, 231, 273). For example, the relative ratio of inhibitory IGFBP-4 to stimulatory IGFBP-5 increases with advancing age, a change that would tend to decrease the endocrine actions of IGFs (222). It is anticipated that future research studies would provide experimental data to demonstrate a role for IGFBPs and their proteases in regulating the endocrine IGF actions depending on the needs of the target tissues.

X. Conclusions

The complexity of the IGFBP system in biological fluids is shown by the presence of six IGFBPs, multiple IGFBP proteases, and the intricate regulation of IGFBPs and IGFBP proteases during various physiological and pathophysiological situations. Although the finding that tissue fluids are enriched with one or more of the IGFBPs suggests a role for the IGFBPs in modulating the actions of IGFs in a tissue-specific manner, the exact roles of the IGFBPs in biological fluids are still poorly understood. Recent development of analytical methods for measuring various IGFBPs, and the potential availability of large amounts of recombinant DNA-derived IGFBPs for animal and human studies, should provide a better understanding of the physiological role of IGFBPs in various tissues.

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