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The Effect of GH Therapy on the Immunoreactive Forms and Distribution of IGFBP-3, IGF-I, the Acid-Labile Subunit, and Growth Rate in GH-Deficient Children

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We have previously shown that the major correlates of growth following growth hormone (GH) therapy in growth hormone-deficient (GHD) children are changes in circulating insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3), suggesting a synergistic interaction between IGF-I and IGFBP-3 *(1)*. The first aim of this project was to examine the molecular forms of IGFBP-3 and the acid-labile subunit (ALS), and to assess the changes in these molecular forms during GH administration to GHD children. Plasma samples from prepubertal GHD patients, prior to therapy and during the first year of GH treatment, were subjected to Western ligand and immunoblot analysis. Densitometric analysis of Western ligand blotting (WLB) showed a 76% increase in IGFBP-3 ($p = 0.02$), but a 56% decrease in 36-kDa IGFBP-2 ($p = 0.03$) during GH therapy. Western immunoblot (WIB) analysis of IGFBP-3 revealed the presence of intact (40- to 45-kDa doublet) as well as a proteolyzed (28-kDa) form of IGFBP-3 in the serum of GHD and healthy children. Both immunoreactive forms of IGFBP-3 increased by 64% during GH therapy (intact $p = 0.003$; proteolyzed $p = 0.0001$). WIB analysis of the ALS showed an 84-to 86-kDa doublet, which increased by 41% with GH therapy ($p = 0.01$). The response to GH therapy, as measured by the height velocity standard deviation score (SDS) adjusted for bone age, correlated with the percent change in total IGFBP-3 ($r = 0.772$, $p = 0.002$ by WIB), intact IGFBP-3 ($r = 0.845$, $p = 0.0005$ by WLB; $r = 0.541$, $p = 0.05$ by WIB), and proteolyzed IGFBP-3 ($r = 0.703$, $p = 0.007$), as well as with the percent change in ALS ($r = 0.813$, $p = 0.014$).

The second aim of this project was to assess the changes in distribution of the immunoreactive forms of IGFBP-3 and IGF-I among the ternary (ALS/IGFBP-3/IGF) complex, the binary (IGFBP-3/IGF) complex, and uncomplexed IGF during the first year of GH therapy, and to explore further the correlation with growth response to GH. Plasma samples, prior to therapy and after the first year of GH treatment, were separated by neutral size-exclusion chromatography and then subjected to IGFBP-3 immunoradiometric assay (IRMA), IGFBP-3 WIB, and IGF-I IRMA analysis. IGFBP-3 increased in both the ternary ($p < 0.0001$) and binary ($p = 0.01$) complexes, but there was a shift in the percentage of IGFBP-3 from the binary to the ternary complex during GH therapy. Both intact and proteolyzed forms of IGFBP-3 were found in both the ternary and binary complexes, but the shift occurred primarily for the proteolyzed (28-kDa) form ($p = 0.001$). There was a significant increase in IGF-I in the ternary ($p = 0.001$) and binary ($p = 0.005$) complexes, but not in uncomplexed IGF-I. The percentage of IGF-I in the ternary complex increased ($p = 0.006$), whereas the percentage of uncomplexed IGF-I decreased ($p = 0.02$), during GH therapy. Growth rate, assessed by the height velocity SDS for bone age, correlated best with the changes in ternary complex IGFBP-3 ($r = 0.72$, $p = 0.01$) and ternary complex IGF-I ($r = 0.56$, $p = 0.10$).

In conclusion, GH treatment of GHD children results in significant increases of intact, proteolyzed, and total IGFBP-3, as well as an increase in ALS, which all correlate with the growth response to GH therapy. In addition, GH treatment results in increases in ternary complex IGFBP-3 and IGF-I, which also correlate with the response to therapy. We suggest that the formation of the ternary complex may be a determining factor in the somatic growth response.

Key Words: GH; IGFBP-3; IGF-I; ALS; growth; ternary complex.

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Introduction

The insulin-like growth factors (IGFs) are recognized as important determinants of somatic growth. The IGFs exist

in the circulation complexed to IGF binding proteins (IGFBPs), which have been shown to increase the half-lives of IGFs, and to modulate their bioactivities and bioavailabilities *(2–8)*. Six high-affinity IGFBPS have been classified; the predominant serum IGFBP is IGFBP-3, a glycosylated protein of 40–45 kDa, which has also been found in a proteolyzed 28-kDa form *(8)*. IGFBP-3 is the only IGFBP that can circulate as part of a large 130- to 150-kDa ternary complex in association with IGF and an acid-labile subunit (ALS) *(3)*. The role of ALS has not been completely determined, but ALS may have a central role in regulating the bioavailability of the IGFs by stabilizing the IGF–IGFBP-3 complex.

In a previous study examining the changes in IGF-I, IGFBP-3, growth hormone binding protein (GHBP), erythrocyte IGF-I receptors, and the growth rate in prepubertal growth hormone-deficient (GHD) children during growth hormone therapy, we have shown that the major correlates of growth are changes in the concentrations of circulating IGFBP-3 and IGF-I *(1)*. We hypothesized that IGFBP-3 acts synergistically with IGF-I in the promotion of growth in GHD, as well as normal children. Administration of IGF-I has been shown to promote growth in GH receptor-deficient (GHRD) children, but the response to IGF-I in GHRD was less dramatic than the growth response to GH in GHD *(9)*. GH therapy of GHD children, however, is associated not only with significant changes in IGF-I, but also in immunoreactive levels of IGFBP-3 *(1,10)*. The purpose of the present study was to assess the changes in the molecular forms of the IGFBPs (particularly IGFBP-3 and its proteolyzed 28-kDa form) and ALS, as well as the changes in distribution of both immunoreactive forms of IGFBP-3 and IGF-I among the ternary (ALS/IGFBP-3/IGF) complex, the binary (IGFBP-3/IGF) complex, and uncomplexed IGF during GH therapy of GHD children, and to determine whether these changes correlate with the growth response.

Results

Western Ligand Blot (WLB) Analysis

To assess the changes in the IGFBPs, samples from the 17 prepubertal GHD patients, prior to therapy and during the first year of GH treatment, were subjected to WLB and Western immunoblot (WIB) analysis. Western ligand blots using iodinated IGF-I and IGF-II revealed a 40- to 45-kDa doublet representing IGFBP-3, as well as a smaller mol-wt band presumably representing 36-kDa IGFBP-2. Smaller mol wt IGFBPs, presumably IGFBP-1 and IGFBP-4, were also detected. IGFBP-1 and IGFBP-4 did not show significant changes during GH therapy (IGFBP-1 levels fluctuate widely during the day and IGFBP-4 bands were very faint). Densitometric analysis of WLB showed a 76% increase in IGFBP-3 and a 56% decrease in 36-kDa IGFBP-2 during the 12 mo of GH therapy (Fig. 1).

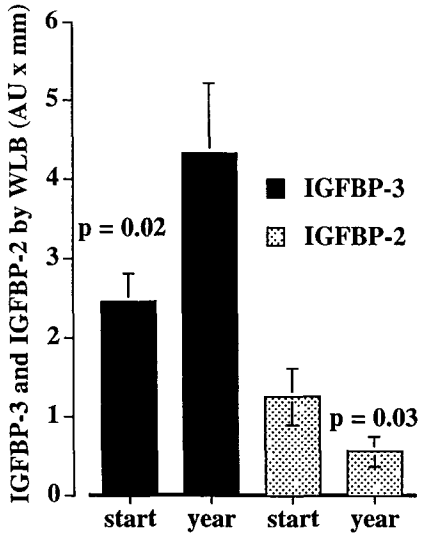


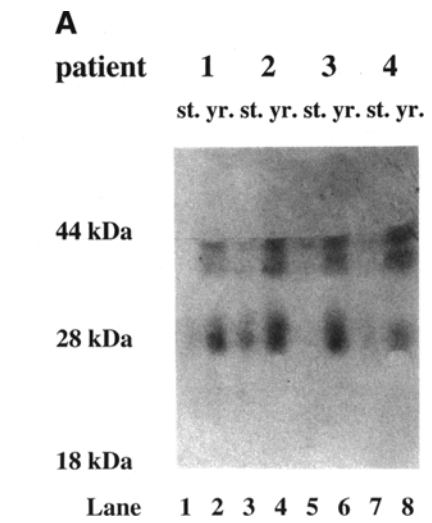
Fig. 1. Changes in GHD plasma IGFBP-3 and IGFBP-2 levels after 1 yr of GH treatment. Five microliters of each pre- and posttreatment sample were subjected to SDS-PAGE and WLB analysis ($n = 17$). Autoradiographs were subjected to densitometric analysis. The means and SEM are shown here.

WIB Analysis, IGFBP-3

In order to ensure that the 40- to 45-kDa doublet was, indeed, IGFBP-3 and to detect the presence of other immunoreactive forms of IGFBP-3, WIB analysis was performed using a specific antibody against human IGFBP-3. WIB analysis of IGFBP-3 revealed the presence of the intact (40- to 45-kDa doublet) as well a proteolyzed (28-kDa) form of IGFBP-3 in GHD and healthy children. The blots of four randomly selected GHD patients (pretreatment lanes 1, 3, 5, and 7; posttreatment lanes 2, 4, 6, and 8) are shown in Fig. 2A. Note the diminished intensity of the 40- to 45-kDa doublet, as well as the 28-kDa band, in the pretreatment samples compared with samples from patients treated with GH for 12 mo. Densitometric analysis of 17 patients demonstrated that the proteolyzed 28-kDa and intact 40- to 45-kDa forms of IGFBP-3 were present in similar proportions. Both immunoreactive forms of IGFBP-3 increased by 64% following 12 mo of GH therapy (Fig. 2B).

Deglycosylation Study

The 28-kDa band may represent an important form of IGFBP-3, although its significance to date is unknown. However, the 28-kDa form has been described in pregnancy serum, fetal serum, and in GHRD serum. To evaluate further the 28-kDa immunoreactive form of IGFBP-3, a deglycosylation analysis was performed (Fig. 3). If the 28-kDa form is a core nonglycosylated IGFBP-3, the molecular weight will not shift with deglycosylation. In normal human serum (lane 3), the 40- to 45-kDa doublet and 28-kDa band are present. The addition of a deglycosylating enzyme (lane 4) results in loss of the 28-kDa band and the appearance of a smaller band of 18 kDa. The 40- to



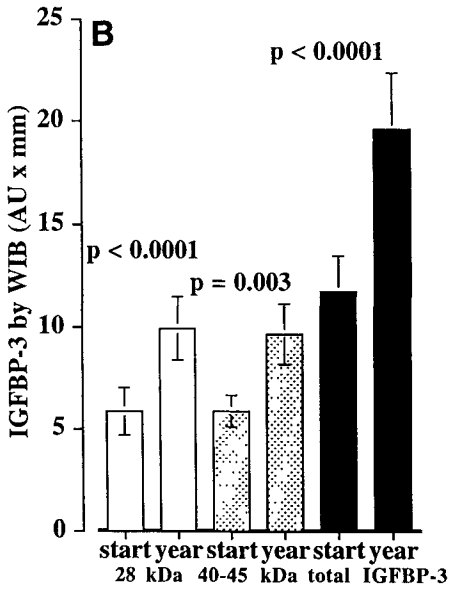


Fig. 2. (A) Changes in plasma IGFBP-3 levels after 1 yr of GH treatment, as assessed by WIB. Five microliters of each pre- and posttreatment sample were evaluated. A representative group of four autoradiographs is shown here. Pretreatment samples are seen in lanes 1, 3, 5, and 7, whereas posttreatment samples are seen in lanes 2, 4, 6, and 8. Molecular-weight markers electrophoresed through the same gel are indicated on the left side of the panel. **(B)** WIB analysis of IGFBP-3 levels in 17 patients. Five microliters of each pre- and posttreatment sample were assessed, and subjected to densitometric analysis. The means and SEM are shown here ($p < 0.0001$ for 28 kDa and total, $p = 0.003$ for 40–45 kDa).

45-kDa doublet is shifted to a 29- to 34-kDa band. Similarly, in plasma of a GHD child (lane 1), the 40- to 45-kDa doublet is deglycosylated to a 29- to 34-kDa band (lane 2), whereas the 28-kDa band is absent and a 18-kDa band is detected (lane 2). Thus, the 28-kDa form of IGFBP-3 is not the core nonglycosylated form of IGFBP-3, based on electrophoretic migration and deglycosylation studies. The 28-kDa IGFBP-3 is a glycosylated 18-kDa IGFBP-3 fragment.

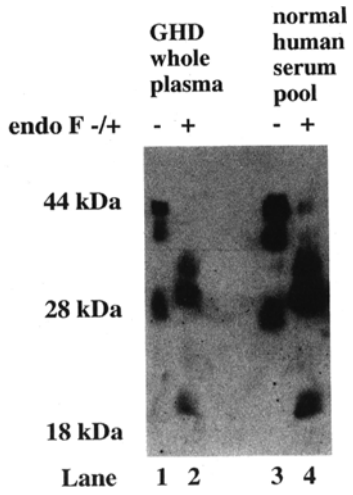


Fig. 3. Deglycosylation of IGFBP-3: intact (endo F "–") vs deglycosylated (endo F "+") GHD whole-plasma and normal human serum pool. Fifty microliters each of a GHD whole-plasma sample and a normal human serum pool were deglycosylated during a 3-h 37°C incubation with 200 μU endo F, following adjustment of sample pH to 5.0. Controls were also run, incubating another set of the same samples with no endo F. All samples were then subjected to SDS-PAGE and WIB analysis in order to compare the molecular weights of the deglycosylated IGFBP-3 to the intact forms.

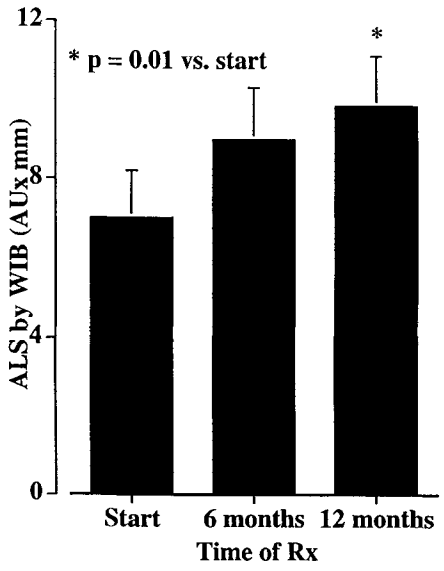


Fig. 4. Changes in GHD plasma levels of ALS. Samples from eight patients were subjected to SDS-PAGE, WIB (using anti-ALS antiserum R00934 from Diagnostics Systems Laboratories), and densitometric analysis. The means and SEM are shown here.

WIB Analysis, ALS

ALS has been reported to be GH-dependent, and is an integral component of IGFBP-3 stability and function in the 130- to 150-kDa ternary complex. Thus, to assess changes in ALS during GH therapy, WIB analysis was performed. A 84- to 86-kDa doublet was detected representing the glycosylated form of ALS, which increased 41% with GH therapy (Fig. 4).

Table 1
Correlations Between Growth Velocity During 1 yr of GH Treatment and the Changes in the Immunoreactive Forms of IGFBP-3 and ALS[a]

	r value	*P* value
Total IGFBP-3 by WIB	0.772	0.002
28-kDa form of IGFBP-3, by WIB	0.703	0.007
40- to 45-kDa form, by WIB	0.541	0.05
40- to 45-kDa form, by WLB	0.845	0.0005
ALS, by WIB	0.813	0.014

[a]The changes in IGFBP-3 and ALS were assessed via densitometric analysis of Western ligand (WLB) and immunoblots (WIB) and expressed as percent change (1 yr minus start, divided by start). Growth rate was expressed as the height velocity standard deviation score adjusted for bone age (HVSDSBA). Linear regression was performed comparing the changes in IGFBP-3 and ALS with the HVSDSBA during 1 yr of GH treatment. Statistical analysis reveals a significant correlation between growth rate and the change in both immunoreactive forms of IGFBP-3 as well as ALS.

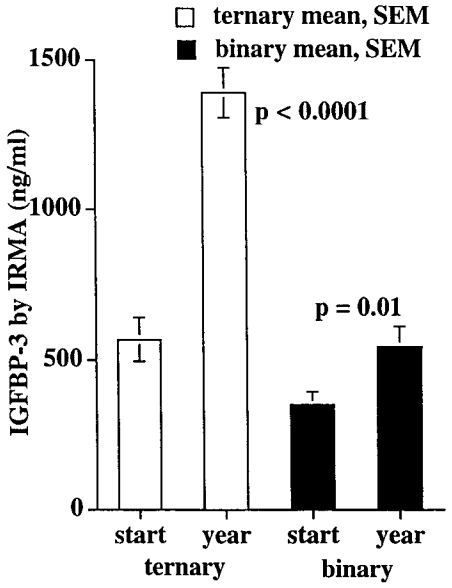


Fig. 5. Mean (± SEM) plasma IGFBP-3 levels in ternary vs binary complex, measured in 12 GHD patients before and after 1 yr of GH treatment. Following size-exclusion chromatography over a Superose-12 column and isolation of the ternary from the binary complex, concentrations were determined via IRMA. IGFBP-3 in the ternary form increased dramatically ($p < 0.0001$), from 568 to 1390 ng/mL. There is a less pronounced increase in the binary form ($p = 0.01$), from 351 to 544 ng/mL.

Growth Response vs Changes in IGFBP-3 and ALS by Western Analysis

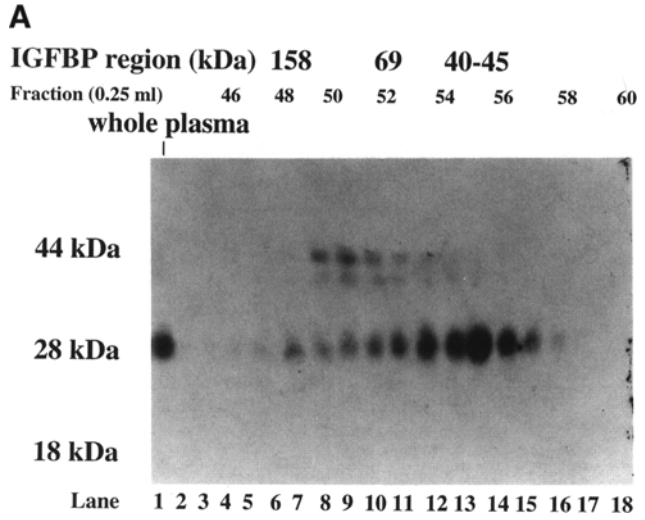
There was a wide variation in growth response to GH treatment, although all children achieved at least a normal growth velocity. The growth response was expressed as the height velocity standard deviation score adjusted for bone age (HVSDSBA), which ranged from 0.2 to 11.9, with a mean of 5.4. We have previously shown that the HVSDSBA during the year of GH therapy correlates with the 12-mo change in serum IGF-I measured by radioimmunoassay (RIA) ($r = 0.72$, $p = 0.005$), as well as with the 12-mo change in serum IGFBP-3 measured by RIA ($r = 0.81$, $p < 0.001$) *(1)*. We were, therefore, interested in determining which molecular forms of IGFBP-3 correlated with the growth response. The response to GH therapy, as measured by the HVSDSBA, correlated with the percent change in 40- to 45-kDa IGFBP-3 (Table 1) by WLB ($r = 0.845$, $p = 0.0005$). The growth response also significantly correlated with the percent change in total ($r = 0.772$, $p = 0.002$), intact 40- to 45-kDa ($r = 0.541$, $p = 0.05$), and proteolyzed 28-kDa ($r = 0.703$, $p = 0.007$) forms of IGFBP-3 as measured by WIB, as well as with the percent change in ALS ($r = 0.813$, $p = 0.014$).

Ternary and Binary Complex IGFBP-3 by IRMA

To define the distribution of IGFBP-3 and IGF-I, IGFBP regions were separated by neutral size-exclusion chromatography into three regions: a 130- to 150-kDa region representing the ternary (ALS/IGFBP-3/IGF) complex, a 40- to 45-kDa region representing the binary (IGFBP-3/IGF) complex and free IGFBP-3, and a 7-kDa region representing uncomplexed IGF. Fractions from within each of the first two regions were pooled and then subjected to IGFBP-3 immunoradiometric assay (IRMA) analysis. The concentration of IGFBP-3 in both the ternary and binary regions increased following the year of GH therapy; however, there was a greater increase in the ternary IGFBP-3 (Fig. 5). This resulted in an increase in the percentage of total IGFBP-3 found in the ternary complex (and a decrease in the percentage found in the binary complex) during GH treatment.

Ternary and Binary Immunoreactive IGFBP-3 by Western Immunoblot

Individual fractions of chromatographed plasma from each patient were subjected to WIB analysis prior to pooling to determine whether the intact (40- to 45-kDa) and proteolyzed (28-kDa) forms of IGFBP-3 were found in the high-mol-wt regions representing the ternary complex or in the lower-mol-wt regions representing the binary complex. A blot from one patient prior to therapy is seen in Fig. 6A, and the blot from the same patient after a year of GH therapy is seen in Fig. 6B. Bands corresponding to the 44-kDa marker in lanes 2–10 represent the intact IGFBP-3 doublet from the ternary complex, whereas bands corresponding to the 44-kDa marker in lanes 11–18 represent the intact IGFBP-3 doublet from the binary complex. Bands corresponding to the 28-kDa marker in lanes 2–10 represent the 28 kDa proteolyzed IGFBP-3 from the ternary complex, whereas bands corresponding to the 28-kDa marker in lanes 11–18 represent proteolyzed IGFBP-3 from the binary



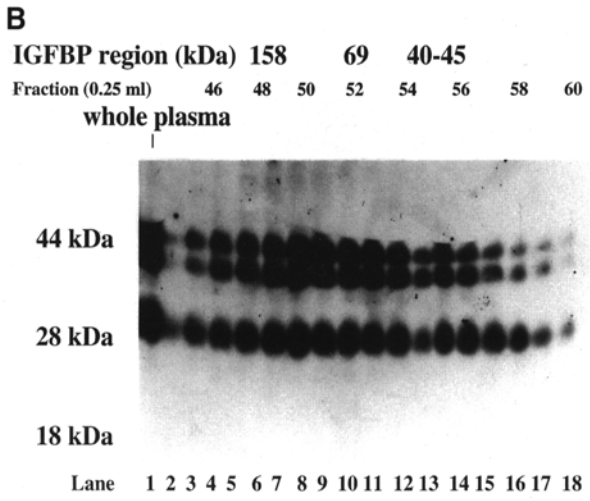


Fig. 6. GHD child plasma drawn pretreatment **(A)** and following 12 mo GH therapy **(B)**. IGFBP-3 characterization by WIB, following neutral size-exclusion chromatography over a Superose-12 column. The mol-wt regions separated through neutral size-exclusion chromatography are indicated across the top of the panel: 158, 69, and 40–45 kDa. The fraction numbers (46–60) are also indicated. The mol-wt markers subjected to electrophoresis through the gel are indicated on the left side of the panel. Comparison of the two blots reveals a greater increase in the ternary (130- to 150-kDa) than in the binary (40- to 45-kDa) region after 12 mo of GH therapy, particularly for the 28-kDa IGFBP-3 variant.

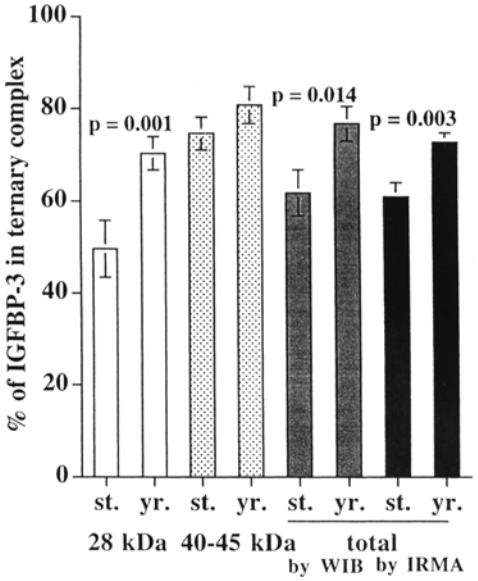


Fig. 7. The distribution of immunoreactive forms of IGFBP-3, between the binary and ternary complex, before and after 1 yr of GH therapy, as assessed by WIB . Data for each immunoreactive form are expressed as percent found in the ternary region (remainder found in the binary region). Densitometric analysis was performed on WIBs (following neutral size-exclusion chromatography) representing the fractionated plasma samples of 13 GHD children. The mean % of the total 28-kDa IGFBP-3 in the ternary complex increased significantly (p = 0.001) from 49.6 to 70.4%, whereas the 40- to 45-kDa form increased to a much lesser extent, from 74.6 to 80.1%. Conversely, the % of 28-kDa IGFBP-3 in the binary complex decreased from 50.4 to 29.6%, and the % of 40- to 45-kDa IGFBP-3 in the binary complex decreased from 25.4 to 19.9%. The percentage of total IGFBP-3 in the ternary region increased significantly (p = 0.01) from 61.8 to 76.8% (decreasing from 38.2 to 23.2% in the binary region) when measured by WIB, and from 60.9 to 72.8% (p = 0.003) when measured by IRMA (whereas total IGFBP-3 in the binary region decreased from 39.1 to 27.2%).

complex. Both intact and proteolyzed forms of IGFBP-3 were found in both the 130- to 150-kDa and 40- to 45-kDa regions prior to and after GH therapy for each patient. During the 12 mo of GH therapy, a shift was seen primarily for the 28-kDa proteolyzed form of IGFBP-3 from the 40- to 45-kDa (binary complex) region to the 130- to 150-kDa (ternary complex) region (Figs. 6A,B). Fractionated plasma from all patients was assessed by WIB and quantitated by densitometric analysis to compare baseline with 12 mo of GH therapy. There was a significant increase in the percentage of total 28-kDa IGFBP-3 found in the ternary complex (and decrease in the percentage of IGFBP-3 found in the binary complex) during GH therapy (Fig. 7). However, there was a smaller shift in the intact 40- to 45-kDa form of IGFBP-3 during GH therapy, since most of the 40- to 45-kDa form of IGFBP-3 was found in the ternary complex even prior to therapy. Quantification of the shift seen in total IGFBP-3 (28 kDa plus 40- to 45-kDa) from the binary to the ternary complex as assessed by the percent change in the WIBs was nearly identical to that measured in the IGFBP-3 IRMA assay (Fig. 7). Thus, both immunoreactive forms of IGFBP-3 are capable of ternary complex formation. However, GH therapy causes a greater shift in ternary complex formation for the 28-kDa variant.

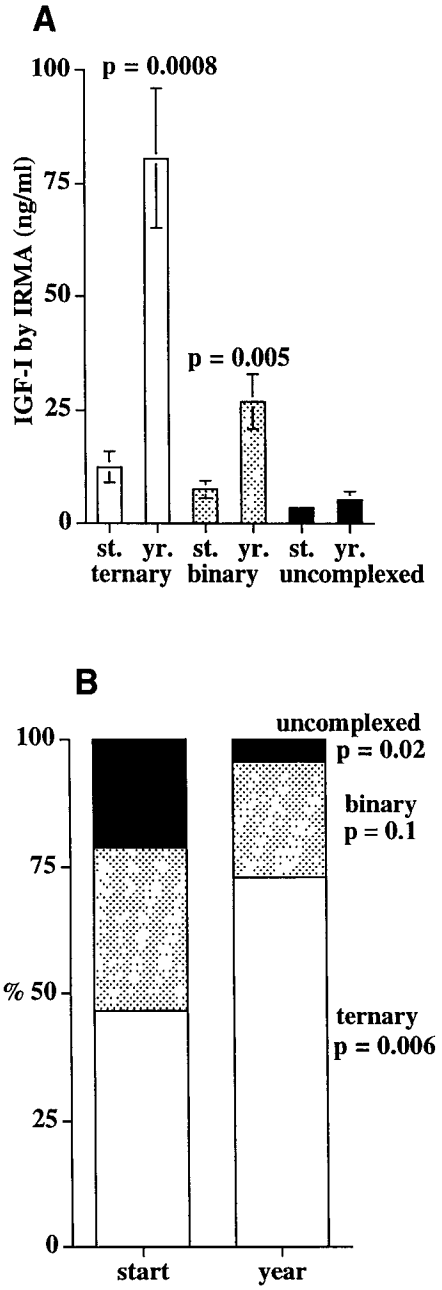


Fig. 8. (**A**) Mean (± SEM) plasma IGF-I levels in ternary, binary, and uncomplexed regions, measured for 10 GHD patients before and after 1 yr of GH treatment. Following neutral size-exclusion chromatography over a Superose-12 column to isolate the three regions, concentrations were determined via IRMA. A highly significant change (p = 0.0008) was observed in IGF-I in the ternary region, rising from 13 to 81 ng/mL. The IGF-I levels also increased significantly (p = 0.005) in the binary region, from 8 to 27 ng/mL, whereas the uncomplexed region remained low and showed no significant increase with treatment. (**B**) Percent IGF-I found in ternary, binary, and uncomplexed regions, before and after 1 yr of GH therapy. Following neutral size-exclusion chromatography to isolate the three regions, levels were determined for 10 patients by IGF-I IRMA, and expressed as a percentage of the combined measures in the ternary, binary, and uncomplexed regions. After 1 yr of GH treatment, there was a significant increase in the % IGF-I found in the ternary complex (p = 0.006) and a significant decrease in the % found in the uncomplexed form (p = 0.02).

Table 2
Correlations Between Growth Velocity During 1 yr of GH Treatment and the Changes in IGFBP-3 and IGF-I in the Ternary (ALS–IGFBP-3–IGF), Binary (IGFBP-3–IGF), and Uncomplexed (IGF) Regions[a]

	r value	p value
IGFBP-3		
Ternary complex	0.72	0.01
Total	0.70	0.02
Binary	0.48	0.1
IGF-I		
Ternary	0.56	0.1
Total	0.49	0.2
Binary	0.22	0.6
Uncomplexed	–0.20	0.6

[a]Measurement of IGFBP-3 and IGF-I was achieved via IRMAs; start values were subtracted from year values. Growth rate was expressed as the HVSDSBA. Linear regression was performed comparing the changes in IGFBP-3 and IGF-I with the HVSDSBA during 1 yr of GH treatment. Statistical analysis reveals a significant correlation between growth rate and the change in both the ternary complex and total IGFBP-3, and a trend for ternary complex IGF-I.

Ternary, Binary, and Uncomplexed IGF-I by IRMA

Fractions from the ternary, binary, and uncomplexed IGF regions were then pooled and rechromatographed under acidic conditions and assayed for IGF-I by IRMA. This revealed a dramatic increase in IGF-I in the ternary (p = 0.001) and binary (p = 0.005) complexes, but not in uncomplexed IGF-I (Fig. 8A). The percentage of IGF-I in the ternary complex increased (p = 0.006), whereas the percentage of uncomplexed IGF-I decreased (p = 0.02) during GH therapy (Fig. 8B).

Growth Response vs the Changes in Ternary, Binary, and Uncomplexed IGFBP-3 and IGF-I by IRMA

We have previously shown *(1)* that the HVSDSBA during the year of GH therapy correlates with the 12-mo change in serum IGF-I measured by RIA (r = 0.72, p = 0.005), as well as with the 12-mo change in serum IGFBP-3 measured by RIA (r = 0.81, p < 0.001). We were, therefore, interested in determining which fractions of IGFBP-3 and IGF-I correlated with the growth response (Table 2). Growth rate, assessed by the HVSDS BA, correlated with the changes in ternary complex IGFBP-3 (r = 0.72, p = 0.01), but to a lesser extent with binary complex IGFBP-3 (r = 0.48, p = 0.10) and total IGFBP-3 (r = 0.70, p = 0.02) as measured by IRMA after plasma separation. There was a trend between the change in ternary complex IGF-I and growth rate (r = 0.56, p = 0.1), but no correlation was found between the changes in binary complex IGF-I (r = 0.22, p = 0.6) and or uncomplexed IGF-I (r = –0.22, p = 0.6).

Discussion

GH therapy of GHD children for 12 mo in our study was associated with increases in IGFBP-3 and decreases in IGFBP-2, as measured by WLB. The decrease in IGFBP-2 during GH therapy is consistent with previous work by Smith et al. *(11)*, who found IGFBP-2 values to be elevated in GHD children and studies in GHD adults *(12)*. In addition, GH administration in cattle results in suppression of IGFBP-2 *(13)*.

The increase in IGFBP-3 was expected, since IGFBP-3 levels are well known to be diminished in GHD and to increase with GH treatment *(1,10)*. The 76% increase in IGFBP-3 as measured by WLB was similar to that seen in our previous study, where IGFBP-3 was measured by RIA, although the change in IGFBP-3 was of greater magnitude (126% increase) when measured by RIA. The WLB measures only the 40- to 45-kDa IGFBP-3, whereas RIA can measure all immunoreactive forms of IGFBP-3. In addition, densitometric analysis of bands from gels may tend to underestimate the change.

Two immunoreactive forms of IGFBP-3 were found by WIB: the intact 40- to 45-kDa doublet, which was also found on WLB, and a proteolyzed 28-kDa form, which was not found on WLB. The two forms of IGFBP-3 were found in similar amounts, and both increased by 64% during GH therapy. Suikkari and Baxter *(14)* demonstrated that the 28-kDa pregnancy form of IGFBP-3 cannot bind iodinated IGF, but is immunoreactive, and this has been extensively supported by other studies *(15–21)*. The 28-kDa form of IGFBP-3 detected in pregnancy serum is thought to result from proteolysis *(15,16)*. We have shown in the present study that both forms of IGFBP-3 are glycosylated. The 40- to 45-kDa form of IGFBP-3 deglycosylates to 29–34 kDa, whereas the 28-kDa form deglycosylates to 18 kDa. The origin of the glycosylated 28-kDa form of IGFBP-3 in fetal serum *(17)*, GHRD *(18)*, urine *(19)*, and in other biologic fluids *(20)* is unknown, yet follows the characteristics of the pregnancy IGFBP-3; it is undetectable by WLB, but is highly immunoreactive. Liu et al. *(21)* have shown that the pregnancy 28-kDa form of IGFBP-3 is capable of forming a 130-kDa complex. In the present study, we have shown the presence of the 28-kDa form of IGFBP-3 in the 130- to 150-kDa region in GHD children pre- and post-GH therapy consistent with reports in GHRD and normal human serum *(18)*, as well as in the serum of normal children *(19)*. Thus, the 28-kDa form of IGFBP-3 is able to bind IGF and ALS, and thus play a role in the modulation of IGF within the ternary complex.

An additional 17.7-kDa form of IGFBP-3 has been recently reported in the urine and serum of GHD children by Spagnoli et al. *(19)*. This form of IGFBP-3 is glycosylated and is able to bind IGF, but like the 28-kDa form, not iodinated IGF. We did not detect this form, and can only speculate that the urinary IGFBP-3 fragment represents alternatively or more completely proteolyzed IGFBP-3. Nonetheless, although the 40- to 45-kDa form of IGFBP-3 has been considered to be the predominant IGFBP-3, the detection of these new smaller forms may alter our current concepts of the modulation of IGF.

The ALS is an 84- to 86-kDa protein, which binds to the binary complex of IGF and IGFBP-3 forming the ternary complex. Formation of the ternary complex may play a key role in regulating the bioavailability of circulating IGFs. ALS, like IGFBP-3, appears to be GH-dependent. ALS levels measured by RIA are reduced in GH deficiency and increased in acromegaly *(22)*, but levels of ALS are low in GH receptor deficiency and do not increase with IGF treatment *(23)*. Levels of ALS, as assessed by WIB in our study, were shown to increase during GH therapy. The increase in ALS in our study was progressive throughout the year of GH therapy. Although the overall increase of 41% was smaller than that observed with IGFBP-3, ALS levels increased dramatically in those patients who grew extraordinarily well in response to GH therapy.

GH therapy in our study resulted in significant increases in both IGFBP-3 and IGF-I in both the ternary and binary complex regions. However, the changes were more dramatic in the ternary complex, resulting in an increase in the percent of total IGFBP-3 and IGF-I found in the ternary complex. Thus, the increases in ALS, both forms of IGFBP-3, and IGF-I all serve to promote ternary complex formation. Interestingly, the increase in the percent total IGFBP-3 found in the ternary complex after GH therapy was accounted for primarily by the shift in the 28-kDa proteolyzed form of IGFBP-3 from the binary (or free IGFBP-3) region to the ternary complex region. Although the reason for this is unclear, we speculate that this may be secondary to differences in affinity between the intact and proteolyzed forms of IGFBP-3 for IGF-I or ALS.

Because changes in IGFBP-3 and, to a lesser extent, IGF-I were the major correlates of growth rate seen in these same patients in a previous study, we were interested to see whether changes in either of the molecular forms of IGFBP-3 or ALS correlated with the growth response. The growth response to GH therapy, as measured by the HVSDSBA, correlated with the percent increase of intact, proteolyzed, and total IGFBP-3, as well as with the percent increase in ALS. Because this suggested that ternary complex formation might be an important factor in the response to GH therapy, we were interested to see which fractions of IGFBP-3 and IGF-I correlated best with the growth response. The growth response correlated best with changes in ternary complex fractions of both IGFBP-3 and IGF-I, although the "p" value for the IGF-I correlation was only 0.1. The IGF-I determinations, however, required not only the initial neutral size-exclusion chromatography, but also rechromatography under acidic conditions, and could only be performed on 10 patients owing to limited sample volume. We speculate that these factors and the difficulty of this

technique, rather than the difference in the assay method (RIA vs IRMA), account for the differences in the IGF-I correlations between this study and our previous study.

These data suggest that both the intact and proteolyzed forms of IGFBP-3, as well as ALS, may interact with IGF-I to determine the somatic growth response. This may have important implications for IGF therapy. We speculate that the formation of the ternary complex is a determining factor in maintaining IGF stability and storage in the circulation, and that immunoreactive forms of IGFBP-3 and ALS potentiate IGF-I action by providing a more stable serum reservoir of bioactive IGF-I. In addition, although there is no evidence for the existence of the ternary complex outside the circulation *(8)*, both forms of IGFBP-3 could also potentiate IGF-I action outside the circulation by assisting in the delivery of IGF-I to its tissue receptors. IGFBP-3 may also have intrinsic activity independent of IGF-I, by preventing IGF-I receptor downregulation *(24)*. IGFBP-3 has been shown to potentiate the action of IGF-I in vivo and in vitro *(8)*. Cell-surface association is considered to result in a significant reduction in affinity of IGFBP-3 for the IGFs, and appears to be required for IGFBP-3 to potentiate IGF action in vitro *(8)*. Because of the suggestion that there is a decreased affinity for IGF-I for smaller mol-wt forms of IGFBP-3 compared with intact IGFBP-3 *(25)*, one might expect differences in the ability of the two immunoreactive forms to potentiate IGF-I activity. A decreased affinity for IGF-I might lead to lesser ternary complex formation for the proteolyzed form of IGFBP-3 compared with the intact form. However, the decreased affinity of the proteolyzed form might be advantageous at the cell surface in promoting somatic growth.

In summary, GH treatment of GHD children results in significant increases in intact, proteolyzed, and total IGFBP-3, as well as an increase in ALS, which all correlate with the growth response to GH therapy. Furthermore, GH treatment results in an increase in ternary complex concentrations of IGFBP-3 and IGF-I, which also correlate with the response to therapy. Data from this study suggest that the formation of the ternary complex may be a determining factor in the somatic growth response.

Subjects, Methods, and Materials

Subjects

Seventeen previously untreated prepubertal GHD children (12 male and 5 female) initially participated in the study. Thirteen of the 17 children are the same subjects previously reported in a study of the changes in IGF-I, IGFBP-3, GHBP, erythrocyte IGF-1 receptors, and growth rate during the first year of GH therapy at a dose of 0.043 mg/kg/d *(1)*. The additional four patients were excluded from the previous study and much of this study because of the inability to calculate accurately the height velocity standard deviation score for bone age: two because of a bone age on the steep portion of the growth velocity curve (one with a bone age <2, one with a bone age >10), another because blood was drawn, but height not measured at 1 yr of treatment, and another because of previous spinal radiation. Subject age at the start of the study ranged from 1.5 to 12.1 yr (mean, 6.2 yr). All responded to insulin-arginine stimulation with a serum GH peak of <10.0 μg/L (range, 1.5–9.8 μg/L: mean, 5.3 μg/L). Their bone ages at the start of the study ranged from 1.0 to 10.3 yr (mean, 4.4 yr). The children were short, with a mean height standard deviation score for chronological age of –2.8, and were growing slowly, with a mean HVSDSBA of –2.2. All patients remained prepubertal during the entire year of the study. Bone age was determined by the method of Greulich and Pyle *(25a)* at the start and after 1 yr of GH therapy.

Consent was obtained from the parents and from the children who were old enough to understand the study. The protocol was approved by the committee on human research at Oregon Health Sciences University, Portland, OR.

Experimental Design

All patients received recombinant Met-hGH (Somatrem, Genentech, South San Francisco, CA) in a dose of 0.043 mg/kg each day. They were examined, weighed, and measured (4 times/visit using a wall-mounted stadiometer) on the start day and after 6 and 12 months of therapy. During these clinic visits, blood was drawn into 5-mL glass tubes containing 72 USP units heparin/tube and centrifuged at 1800 rpm, 4°C, yielding plasma (frozen at –70°C) for assessment of IGF, IGFBPs, and ALS. We have previously published our extensive comparison of serum and plasma IGFBP profiles *(26)*. We found that collection of blood in heparinized tubes affected neither the IGF distribution nor the molecular distribution of IGFBPs. Thus, owing to available sample volume, we have used plasma in the current analysis.

Laboratory Methods

Peptides and Antiserum

Recombinant human IGF-I was provided by Bachem (Torrance, CA). IGF-II was provided by Eli Lilly Research Laboratories (Indianapolis, IN). Both peptides were iodinated for use in WLB by a modification of the chloramine T method *(27)* to SAs of 350–500 μCi/μg. IGFBP-3 antiserum used for WIB analysis was generated in our laboratory *(28)*. ALS antiserum was generously provided by Diagnostic Systems Laboratories, Webster, TX.

WLB Analysis

Plasma samples collected at 0 and 12 mo of treatment were diluted 1:10 in 0.05 *M* Tris and subjected to Western ligand blot analysis, as described by Hossenlopp et al. *(29)*. Fifty microliters of each diluted sample were added to 50 μL nonreducing SDS-dissociation buffer (0.5 *M* Tris,

pH 6.8; 69% glycerol; 4% sodium dodecyl sulfate), loaded onto a 1 mm discontinuous SDS polyacrylamide gel and electrophoresed through a 4% stacking gel and 10% separating gel *(30)* at 50 V overnight. Molecular-weight markers in SDS-dissociation buffer containing 1 m*M* dithiothreitol as a reductant were also electrophoresed. Proteins were then electrotransferred from gels to 0.45 U nitrocellulose (Schneider and Schuell) at 0.2 A for 1.5 h with a Hoefer Semi-dry Transphor unit (San Francisco, CA), following the method of Towbin et al. *(31)*. The nitrocellulose-bound proteins were then treated with 3% NP40 for 30 min and 1% BSA for 2 h. Each treatment was carried out at room temperature in a Tris-saline buffer (0.1 *M* Tris, 0.15 *M* NaCl; pH 7.4). The treated nitrocellulose filters were probed with radiolabeled IGF-I and IGF-II (one million cpm/peptide/filter) overnight. They were then washed extensively in 1% Tween-20, air-dried, and exposed to X-ray film (Kodak X-OMAT AR) in the presence of Cronex Hi-Plus intensifying screens (DuPont) for 4 d at –70°C. After the film was developed, band intensity was assessed by densitometric analysis.

WIB Analysis, IGFBP-3

Each plasma sample was subjected to nonreducing gel electrophoresis and electrotransferred as described above. After being treated with 1% BSA for at least 4 h in a buffer containing 0.1 *M* Tris and 0.15 *M* NaCl at pH 7.4, the nitrocellulose was incubated overnight with anti-IGFBP-3g1 *(13)* in the same 1% BSA saline buffer. The nitrocellulose was then washed two times for 10 min in 0.1% Tween-20. Following a 2-h incubation with goat antirabbit IgG conjugated with horseradish peroxidase, the nitrocellulose was washed again in 0.1% Tween-20 buffer (2 × 10 min), treated with 10 mL each of enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) for 1 min, rinsed quickly with 0.1% Tween-20, and exposed to X-ray film for 1 h.

WIB Analysis, ALS

In order to assess the change in ALS levels over the course of GH therapy, similar analysis was performed on 0-, 6-, and 12-mo samples from eight randomly selected GHD patients. Ten microliters of each sample were added to 10 μL of nonreducing SDS-dissociation buffer, loaded onto a 1-mm 8.5% polyacrylamide gel and subjected to overnight electrophoresis. Proteins were then electrotransferred to nitrocellulose and treated with 1% BSA in buffer as described for IGFBP-3 above. The nitrocellulose was incubated with a 1:500 dilution of anti-ALS antiserum (R00934) generated by Diagnostic Systems Laboratories (Webster, TX). The nitrocellulose was then treated with goat antirabbit antibody, enhanced chemiluminescence reagents, and exposed to X-ray film as described for IGFBP-3 above. As with the ligand blots, band intensity was determined by densitometric analysis of the developed film.

Deglycosylation Study

A normal human serum pool and whole plasma from one GHD patient were diluted 1:25 in saline buffer (50 m*M* NaH2PO4, 158 m*M* NaCl, 0.02% Na Azide, pH 7.4). Each sample was divided into two 50-μL aliquots, and pH was adjusted to 5.0 with 20-μL 0.1 *N* HCl. One of each pair of aliquots was deglycosylated by the addition of 200 μU endo F (CALBIOCHEM, La Jolla, CA) and a 3-h incubation at 37°C. The remaining aliquots were left as controls and merely incubated with the others. Following incubation, 30 μL SDS loading buffer (*see* Western ligand blot analysis) were added to each aliquot, in order to stop the Endo F reaction. Each sample was stored overnight at 4°C, and then subjected to Western immunoblot analysis for IGFBP-3, as described above.

Separation of Plasma by Neutral Size-Exclusion Chromatography

Two hundred microliters from each plasma sample were delipidated by extraction with an equal volume of freon (1, 1, 2-trichloro-1, 2, 2-trifluoroethane). Separation of the 130- to 150-kDa ternary complex (ALS–IGFBP-3–IGF), 40- to 45-kDa binary complex (IGFBP-3–IGF), and free IGF was achieved via chromatography through a Superose-12 column, using a Pharmacia fast performance liquid chromatography (FPLC) system in buffer (50 m*M* NaH2PO4, 158 m*M* NaCl, 0.02% Na azide; pH 7.4) with a flow rate of 0.5 mL/min. The column was precalibrated with gel-filtration standards: thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B12, 1.35 kDa (Bio-Rad Laboratories, Hercules, CA); as well as BSA, 69 kDa. 0.25-mL fractions from plasma were frozen at –70°C before their use in Western immunoblot IGFBP-3 analysis as described above, and IGFBP-3 and IGF-I IRMA analysis as described below.

IGFBP-3 Quantitation

A ternary and binary complex pool was created for 12 of the 13 chromatographed plasma samples (one was excluded owing to insufficient sample volume) as follows: the fraction number that corresponded to the elution of 69 kDa BSA when standards were run was considered the dividing point between the elution of the 130- to 150-kDa form and the 40- to 45-kDa form of IGFBP-3. FPLC fractions preceding and following this 69-kDa fraction were set aside to represent the ternary and binary regions, respectively. (Western blotting reveals that this number of fractions encompasses the bulk of eluted IGFBP-3.) Twenty-microliter aliquots per fraction within each region were pooled. These pooled FPLC fractions, representing the 130- to 150-kDa (ternary) and 40- to 45-kDa (binary) regions of each sample, were assayed for IGFBP-3 by use of IRMA kits generously supplied by Diagnostic Systems Laboratories. Results were multiplied by the appropriate factor in order to represent concentration per milliliter of whole plasma, compensating

for the fact that only 200 µL of each plasma sample were chromatographed, and only 20 µL of each 250-µL fraction were assayed.

IGF-I Quantitation

The distribution of IGF among ternary, binary, and uncomplexed regions was assessed in a subset of 10 GHD patients, before and after 1 yr hGH treatment. Three pools, corresponding to the differently complexed regions, were created in the same manner described above under IGFBP-3 quantification. The fraction corresponding to 69 kDa of BSA and the fraction corresponding to 17 kDa of myoglobin served as dividing points. Fraction pools were assayed for IGF-I using IRMA kits supplied by Diagnostic Systems Laboratories. As with the IGFBP-3 quantitation, reported values have been multiplied by a common factor in order to represent ng/mL of whole plasma.

Statistical Analysis

Statistical comparisons of the baseline, 6 mo, and year determinations were performed by one-way repeated analysis of variance, followed by paired "*t*" tests where significant ($p < 0.05$) differences were found. Growth rate was expressed as the HVSDSBA over the 12-mo period and was calculated using Growth Base III (MedicaLogic, Portland, OR). Correlations among IGFBP-3, ALS, and IGF-I levels and growth rate were evaluated by linear regression analysis. A "*p*" value < 0.05 was considered to be statistically significant.

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References

1. Mandel, S., Moreland, E., Nichols, V., Hanna, C., and LaFranchi, S. (1995). *J. Clin. Endocrinol. Metab.* **80,** 190–194.
2. Baxter, R. C. (1988). *Comp. Biochem. Physiol.* **91,** 229–235.
3. Baxter, R. C. and Martin, J. L. (1989). *Prog. Growth Factor Res.* **1,** 49–68.
4. Holly, J. M. P. and Wass, J. A. H. (1989). *J. Endocrinol.* **122,** 611–618.
5. Rosenfeld, R. G., Lamson, G. L., Pham, H., Oh,Y., Conover, C., DeLeon, D. D., et al. (1991). *Recent Prog. Hormone Res.* **46,** 99–159.
6. Shimasaki, S. and Ling, N. (1991). *Prog. Growth Factor Res.* **3,** 243–266.
7. Kelly, K. M., Oh, Y., Gargosky, S. E., Gucev, Z., Matsumoto, T., Hwa, V., et al. (1996). *Int. J. Biochem.* **28,** 619–637.
8. Jones, J. I. and Clemmons, D. R. (1995). *Endocr. Rev.* **16,** 3–34.
9. Rosenbloom, A. L., Guevara-Aguirre, J., Martinez, A. L., Martinez, V., Vasconez, O., Rosenfeld, R. G., et al. (1995). *Pediatr. Res.* **37,** 97A.
10. Blum, W. F., Ranke, M. B., Kietzmann, K., Gauggel, E., Zeisel, H. J., and Bierich, J. R. (1990). *J. Clin. Endocrinol. Metab.* **70,** 1292–1298.
11. Smith, W. J., Nam, T. J., Underwood, L. E., Busby, W. H., Celnicker, A., and Clemmons, D. R. (1993). *J. Clin. Endocrinol. Metab.* **77,** 1294–1299.
12. Clemmons, D. R., Busby, W. H., and Snyder, D. K. (1991). *J. Clin. Endocrinol. Metab.* **73,** 727–733.
13. Cohick, W. S., McGuire, M. A., Clemmons, D. R., and Bauman, D. E. (1992). *Endocrinology* **130,** 1508–1514.
14. Suikkari, A. M. and Baxter, R. C. (1991). *J. Clin. Endocrinol. Metab.* **73,** 1377–1379.
15. Hossenlopp, P., Segovia, B., Lassarre, C., Roghani, M., Bredon, M., and Binoux, M. (1990). *J. Clin. Endocrinol. Metab.* **71,** 797–805.
16. Giudice, L. C., Farrell, E. M., Pham, H., Lamson, G., and Rosenfeld, R. G. (1990). *J. Clin. Endocrinol. Metab.* **71,** 806–817.
17. Bang, P., Stangenberg, M., Westgren, M., and Rosenfeld, R. G. (1994). *Growth Regul.* **4(2),** 68–76.
18. Gargosky, S. E., Wilson K. F., Fielder, P. J., Baxter, R. C., Vacarello, M. A., Diamond, F. B., et al. (1993). *J. Clin. Endocrinol. Metab.* **77,** 1683–1689.
19. Spagnoli, A., Gargosky, S. E., Spadoni, G. L., MacGillivary, M., Oh, Y., Boscherini, B., et al. (1995). *J. Clin. Endocrinol. Metab.* **80,** 3668–3676.
20. Gargosky, S. E., Pham, H. M., Wilson K. F., Liu, F., Giudice, L. C., and Rosenfeld, R. G. (1992). *Endocrinology* **131,** 3051–3060.
21. Liu, F., Baxter, R. C., and Hintz, R. L. (1992). *J. Clin. Endocrinol. Metab.* **75,** 1261–1267.
22. Baxter, R. C. (1990). *J. Clin. Endocrinol. Metab.* **70,** 1347–1353.
23. Labarta, J., Gargosky, S. E., Simpson, D., Aguirre, J. G., and Rosenfeld, R. G. (1997). *Clin. Endocrinol.* **47,** 657–666.
24. Conover, C. A. and Powell, D. R. (1991). *Endocrinology* **129,** 710–716.
25. Binoux, M., Roghani, M., Hossenlopp, P., Hardouin, S., and Gourmelen, M. (1991). *Acta Endocrinol.* **124(Suppl. 2),** 41–47.
25a. Greulich, W. W. and Pyle, S. I. (1950). *Radiologic Atlas of Skeletal Development of the Hand and Wrist.* Stanford University Press, Stanford, CA.
26. Mandel, S., Moreland, E., Rosenfeld, R. G., and Gargosky, S. E. (1996). *Endocrine* **5,** 1–8.
27. Van Obberghen-Schilling, E. and Pouyssegur, J. (1983). *Exp. Cell Biol.* **151,** 207–214.
28. Gargosky, S. E., Pham, H. P., Wilson, K. F., Liu, F., Giudice, L. C., and Rosenfeld, R. G. (1992). *Endocrinology* **131,** 3051–3060.
29. Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986). *Anal. Biochem.* **154,** 138–143.
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
31. Towbin, H., Staehlin, T., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.