

Measurement of IGF-1, IGFBP-3 and Free IGF-1 Levels by ELISA in Growth Hormone (GH) Deficient Children Before and After GH Replacement

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Serum insulin-like growth factor-1 (IGF-1) levels reflect the 24-hour growth hormone (GH) secretion. They are high in situation of GH excess and low in GH deficiency. Most of the IGF-1 in circulation is bound to the IGF binding protein, of which six classes have been identified (IGFBP-1 to IGFBP-6).^{1,2} However, IGFBP-3 is the one that mostly depends on GH status. It is believed that IGFBP-3 prolongs the half-life of IGF-1 and modulates the biological activity of IGF-1.³ Physiologically, IGF-1 mostly binds with IGFBP-3 and forms a ternary complex with an acid-labile subunit resulting in a high and more stable molecular mass of 150 kDa. This stable complex cannot escape from the circulation. A few percent of IGF-1 circulate in the free form. However, this form is believed to have more potent biological action than the bound form similar to the free form of thyroxine and is able to cross the capillary boundaries and reaches the target tissues.⁴ IGFBP-3 is cleaved into fragments by IGFBP-3 protease enzyme in some pathological conditions in order to normalize

SUMMARY Serum insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3) levels reflect the growth hormone (GH) status. A few percent of IGF-1 circulate in a free form which is believed to represent the IGF biological activity. We retrospectively studied the changes of serum IGF-1, serum IGFBP-3, and plasma free IGF-1 levels in growth hormone deficient (GHD) children before and after treatment with recombinant human growth hormone (rhGH) for a period of 6 months and 1 year. Twenty-one GHD children (16 boys and 5 girls) who had the mean chronological and bone ages of 7.7 ± 0.7 and 4.8 ± 0.6 years, respectively, were treated with a mean rhGH dose of 1.66 ± 0.42 U/m² body surface area/week. Serum IGF-1 level increased from 162.5 ± 42.9 ng/ml before treatment to 252.8 ± 49.5 ng/ml ($p = 0.007$) and 282.7 ± 86.9 ng/ml after treatment for 6 months and 1 year, respectively. Plasma free IGF-1 also increased from 0.38 ± 0.30 ng/ml before treatment to 1.21 ± 0.30 ($p = 0.001$) and 1.17 ± 0.42 ng/ml after 6 months and 1 year of treatment. However, serum IGFBP-3 did not significantly increase after treatment. In addition, the free/total IGF-1 ratio decreased after treatment with rhGH. The height velocities at 6 months and 1 year after treatment were negatively correlated with plasma free IGF-1 before treatment. In conclusion, therefore, plasma free IGF-1 levels could serve as a good predictor of growth hormone responses. Furthermore, their circulating levels would be modified by serum IGF-1 status, and possibly, IGFBP-3 protease activity.

the free IGF-1 level and maintain the IGF biological action such as in post surgical conditions⁵ and during severe illness.⁶

Serum levels of IGF-1 and IGFBP-3 in growth hormone-deficient (GHD) children have been studied worldwide. However, to the best of our knowledge, the studies of free IGF-1 were not so extensive and the results were not yet conclusive. In this study, we aimed to

look at the free IGF-1 levels in GHD children, the pattern of free IGF-1 after GH replacement and the relationship of free IGF-1 with other growth parameters.

MATERIALS AND METHODS

Patients

Twenty-one children (16

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boys and 5 girls) with GH deficiencies were retrospectively studied in this study. The means \pm SEM of chronological age (CA) and bone age (BA) at diagnosis were 7.7 ± 0.7 years and 4.8 ± 0.6 years, respectively. The diagnosis of GH deficiency was made by two standard GH provocative tests (ITT, clonidine or glucagons) and the peak GH level of 10 ng/ml was considered the cut-off point. Complete GHD was diagnosed when the peak GH was less than 5 ng/ml; and partial GHD, when the peak GH was between 5-10 ng/ml. Their other endocrine functions such as thyroid function and adrenal function were normal. The children were treated with recombinant human growth hormone with a mean dose of 11.66 ± 0.42 U/m² body surface area/week.

Blood sampling

One milliliter of serum for IGF-1 and IGFBP-3 measurement and one milliliter of plasma for free IGF-1 measurement were routinely collected at the time of the GH provocative test, six months and one year after treatment with recombinant human growth hormone. All blood samples were stored at -20°C until they were analyzed.

Serum analysis for IGF-1, IGFBP-3 and plasma analysis for free IGF-1

Serum IGF-1 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic System Laboratories, Inc., Webster, TX, USA). The assay was an enzymatically amplified "one-step" sandwich-type immunoassay. Briefly, the serum samples were extracted with ethanolic HCl solution in order to remove IGF binding proteins and then diluted with bovine serum albumin (BSA) buffer. The standards, controls and the diluted unknowns were incubated with anti-IGF-1 antibody that

was labeled with the horseradish peroxidase (HRP) enzyme in micro-titration wells coated with another anti-IGF-1 antibody. Incubation was at room temperature for 2 hours. After the incubation and washing, the wells were incubated with the tetramethylbenzidine (TMB) substrate at room temperature for 10 minutes. The reaction was stopped with 0.2 M sulfuric acid. The degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The intra-assay coefficients of variation (CVs) were 7.1% (at 26.47 ± 1.87 ng/ml), 4.5% (at 48.36 ± 2.15 ng/ml), and 6.5% (at 169.67 ± 11.01 ng/ml). The inter-assay CVs were 8.8% (at 42.94 ± 3.79 ng/ml), 4.8% (at 132.61 ± 6.35 ng/ml), and 6.4% (at 379.12 ± 24.38 ng/ml).

Serum IGFBP-3 levels were measured by the similar procedures mentioned above using the commercial kit (Diagnostic System Laboratories, Inc.). It was found that the intra-assay CVs were 9.6% (at 4.62 ± 0.44 ng/ml), 9.5% (at 27.43

± 2.60 ng/ml), and 7.3% (at 74.40 ± 5.41 ng/mL). The inter-assay CVs were 11.4% (at 5.64 ± 0.64 ng/ml), 10.4% (at 25.13 ± 2.60 ng/ml), and 8.2% (at 65.55 ± 5.40 ng/ml).

Plasma free IGF-1 levels were similarly measured using another commercial kit (Diagnostic System Laboratories, Inc.). It was found that the intra-assay CVs were 4.80% (at 0.20 ± 0.01 ng/ml), 3.61% (at 0.83 ± 0.03 ng/ml) and 3.74% (at 1.87 ± 0.07 ng/ml). The inter-assay CVs were 11.1% (at 0.18 ± 0.02 ng/ml), 10.1% (at 0.80 ± 0.08 ng/ml) and 6.2% (at 2.09 ± 0.13 ng/ml).

Statistical analyses

The results were shown as means \pm SEM. Nonparametric statistical analysis was performed using the Statistical Package for Social Sciences (SPSS). The relationships between the different variables were calculated by linear regression analysis and $p < 0.05$ was considered significant.

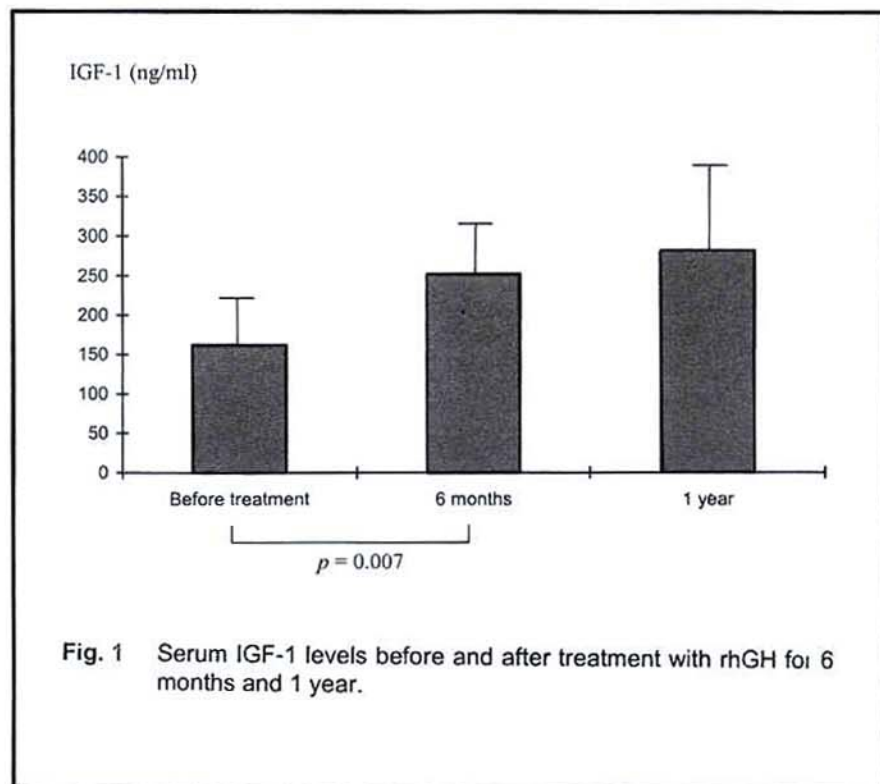


Fig. 1 Serum IGF-1 levels before and after treatment with rhGH for 6 months and 1 year.

RESULTS

All patients responded well to GH replacement. The mean height velocity (HV) was significantly increased from 4.1 ± 0.4 to 8.3 ± 0.8 cm/year ($p < 0.001$) at 6 months, and to 8.0 ± 0.6 cm/year ($p = 0.001$) at 1 year of treatment. The CA/BA ratio changed from 1.73 ± 0.11 before treatment to 1.56 ± 0.14 at one year of treatment with the p value of 0.05.

Serum IGF-1, serum IGFBP-3 and plasma free IGF-1

The mean serum IGF-1 level increased from 162.5 ± 42.9 ng/ml before treatment to 252.8 ± 49.5 ng/ml ($p = 0.007$) after 6 months of treatment, but had no significant increase at 1 year thereafter (282.7 ± 86.9 ng/ml) (Fig. 1).

The mean serum IGFBP-3 level increased from $3,011.5 \pm 417.9$ ng/ml to $3,773.3 \pm 390.7$ ng/ml ($p = 0.16$) and to $3,374.8 \pm 430.1$ ng/ml ($p = 0.9$) after 6 months and 1 year of treatment, respectively (Fig. 2).

The mean plasma free IGF-1 level increased from 0.38 ± 0.06 ng/ml before treatment to 1.21 ± 0.30 ng/ml ($p = 0.001$) after 6 months of treatment, but had no significant increase 1 year thereafter (1.17 ± 0.42 ng/ml) (Fig. 3).

The mean IGF-1 level in the complete GHD children ($N = 11$) was significantly lower than that in the partial GHD children ($N = 10$) (108.08 ± 47.37 and 222.43 ± 71.26 ng/ml, $p = 0.04$), but there was no significant difference demonstrated for plasma free IGF-1 and serum IGFBP-3.

The free/total IGF-1 ratio decreased from $1.34 \pm 0.4\%$ before treatment to $0.72 \pm 0.18\%$ and to $0.72 \pm 0.35\%$ after 6 months and 1

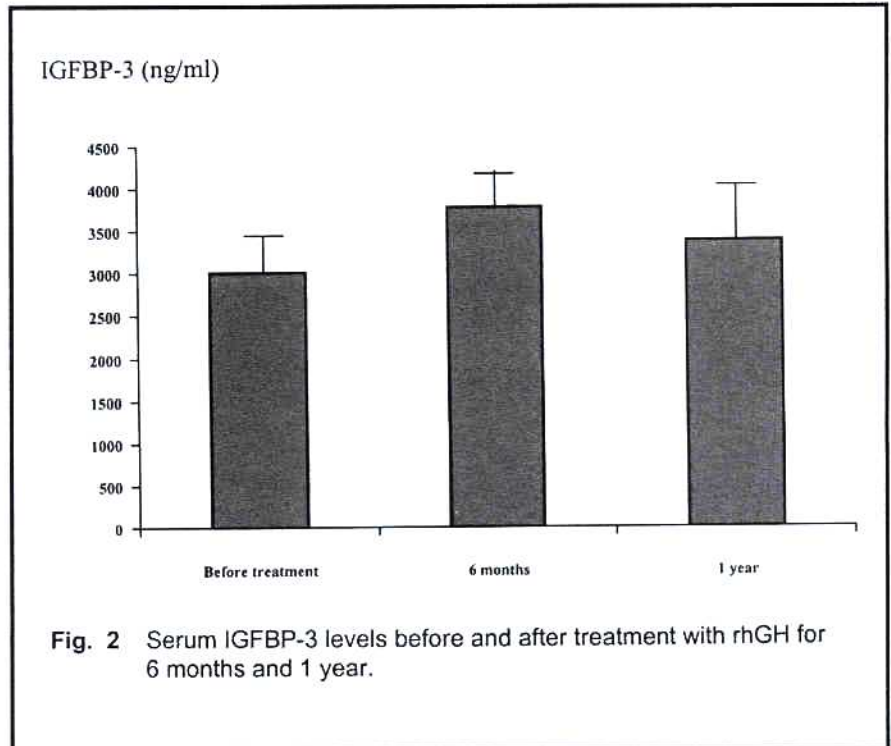


Fig. 2 Serum IGFBP-3 levels before and after treatment with rhGH for 6 months and 1 year.

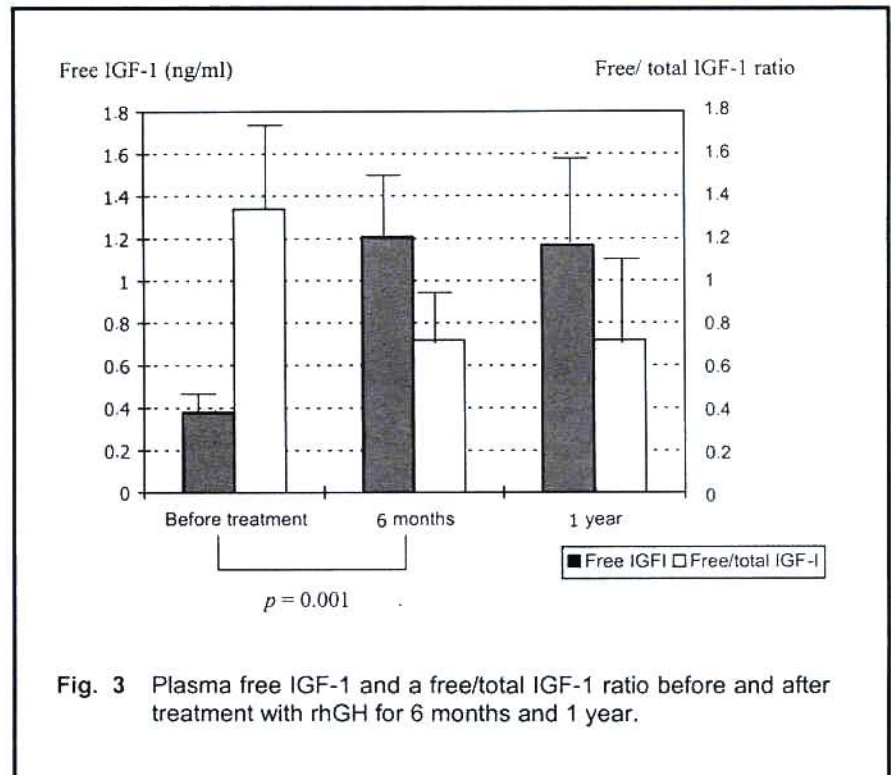


Fig. 3 Plasma free IGF-1 and a free/total IGF-1 ratio before and after treatment with rhGH for 6 months and 1 year.

year of treatment but there were not significantly different (Fig. 3).

Relationships between serum IGF-1, serum IGFBP-3 and plasma free IGF-1 and height velocity (HV) during GH treatment

Before treatment, serum

IGF-1 levels were positively correlated with serum IGFBP-3 ($r = 0.62$, $p = 0.003$) but not with plasma free IGF-1. However, the free/total IGF-1 ratio was negatively correlated with serum IGF-1 ($r = -0.61$, $p = 0.009$). Free IGF-1 had no correlation with the height velocity before treatment.

The HVs at 6 months and 1 year after treatment were negatively correlated with plasma free IGF-1 before treatment, with $r = -0.61$, $p = 0.009$ and $r = -0.67$, $p = 0.009$, respectively, but not with serum IGF-1 and IGFBP-3.

The serum IGF-1, IGFBP-3 and plasma free IGF-1 at 6 months had no significant correlation with HV at 6 months.

DISCUSSION

Measurement of plasma free IGF-1 detected both an unbound form and a readily dissociable form of IGF-1, representing 1-2% of total IGF-1.^{4,7,8} However, free IGF-1 was believed to be a biologically active component of IGF-1. Physiologically, the serum level of free IGF-1 was high in neonates⁸ and adolescents.⁴ The findings agree with the acceleration of height during these periods. Furthermore, the free IGF-1 would also be controlled by IGF-binding protein (IGFBP)-3 and IGFBP-3 protease enzyme. The IGFBP-3 protease enzyme activity increased in both physiological conditions, such as pregnancy, and some pathological conditions. This enzyme would cleave the IGFBP-3 into small fragments, and hence would attenuate the stability of ternary complex, producing more free IGF-1. Free IGF-1 levels have been proven to be high in boys with precocious puberty⁹ and obese adults.¹⁰ Therefore, the height velocity was accelerated in boys with precocious puberty, and the low GH level in obesity was due to a negative feedback mechanism.

The results of our study showed that the serum IGF-1 and plasma free IGF-1 significantly increased after treatment with GH for a period of 6 months, but they did not significantly change after 1 year of treatment. However, serum IGFBP-3 levels were not signifi-

cantly different after 6 months and 1 year of treatment. The findings are similar to the study conducted by Kawai *et al.*¹¹ which showed that the serum IGF-1 and free IGF-1 after 1 month of treatment with GH increased more than the serum IGFBP-3; and the maximal free IGF-1 level was seen after 6 months of GH therapy. This suggested that the direct effect of GH on IGF-1 production was more than the IGFBP-3 production, and the effect would be sustained after 6 months of treatment. The use of serum IGF-1 and IGFBP-3 as a screening tool for the diagnosis of GHD has been studied worldwide, although the results were discrepant, especially in the cases of partial GHD. Probably, the use of free IGF-1 as a screening method had no more merit than the use of total IGF-1 and IGFBP-3.^{4,12} Moreover, a study in adults showed the sensitivities of IGF-1 and free IGF-1 in the diagnosis of acromegaly were 100% and 95%, respectively. In contrast, the sensitivity in diagnosing GHD was only 41% for IGF-1 and 35% for free IGF-1.¹³ In our study, however, there was no difference for free IGF in complete and partial GHD; and the free/total IGF-1 ratio was negatively correlated with total IGF-1. In addition, there was no relationship between free IGF-1 and pre-treatment HV, which was similar to a previous study.¹¹ The results could be explained by an adaptive mechanism of IGF-1 and free IGF-1. A few studies were researching the free IGF-1 after GH treatment, and some of them were done in adults with GHD.¹³⁻¹⁵ In our study, however, we observed the changes of serum IGF-1, IGFBP-3 and plasma free IGF-1 in GHD children who were treated with GH for 6 months and 1 year. Plasma free IGF-1 levels increased significantly after 6 months of GH replacement and then remained rather stable. Additionally, a free/total IGF-1 ratio decreased after 6 months of GH re-

placement, then also remained stable, or otherwise showed no statistical difference. The free/total IGF-1 ratio in GHD adults was higher than that of the control, but the changes of this ratio after GH replacement were not mentioned.¹⁴ In GHD, free IGF-1 would be adapted, as much as possible, in order to stabilize a normal growth and maintain a normal metabolism. This might be due to the increase in IGFBP-3 protease activity which resulted in the instability of the ternary complex, and hence the release of free IGF-1. A previous study by Lassare C *et al.* found that the estimated proportion of proteolyzed IGFBP-3 was 37% in normal subjects, more than 50% in GHD patients and only 15% in acromegalic patients.¹⁶ However, some studies showed the different results.^{17,18} The regulation of proteolytic enzyme was adapted to circulating levels of IGF-1, i.e., bioavailability increased when its concentration was lower. In addition, IGF-1 could also be bound with other IGFBPs such as IGFBP-1, but it had less stability than that bound with IGFBP-3. The previous studies showed that IGFBP-1 levels were high in GHD¹⁹ and idiopathic short stature²⁰ and that these high IGFBP-1 levels could modify IGF-1 bioavailability. The measurement of IGFBP-1 was not performed in our study, but a study in adults with GHD showed no significant difference with the normal control group.¹⁴ After treatment, the protease enzyme activity might decrease and result in a more stable ternary complex. GH increased the IGF-1 production, especially in the liver; and IGF-1 expression was shown in the hepatocyte, whereas IGFBP-3 mRNA was exclusively expressed in adjacent endothelial cells.²¹ We postulated that GH had a direct effect on hepatic IGF-1 production; however, free IGF-1 would be modified by IGF-1 and IGFBP-3 protease enzyme. Although the to-

tal number of the circulating free IGF-1 was increased, the ternary complex was more stable after GH treatment. Therefore, most of the produced IGF-1 was in the bound form, causing a decreased ratio of free/total IGF-1. The IGFBP-3 changes would not be high because the IGFBP-3 quantitative assay would detect both the intact and fragmented form of IGFBP-3. The regulation of IGFBP-3 levels by GH was, presumably, indirect and most likely to be mediated by IGF-1, as demonstrated by the marked reduction in circulating IGFBP-3 levels in liver-specific IGF-1 gene-deleted mice despite elevated GH levels.²² Treatment with IGF in GH resistant mice decreased the IGFBP-3 protease activity and restored the intact IGFBP-3.²³

Plasma free IGF-1, before treatment, had a good correlation with the growth response to GH treatment, after 6 months and 1 year of treatment. However, the increment in plasma free IGF-1 did not have such a good correlation, which was different from the previous report. This suggested that the one-month change of free IGF-1 could be a tool to predict the growth response at one year.¹¹ The results of our study were similar to other previous ones: the total IGF-1 level could not be used to predict the growth response.^{24,25}

In conclusion, GH could influence IGF-1 production, and possibly, the stability of the ternary complex. But plasma free IGF-1 in the circulation would be modified by the IGF-1 status to stabilize the normal metabolism. The free IGF-1 could serve as a good predictor of the growth response to GH therapy in GHD children.

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