Western Ligand Blot Assay for Human Growth Hormone-Dependent Insulin-Like Growth Factor Binding Protein (IGFBP-3): The Serum Levels in Patients with Classical Growth Hormone Deficiency

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Abstract. The insulin-like growth factors I and II (IGFs), important growth factors both in vivo and in vitro, are known to have at least six binding proteins (IGFBP-1–6). In human serum, IGFBP-3 is a major binding protein and is considered to be GH-IGF-I-dependent. We have established a Western Ligand Blot (WLB) assay for IGFBP-3. The method is a densitometric analysis of IGFBP-3 bands on a film of WLB. The IGFBP-3 levels of patients with classical growth hormone deficiency (GHD, 5 isolated and 10 multiple hormone deficiencies with appropriate therapy) were studied. Before puberty there is no overlap between control (n=31) and the patients with GHD (n=10). However, IGFBP-3 levels of two of five puberal patients with GHD were within the normal range (n=16). We think that measurement of serum IGFBP-3 is a useful diagnostic marker for GHD, especially before puberty.

Key words: Western ligand blot, Insulin-like growth factor binding protein-3 (IGFBP-3), Growth hormone deficiency.

THE INSULIN-LIKE growth factors I and II (IGFs) belong to a family of peptide hormones that include relaxin and insulin, and share a high degree of structural similarity with proinsulin [1]. IGFs have direct effects on the proliferation of many tissues and cell types, both in vivo and in vitro [2]. The IGF-I is considered the main mediator of growth hormone action on somatic growth, and its measurement in plasma and serum plays a diagnostic role [3] in growth hormone deficiency (GHD) and in acromegaly.

In plasma, the IGFs have been shown to be bound to binding proteins [4]. Recently, six binding proteins (BP) have been cloned and sequenced, IGFBP-1–6 [5, 6, 7, 8]. Among these BPs, human IGFBP-3 (hIGFBP-3) is the most predominant BP in human serum [9, 10]. Upon western ligand blot (WLB), it is a doublet having a size of 40–44 kD and well characterized in immunoprecipitation and deglycosylation studies [9, 11]. The IGFBP-3 doublet, which is recognized immunologically [9, 11], is thought to reflect a different degree of glycosylation, because it goes down to a single band of 29 kD, when deglycosylated [11]. Human IGFBP-3 complex is thought to be a reservoir of IGFs and to regulate the availability of the free IGFs in plasma and interstitial tissue [9]. Human IGFBP-3 is reported to be GH-IGF-I-dependent because its level is decreased or increased in patients with GHD [10, 12, 13, 14].
and dwarfism with GH insensitivity [15], or acromegaly [12], although we preliminarily reported some overlap between the values for control adults and patients with GHD [10].

In this study, we have established a WLB assay for hIGFBP-3, by using densitometric analysis of hIGFBP-3 bands after WLB, and measured the hIGFBP-3 levels in patients with classical GHD.

Materials, Subjects and Methods

Materials

[125I] recombinant human IGF-1 was kindly given by Fujisawa pharmaceutical Co., Ltd. (Osaka, Japan). Iodination of recombinant IGF-1 was done commercially (Amersham Co., Ltd.) with sodium [125I] iodide and a gentle oxidative process. The specific activity was 2000 Ci/mmol.

Subjects

Serum was obtained in the morning after informed consent. Subjects included six healthy adult male volunteers (30–37 years old), forty-seven healthy children (4–15 years old, 31 prepubertal and 16 pubertal children, 22 male and 25 female, grade of obesity; 0.8±9.5%), and fifteen untreated patients with classical GHD (5–23 years old, 10 prepubertal and 5 pubertal; Tanner III–V, bone age; 1.5–15 years, 7 male and 8 female, grade of obesity; 4.9±10.3%, 5 isolated GHD and 10 GHD with other hormonal deficiencies). The definition of classical GHD is as follows; all the peak GH levels are less than 5 ng/ml in two or three different GH provocation tests. Other hormonal deficiencies were treated, if any, with appropriate therapies such as thyroid hormone, glucocorticoid and sex hormones, etc. Pooled control sera for adult periods (n=6, age 30–37) were obtained from the above healthy subjects of normal stature (within mean±2SD). In our study, pooled adult serum was used for the absolute standard for densitometry.

Western Ligand Blots (WLB)

Serum samples (2 μl) were electrophoresed on 10% polyacrylamide-SDS gels at 50v overnight. The size fractionated proteins were electroblotted onto nitrocellulose for 2 h. The filter immobilized proteins were incubated with 1×10^6 cpm of [125I] IGF-I overnight at 4°C. The incubated filter was exposed to film for 5–10 days, as previously described [16, 17].

Densitometric analysis

IGFBP-3 bands on films after WLB were scanned with a MODEL 300A Computing Densitometer (Tomy Co., Ltd.). The densitometric values for IGFBP-3 bands for healthy controls and patients with GHD were expressed as a percentage of the value of adult pooled control serum (BP-3-D%).

Statistics

All data were expressed as the mean±SD. Statistical comparisons were made by Student’s unpaired and paired t-tests, and linear regression analysis. P<0.05 was regarded as significant.

Results

WLB assay for IGFBP-3 (densitometric analysis)

1) The doublets of IGFBP-3 bands (Mr=about 40, 42 kD) were shown on WLB (data, not shown). The intraassay and interassay coefficients of variation (CV) of densitometric values for IGFBP-3 bands were 3–5% (n=7, 8, 8, on different concentrations) and 7–9% (n=13, 13, 12, on different concentrations), respectively.

2) Three dilution tests (with three different sera) were performed (Fig. 1 is a representative film). Each test revealed a significant linear relationship (r=0.90, 0.92, 0.95; P<0.001) between the degree of dilution and densitometric values for IGFBP-3 bands.

Individual prepubertal and pubertal values for healthy controls

The individual BP-3-D% is shown in Fig. 2. BP-3-D% in healthy children did not increase significantly from the age of 4 until the onset of puberty. There was no significant difference between prepubertal and adult healthy controls in the individual BP-3-D%. Individual values for
Fig. 1. Western ligand blot (WLB) of differently diluted adult pooled control sera (with the same loading volume). Lanes a, a’ are WLB using undiluted sera. Lanes b, b’, c, c’ and d, d’ are WLB with 2x, 10x, 20x diluted sera, respectively.

Fig. 2. Relationship between age and BP-3-D%.

Healthy control BP-3-D% during prepuberty were 94.4±25.2% (n=31, 55.0–146.8%), which are shown as a square in the left part of Fig. 3.

The individual BP-3-D% values for healthy controls were significantly greater (P<0.001) during puberty than during prepubertal and adult periods. Individual values for healthy control BP-3-D% during puberty were 146.0±37.3% (n=15, 98.0–216.1%), shown as a square in the right part of Fig. 3.

Fig. 3. BP-3-D% of controls and patients with GHD.

The squares and closed circles (●) denote the normal BP-3-D% range and BP-3-D% of patients with GHD, respectively. The left and right parts are separated to show the group before puberty and after puberty, respectively.
BP-3-D% of patients with classical GHD

Figure 4 shows representative WLB for untreated classical GHD. Pubertal patients with GHD (lanes b, c) have much stronger IGFBP-3 bands than prepubertal patients with GHD (lanes d, e, f, g and h). Figure 3 shows BP-3-D% in healthy controls (squares) and the patients (circle), during prepuberty and puberty. This BP-3-D% was significantly lower in the patients with GHD than in healthy controls both during prepuberty and puberty (P<0.001, P<0.05, respectively). There was no overlap of the BP-3-D% between the patients (n=10, 15.7–48.3%) and healthy controls (n=31, 55.0–143.6%) before puberty (in the left part of Fig. 3), whereas there was a definite overlap between the patients (n=5, 84.0–120.5%) and healthy controls (98.0–216.4%) during puberty (in the right part of Fig. 3). The patients' BD-3-D% values correlated with both their chronological age and bone age (r=0.96; P<0.001, r=0.93; P<0.001, respectively), suggesting that IGFBP-3 levels for the patients with GHD increase with age.

Figure 5 shows four sets of WLB for untreated and treated patients with GHD (the duration of the treatment is from 1 month to 11 years). The sets of BP-3-D% values without and with GH treatment (0.5 U/kg/week, 3–7 X/week) are 1) 84.1%, 86.2% (treatment duration; 11Y), 2) 16.3%, 26.0% (1M), 3) 48.2%, 53.1% (3M), 4) 30.2%, 37.9% (1M), respectively. Patient #1 is pubertal and patients #2, 3, 4 are prepubertal. BP-3-D% increased significantly (P<0.05) after starting GH therapy, although the increase in IGFBP-3 levels was too small to reach the normal value.

fig. 4. Representative WLB of patients with GHD. Lanes a and b-h show a pooled adult control and patients with classical GHD, respectively. Lanes b, c and lanes c, d, e, f, g, h are WLB for pubertal and prepubertal patients with GHD, respectively.
Discussion

The results of the intra- and inter-assay CV and the dilution tests showed that this measurement of hIGFBP-3 (WLB assay for hIGFBP-3) was quite accurate. This finding is consistent with results reported by Deil [18], who suggested that densitometric analysis of IGFBP-3 correlated well with IGFBP-3 radioimmunoassay (RIA).

IGFBP-3 RIA does not reflect the physiological condition during pregnancy, whereas the western ligand blot may give us physiological values in that condition [19, 20, 21]. This discrepancy was explained by proteolysis of IGFBP-3 in pregnancy [20, 21]. The proteolysed fragments are considered to be detected by the IGFBP-3 RIA antibody. Some authors reported the existence of the protease for IGFBP-3 in other pathological conditions including in patients with GHD [22, 23]. Considering possible proteolysis of IGFBP-3 in other conditions, it is better to measure IGFBP-3 by using WLB-assay in addition to RIA, especially when measuring non-proteolised IGFBP-3.

We previously reported lower IGFBP-3 levels of GHD on WLB than adult control values, although we also showed that there were wide variations in IGFBP-3 levels of GHD [10] which resulted in some overlap with adult control values. Our present GHD results, which are quite similar to our previous preliminary results [10], are the following: (1) BP-3-D% values in patients with classical GHD during both prepuberty and puberty were significantly lower than in puberty-matched controls. This result may mean a de-
pendency of IGFBP-3 on the GH-IGF-I axis, as suggested in previous reports [12, 13, 14, 15]. The patients had wide variations in IGFBP-3 levels, leading to some overlap of BP-3-D% between patients with GHD and controls, especially during puberty. The reason for the overlap is unknown at present. Among our patients, BP-3-D% in patients with GHD correlated with both their chronological age and bone age. Furthermore, there was no overlap of BP-3-D% between the patients and controls before puberty, whereas there was a definite overlap between the patients and controls during puberty (Fig. 4). Thus, age of more specifically, puberty (gonadal steroids) may be one of regulators of IGFBP-3 and gonadal steroids during puberty may increase IGFBP-3 levels even in patients with classical GHD, suggesting this increase in IGFBP-3 is GH-IGF-I independent.

The reason why post-GH treatment BP-3-D% does not increase to the normal range is unknown at present. This might imply a limitation in our GH treatment for patients with GHD. Another possibility is that some period of treatment, at least more than one month, is needed for the IGFBP-3 level to reach the normal range, remembering that two of our four periods of treatment were about one month long. The IGFBP-3 levels before and after GH treatment remain to be extensively measured, since our paired samples numbered only four sets.

References


