NOTE

Absence of Proteolysis of Insulin-like Growth Factor Binding Protein-3 in Serum from Patients with Growth Hormone Deficiency

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Abstract. Insulin-like growth factor-I (IGF-I) is predominantly bound to IGF binding protein-3 (IGFBP-3), and free form of IGF-I (fIGF-I) may be bioactive in the circulation. Proteolysis of IGFBP-3, as reported in pregnant serum, results in the lowering of the affinity for IGF-I, thereby increasing the ratio of fIGF-I to total IGF-I (f/t IGF-I ratio). Conflicting results have been reported regarding the relationship between the proteolysis and growth hormone (GH)-IGF-I axis. Proteolysis of IGFBP-3 was previously reported to be present late at night in serum from pediatric subjects with GH receptor dysfunction (GHRD or "Laron-type dwarfism"). Recently, it was reported that proteolysis of IGFBP-3 could not be detected in adult patients with GH deficiency (GHD). The purpose of this study was to investigate the possible relationship between proteolysis of IGFBP-3 and GH in patients with GHD including pediatric cases. Here, proteolysis of IGFBP-3 measured by Western immunoblotting (ages 4–25 years; n = 11) and f/t IGF-I ratio measured by immunoradiometric assay (ages 4–25 years; n = 10) were studied in patients with GHD, which is similar to GHRD in terms of lowered GH function. There was no significant proteolysis of IGFBP-3 in the sera from the 11 patients with GHD. No proteolysis of IGFBP-3 was observed during a 24 hour period in sera obtained every two hours from two patients with GHD. f/t IGF-I ratio was not increased in plasma from the 10 patients with GHD. Our data suggest that proteolysis of IGFBP-3 is independent of the GH-IGF-I axis.

Key words: Insulin-like growth factor-I (IGF-I), IGF binding protein-3 (IGFBP-3), Proteolysis of IGFBP-3, Growth hormone deficiency (GHD), Western immunoblotting, Free/total IGF-I ratio

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physiological conditions, proteolysis of IGFBP-3 was detected in sera during pregnancy [7-9] and early infancy [10], which may indicate that increased fIGF-I plays a role of the growth of the uterus, placenta, and infants. In catabolic conditions, the proteolysis was detected in sera during post-surgery [11, 12] and acute diseases [13], suggesting that fIGF-I plays an important role in recovery from the catabolic state and tissue damage.

It is controversial whether proteolysis of IGFBP-3 in the circulation is associated with growth hormone (GH)-IGF-I axis. Lassarre et al. found that proteolyzed IGFBP-3 was increased in serum from adult patients with GH deficiency (GHD), compared to normal subjects [14]. Skjaerbaek et al. recently reported that proteolysis of IGFBP-3 was not observed in serum from adult patients with GHD [15]. Proteolysis of IGFBP-3 was reported to decrease in serum from hypophysectomized (hypo)-rat [16]. Increased proteolysis of IGFBP-3 in serum from pregnant hypo-rat was not observed compared to non-pregnant hypo-rat [17] either.

Proteolysis of IGFBP-3 at pediatric ages in terms of GH-function has been studied only by Cotterill et al. [18]. They reported that proteolysis of IGFBP-3 was detected late at night (from 2000 to 0100 h) in sera from two pediatric patients with GH receptor dysfunction (GHRD) [18]. Skjaerbaek et al. [15] suggested that low levels of tIGF-I may activate the proteolysis in the circulation, which leads to increase in fIGF-I levels only in the pediatric condition of low GH function. The aim of our study is to investigate whether proteolysis of IGFBP-3 is dependent on GH-IGF-I axis, by using serum from patients with GHD including pediatric cases. Here, proteolysis of IGFBP-3 in sera from patients with GHD in which the condition is similar to GHRD in terms of low IGF-I synthesis was measured by Western immunoblotting [13]. f/t IGF-I ratio was also measured as a marker for the proteolysis in plasma from the patients with GHD by immunoradiometric assay (IRMA) for free and total IGF-I [19, 20].

Materials and Methods

Materials

Horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin whole antibody (from donkey) and Western blotting detection reagents were purchased from Amersham (Tokyo, Japan). The first antibody, namely anti-glycosylated IGFBP-3 antibody (from rabbit), was provided by Dr. R. G. Rosenfeld (Oregon Health Sciences University, Oregon, U.S.A.). Recombinant human IGF-I (rhIGF-I) was purchased from Toyobo (Osaka, Japan).

Subjects and Sample collection

Twelve patients with hypopituitarism including GHD (ages 4-25 years, male/female=3/8; stimulated GH peaks <5 ng/ml) were studied. These patients were not being treated with GH at the time of this study, although other hormone deficiencies were appropriately treated. Diagnosis of these patients is as follows: idiopathic GHD, male/female=2/2; craniopharyngioma, male/female=0/2; germinoma, male/female=0/2; perinatal-onset hypopituitarism after breech delivery, male/female=1/1; pituitary adenoma, male/female=0/1. IGFBP-3 levels in the subjects were less than the fifth percentile for each age, as was reported previously [21]. The sera from the patients were taken in the morning after breakfast. An intra-daily (0000-2400 h) variation of proteolysis of IGFBP-3 (every two hours) was studied in two patients with GHD (ages 4 and 14 years; male/female=1/1; diagnosis, craniopharyngioma and perinatal-onset hypopituitarism after breech delivery, respectively). Written informed consent was obtained from the patients and/or their parents or legal guardians before participating in this study.

Western immunoblotting

Proteolysis of IGFBP-3 was measured by Western immunoblotting [13]. Each sample (0.002 ml) was subjected to SDS-PAGE (12%) directly or after incubation with 0.002 ml of control serum at 37°C for two hours. The gel was transferred to nitrocellulose, which was incubated with the anti-IGFBP-3 antibody and then the HRP-linked second antibody (rat anti-rabbit gamma-globulin antibody). The complexes of the HRP-linked antibody were detected by chemiluminescence method (Western blotting detection, Amersham).
Densitometric analysis of Western immunoblotting

Proteolysis of IGFBP-3 was quantified by densitometric analysis (DM-303 digital densitrol, ADVANTEC). In pregnant serum, the activities were generally more than 50%. In normal control subjects, all the activities were less than 10%, which is called absent in this paper [13].

Analysis of f/t IGF-I ratio

fIGF-I was measured by IRMA as previously described [19, 20]. The assay was specially designed not to disturb the equilibrium between free and bound IGF-I. The EDTA plasma samples were used without dilution, additives or preseparation. Sample (0.2 ml) of plasma or rhIGF-I was added to the tube containing anti-IGF-I monoclonal antibody (MAB 39-11, Yuka Medias, Ibaraki, Japan) coated to a polystyrene bead. After five minutes incubation at 37°C, the bead was washed three times with distilled water and [125I] anti-IGF-I monoclonal antibody (MAB 39-12; 200000 cpn in 0.2 ml) was added for an additional three hours at room temperature. The bead was then washed and counted in an automatic gamma counter. The functional range of assay was 0.03 to 10.00 ng/ml with interassay coefficients of variation and intraassay coefficients of variation of less than 10%.

tIGF-I was measured using the same agents as in the fIGF-I, with the addition of an extraction step and modifications of the procedure [19, 20]. Plasma samples (0.025 ml) were extracted with 0.5 ml of 12.5% (vol/vol) absolute ethanol in 0.1 N HCl to minimize interference from IGFBPs. Extracted samples (0.025 ml) were then mixed with 0.3 ml of the iodinated detection antibody (39-12) and capture antibody (39-J1)-coated bead for two hours at room temperature. The bead was then washed twice with distilled water and counted. The functional assay range was 6.3 to 2100.0 ng/ml with interassay coefficients of variation and intraassay coefficients of variation of less than 10%.

Statistics

Descriptive data are given as mean ± SD. Statistical comparisons were performed with Mann-Whitney U-test. P-values less than 0.05 were considered to be significant.

Results

Proteolysis of IGFBP-3 was not detected by Western immunoblotting in patients with GHD. Fig. 1 shows proteolysis of IGFBP-3 in serum from one of the 11 patients with GHD (age, 6 years). The major band of intact IGFBP-3 (MW=40 kDa) and some bands of degraded IGFBP-3 (MW≤30 kDa) were seen in all lanes. Compared to control serum which was age-matched with the patient (control serum; lane 1), the decreased band of intact IGFBP-3 was detected in pregnant serum incubated with control serum (lane 3), suggesting that proteolysis of IGFBP-3 was not detected by Western immunoblotting in patients with GHD.
IGFBP-3 is present in pregnant serum. The ratio of intact IGFBP-3 and degraded IGFBP-3 in serum from the patient with GHD (lane 4) was quantitatively similar to those of control serum alone (lane 1). Neither decreased intact IGFBP-3 nor increased proteolyzed IGFBP-3 was observed in the GHD sample after the incubation with control serum (lane 5), indicating the absence of proteolysis of IGFBP-3 in the patient with GHD. The proteolysis in sera from the other 10 patients with GHD was absent (data not shown). The band of intact IGFBP-3 in GHD (lane 4) were less dense than in control serum alone (lane 1).

Absence of proteolysis of IGFBP-3 in all the 11 patients with GHD was confirmed by densitometric analysis (Fig. 2). There was no significant difference in the ratios between GHD (G; 1.05 ± 8.64%) and normal control subjects (C; 2.36 ± 3.15%), although a significant difference was noted between G and pregnant subjects (P; 75.47 ± 12.20%). The proteolysis of IGFBP-3 was not observed in G.

Proteolysis of IGFBP-3 was not observed at any time in the two patients with GHD (ages 4 and 14 years). Representative data of one of the patients with GHD (1800-0400 h, lanes 4, 5, 6, 7, 8, 9; without incubation, lanes 10, 11, 12, 13, 14, 15; with incubation) are shown in Fig. 3. Proteolysis of IGFBP-3 was considered as absent (lanes 10, 11, 12, 13, 14, 15) by densitometric analysis. Similar data were obtained at other time periods in this patient or in another patient with GHD (data not shown).

All f/t IGF-I ratios in GHD were generally in the normal range. f/t IGF-I ratios in plasma from 10 patients with GHD (G) are shown in Fig. 4, together with the data of normal comparison populations [10, 20]. The normal comparison populations included C (ages 4-25 years; N=36; non-pregnancy; 0.79 ± 0.16%). As positive control, the high ratios in during pregnancy (P; ages 22-35 years; N=17; 10-38 weeks gestation; 2.53 ± 0.22%) is shown [10]. There was no significant difference in the ratios between G and C, but there was a significant difference between G and P, which is consistent with the fact that proteolysis of IGFBP-3 was absent. All the levels of IGF-I and IGF-I in the patients were less than the fifth percentile for each age, which is consistent with our previous report [20].
Discussion

It is conjectured that f/t IGF-I ratio is a marker for proteolysis of IGFBP-3. In this study, the results of this ratio were shown to be consistent with those of proteolysis of IGFBP-3 as determined by Western immunoblotting. Recently, we have reported that there was an increased f/t IGF-I ratio in plasma of pregnant women who had proteolysis of IGFBP-3 [9], and that there was a direct correlation between proteolysis of IGFBP-3 and f/t IGF-I ratio [22]. f/t IGF-I ratio may be reflected by other IGFBPs excluding IGFBP-3, although IGF-I is predominantly bound to IGFBP-3 in the circulation.

Our data indicate that proteolysis of IGFBP-3 is not dependent on the GH-IGF-I axis, based on the results of Western immunoblotting and the relatively normal f/t IGF-I ratios in the subjects with GHD. The mechanism(s) of increase in the proteolysis during pregnancy [7-9], post-surgery [11, 12], acute diseases [13] and early infancy [10] is unknown. However, our results suggest proteolysis of IGFBP-3 is not upregulated by low function of GH or IGF-I, which is consistent with a recent report [15].

In low GH function, two controversial results about the proteolysis of IGFBP-3 were previously reported [14, 15, 18]. In human studies, Skjaerbaek et al. reported that proteolysis of IGFBP-3 was not detected in adult patients with GHD [15], which is consistent with our data. However, two other groups, Lassarre et al. and Cotterill et al., reported that the proteolysis was observed in adult patients with GHD [14] and in two children with GHRD [18], respectively. The reason for the difference between conflicting data is unknown. Some possible reasons were the differences in age, nutritional state, and catabolic conditions in the subjects.

Proteolysis of IGFBP-3 was absent at all times in the two patients with GHD in this study, whereas the presence of proteolysis of IGFBP-3 from 2000 to 0100 h in two patients with GHRD in childhood was reported [18]. Proteolysis of IGFBP-3 ought to be detected at any time during the day, if low function of GH-IGF-I axis leads to the compensatory increase in proteolysis of IGFBP-3, thus resulting in incremental increase of fIGF-I levels. It remains to be clarified whether proteolysis of IGFBP-3 is detected late at night in other patients with GHD or GHRD.

References


