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COMPARISON OF WESTERN BLOT ANALYSES IN NATIVE AND ACID-ETHANOL-EXTRACTED SERUM FOR DETECTING BIG IGF-II IN PATIENTS WITH NON-ISLET CELL TUMOR HYPOGLYCEMIA

Riko Kashima, Hiroki Fujita, Hitomi Otomo, Yuki Fukuoka and Yuichiro Yamada

Department of Endocrinology, Diabetes and Geriatric Medicine, Akita University Graduate School of Medicine

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Abstract

Non-islet cell tumor hypoglycemia (NICTH) triggered by high molecular weight insulin-like growth factor (IGF)-II (big IGF-II) is a rare cause of recurrent hypoglycemia. Western blot analysis is thought to be a best method to detect the presence of the big IGF-II in serum or tumor of patients with NICTH. However, its detailed protocols regarding treatment of human serum samples have not been reported. To determine how human serum samples should be treated in this analysis, we performed western blot analyses for detecting big IGF-II in native and acid-ethanol-extracted serum samples obtained from three patients with recurrent hypoglycemia and hypoinsulinemia who were clinically diagnosed as NICTH. In abdominal computed tomography examination, bulky tumor which occupies abdominopelvic cavity was found in two cases of these patients, and renal tumor was detected in the other case. Through western blot analyses in these three cases, we found that big IGF-II is more clearly visualized in acid-ethanol-extracted serum rather than native serum. The present results suggest that the acid-ethanol-extraction method in western blot analysis for detecting big IGF-II would facilitate the diagnosis of big IGF-II producing NICTH.

Key words : Insulin-like growth factor-II, Western blot, Acid-ethanol extraction, Non-islet cell tumor hypoglycemia, Recurrent hypoglycemia

Introduction

Insulin-like growth factor (IGF)-II, which is a 7.5 kDa peptide with homology to insulin, exerts a blood glucose-lowering effect through interacting directly with insulin receptor[1]. In normal sera, IGF-II circulates forming a ternary 150 kDa complex with IGF binding protein (IGFBP)-3 and acid-labile subunit[2]. The ternary complex of 7.5 kDa normal IGF-II does not pass easily through vascular walls and access the insulin receptor of target tissues, because of its relatively high molecular weight[3]. However, in some patients with non-islet cell tumor hypoglycemia (NICTH), a high molecular weight IGF-II (big IGF-II) of 10-15 kDa is often produced in tumors by abnormal processing of pro-IGF-II[4]. Since the big IGF-II secreted from tumors into sera circulates mainly in a 50 kDa binary complex with IGFBP-2, which can cross capillary barrier and render its IGF-II bioavailable to insulin target tissues, the patients with abnormal

Correspondence : Hiroki Fujita
Department of Endocrinology, Diabetes and Geriatric Medicine, Akita University Graduate School of Medicine,
1-1-1 Hondo, Akita 010-8543, Japan
Tel : 81-18-884-6769
Fax : 81-18-884-6449
E-mail : hirofuji@gipc.akita-u.ac.jp

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big IGF-II tend to develop severe hypoglycemia[5-7].

Currently, the diagnosis of NICTH triggered by the big IGF-II is carried out based on its presence in serum or tumor. The best method to detect the big IGF-II is thought to be western blot analysis. Unfortunately, there are only a few articles which described the detailed analysis method. In western blot analysis for detecting specific serum protein, native serum samples are often used because of saving time and effort. However, human serum contains a large amount of high molecular weight proteins, which may interfere with western blot analysis of low molecular weight proteins and produce erroneous results. Therefore, we should carefully prepare serum samples in western blot analysis of serum hormones with low molecular weight. Previously, acid-ethanol-extracted serum samples were used in a radioimmunoassay for serum levels of some hormones with low molecular weight such as IGF-I, IGF-II, and somatostatin[7]. Considering such background, we tested which one of native or acid-ethanol-extracted serum samples is more appropriate for western blot analysis of IGF-II that is a 7.5 kDa peptide. We here describe how we should treat the serum samples in western blot analysis for detecting big IGF-II, and also report the results of big IGF-II analysis in three cases of NICTH.

Methods

Patients and serum samples

Western blot analysis for IGF-II was performed in serum samples collected from three patients who were hospitalized for loss of consciousness due to hypoglycemia. Informed consent for western blot analysis for IGF-II was obtained from all patients. This study was approved by the Ethics Committee of Akita University. All procedures performed were in accordance with the Helsinki Declaration.

Western blot analysis

Western blot analysis for IGF-II in acid-ethanol-extracted serum was compared with that in native serum. The detailed method of acid-ethanol extraction is

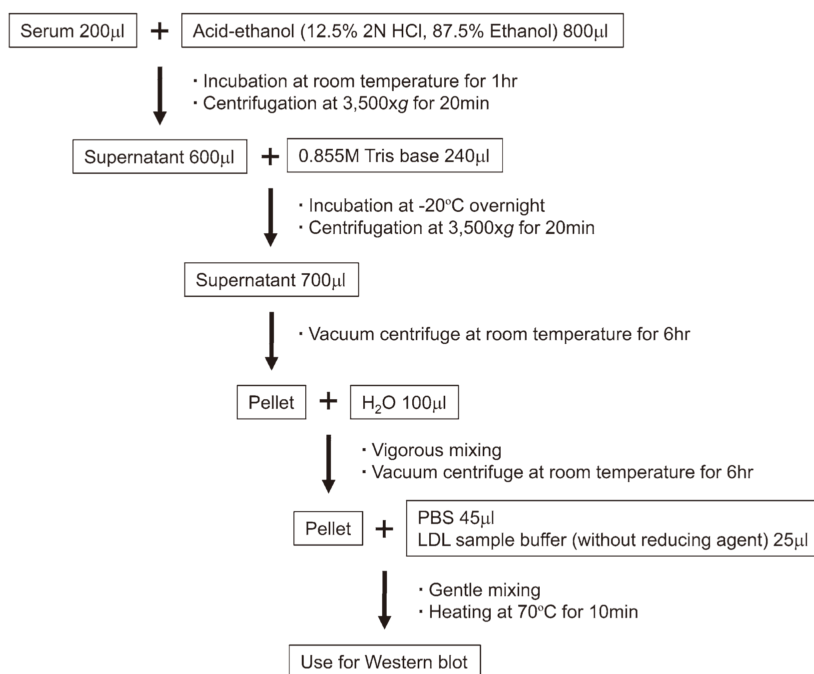

Fig. 1. The method of acid-ethanol extraction and sample preparation in western blot analysis for detecting serum IGF-II.

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summarized in Fig. 1. First, 800 μl of acid-ethanol (12.5% 2N HCl, 87.5% ethanol) was added to 200 μl of serum. The mixture was incubated at room temperature for 1 hr and centrifuged at 3,500 × *g* for 20 min. Then, 600 μl of supernatant was neutralized with 240 μl of 0.855 M Tris base and incubated at −20°C overnight. After centrifuged at 3,500 × *g* for 20 min, 700 μl of supernatant was dried by vacuum centrifuge. One hundred microliter of H_2O was added to the pellet, and then the mixture was dried again by vacuum centrifuge. Finally, the concentrated samples were mixed with 45 μl of phosphate buffered saline (PBS) and 25 μl of lithium dodecyl sulfate (LDS) sample buffer (4× ; Thermo Fisher Scientific, Waltham, MA, USA) without reducing agent, heated at 70°C for 10 min, and used for Western blot. Native serum was diluted 1 : 50 with PBS. Eighteen microliter of the diluted native serum was mixed with 6 μl of LDS sample buffer (4×), heated at 70°C for 10 min, and used for western blot.

Ten microliter of acid-ethanol-extracted or native serum protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, the membranes were reacted with mouse anti-IGF-II monoclonal antibody (1 : 1,000, Millipore, Billerica, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1 : 5,000 ; DakoCytomation, Glostrup, Denmark). The reactions were visualized using ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Results

Three patients (Case #1-3) were hospitalized for loss of consciousness due to hypoglycemia. Table 1 shows clinical characteristics and biochemical data in the patients on admission. All the patients exhibited reduced levels of plasma insulin and C-peptide relative to plasma glucose. Through abdominal computed tomography (CT) examination, two patients, cases #1 and #3, were found to have bulky tumor which occupies abdominopelvic cavity (Fig. 2A, C). In case #2, left renal tumor was observed via abdominal CT test (Fig. 2B). All the three patients exhibited normal levels of pituitary and adrenal hormones and no abnormality in pancreas via CT examination (data not shown). Based on such endocrine and

Table 1. Biochemical parameters on admission

	Case #1	Case #2	Case #3
Age	64	84	31
Gender	female	female	male
Plasma glucose (mg/dl)	79	42	48
Serum insulin (μU/ml)	< 0.5	< 0.5	< 0.5
Serum C-peptide (ng/ml)	< 0.20	< 0.20	0.89

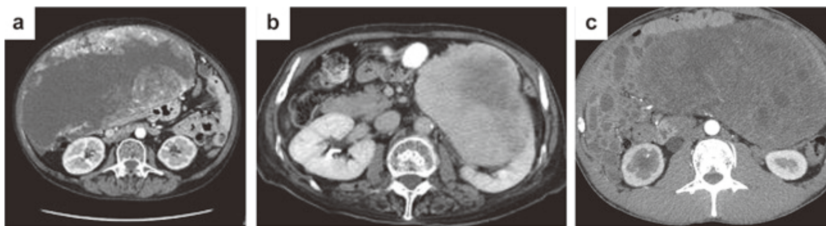

Fig. 2. Findings in abdominal computed tomography scan in three patients with NICTH. (a) A bulky tumor histopathologically diagnosed as gastrointestinal stromal tumor (GIST) in case #1. (b) A left renal tumor in case #2. (c) A bulky tumor histopathologically considered to be solitary fibrous tumor (SFT) in case #3.

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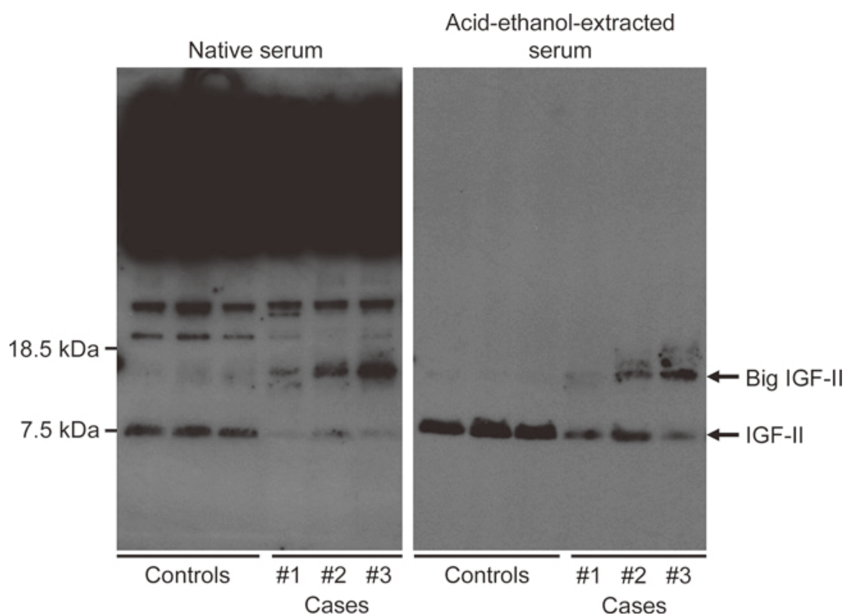

Fig. 3. Western blot analysis for IGF-II in native and acid-ethanol-extracted serum samples obtained from three healthy control subjects and three patients with NICTH (cases #1-3).

CT test findings, the diagnosis of NICTH caused by production of big IGF-II was suspected in these patients. We examined whether or not the patients' tumors produce big IGF-II and release it into the blood stream.

To clearly visualize big IGF-II band by western blot analysis, we tested several methods for pre-treating serum samples. As a healthy control, serum samples obtained from three authors in this article were used in western blot analysis. Fig. 3 shows the results of western blot analysis in native and acid-ethanol-extracted serum samples. We confirmed that all three patients have big IGF-II in serum and also show reduced levels of normal IGF-II relative to healthy controls. Regarding this analysis method of IGF-II and big IGF-II, it is noteworthy that more clear bands of normal and big IGF-II were observed in western blot performed using acid-ethanol-extracted serum samples. In contrast, multiple bands were seen above specific IGF-II bands in western blot carried out using native serum samples, possibly due to non-specific binding of primary antibody to some serum proteins. Thus, we found that it is difficult to identify big IGF-II by western blot analysis in native serum samples.

The bulky tumors in cases #1 and #3 were removed by surgery and histopathologically diagnosed as gastrointestinal stromal tumor (GIST) and solitary fibrous tumor (SFT), respectively. Because the case #2 did not hope to receive surgery, histopathological examination was not performed for the renal tumor. After surgery for GIST in case #1 and SFT in case #3, the patients had an amelioration of the spells of hypoglycemia. In case #2, the treatment with dexamethasone was effective to reduce hypoglycemic symptoms.

Discussion

NICTH is regarded as a rare cause of hypoglycemia. Since the close link of big IGF-II to NICTH was first found by Daughaday *et al.* in a case with fibrosarcoma[8], the cases with NICTH caused by multiple tumors such as GIST in the abdomen and pelvis[9], SFT of the pleura[10], and gastric cancer[11], adrenocortical carcinoma[12], and also by sarcoidosis of the spleen[13] have been reported. In most of these reported cases, big IGF-II was detected in their sera. Currently, routine diagnostic tests for big IGF-II producing NICTH have not been established, and

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therefore it appears to be relatively difficult to confirm this disease. Previously, serum IGF-II levels were measured for the diagnosis of NICTH. However, several clinical studies revealed that mean value of serum IGF-II in patients with NICTH was not significantly different from that in normal subjects[14,15]. Therefore, it may be difficult to confirm big IGF-II producing NICTH by only measurement of serum IGF-II level. Collectively, it is conceivable that the best way for the diagnosis of big IGF-II producing NICTH would be directly detecting big IGF-II in sera or tumors by western blot analysis.

To our knowledge, there is no article which describes the detailed method for treatment of human serum samples in western blot analysis for detecting big IGF-II. Therefore, we here determined how human serum samples should be treated in this analysis. As shown in Fig. 3, our analysis results clearly demonstrate that the presence of big IGF-II can be better confirmed in acid-ethanol-extracted serum rather than native serum. Furthermore, our data suggest that western blot analysis performed using native serum samples may provide confusing data, because multiple non-specific bands are observed in this method. Both normal and big IGF-IIs exist forming complexes with IGFBPs in serum. Therefore, a major problem is considered to be the interference of IGFBPs in this analysis. To circumvent this problem, pretreatment of serum samples appears to be useful for not only western blot analysis but also various immunoassays for IGFs. For example, in human serum IGF radioimmunoassay (RIA), it has been shown that extracting serum IGF by using acid-ethanol before measurement is needed to avoid detecting false values[16]. To physically separate IGF-IIs from IGFBPs present in serum samples, the acid-ethanol extraction currently appears to be a best method, although this technique is relatively inconvenient and time-consuming.

The most common cause of hypoglycemia is well known to be a diabetic treatment with blood glucose-lowering agents such as sulfonylurea or insulin, followed by insulinoma and endocrine deficiencies[17]. When these diseases are excluded as a cause of hypoglycemia, and also when insulin secretion is suppressed despite the occurrence of repeated hypoglycemia, we should focus on NICTH as a differential diagnosis of hypoglycemia. Through the present analyses, we conclude that western blot analysis using acid-ethanol-extracted serum samples is more suitable for precisely detecting big IGF-II, and that this method would facilitate the diagnosis of big IGF-II producing NICTH.

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Disclosure

The authors state that they have no conflict of interest.

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