Study of serum big-insulin-like growth factor (IGF)-II and IGF binding proteins in two patients with extrapancreatic tumor hypoglycemia, using a combination of Western blotting methods

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Abstract
Extrapancreatic tumor hypoglycemia (EPTH) is associated with increased amounts of high-molecular-weight precursor forms of insulin-like growth factor (IGF)-II (‘big-IGF-II’) that have a primary role in the pathophysiology of hypoglycemia. In the present study, using Western ligand and immunoblotting methods, we investigated IGF-binding proteins (IGFBPs), IGFBP-3 proteolysis and big-IGF-II in pre- and postoperative serum from two patients with EPTH due to benign pleural fibroma. In the preoperative serum, IGFBP-3 was reduced and IGFBP-2 was increased compared with that from an age-matched healthy control. IGFBP-3 proteolysis was dramatically reduced in one patient, whereas no major alteration was observed in the other (9% and 120% of control serum, respectively). IGFBPs progressively returned to a subnormal pattern in postoperative serum, whereas IGFBP-3 proteolysis remained greater than in preoperative serum in both patients at days 14 and 90 after surgery. High-molecular-weight forms of IGF-II predominate in EPTH serum (65% and 57% of total IGF-II immunoreactivity in patients 1 and 2, respectively, compared with 25% in control serum). Two forms, of molecular mass 10 and 12 kDa (‘standard big-IGF-II’) were present in both EPTH and control sera, whereas two additional forms, of molecular mass 15 and 18 kDa (‘big big-IGF-II’) were observed in EPTH sera only. Big big-IGF-II represented 72% and 55% of total high-molecular-weight forms of IGF-II in the two EPTH sera, respectively. All big forms of IGF-II disappeared from the serum as early as 6 h after surgery. This study shows that combination of simple Western blotting methods, available routinely in most laboratories, should prove useful in providing reliable physiopathological information in EPTH.

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Introduction
Extrapancreatic tumor hypoglycemia (EPTH) is a rare cause of organic hypoglycemia. Tumors associated with EPTH overexpress the insulin-like growth factor (IGF)-II gene, and secrete into the circulation increased concentrations of 10–15-kDa forms of IGF-II, known as big-IGF-II. These high-molecular-weight forms of IGF-II represent partially processed pro-IGF-II and are usually detected by gel filtration (1, 2). In normal individuals, about 80% of the circulating IGF is carried in ternary complexes of about 150 kDa, known as big-IGF-II. These high-molecular-weight forms of IGF-II are usually detected by gel filtration (1, 2). In normal individuals, about 80% of the circulating IGF is carried in ternary complexes of about 150 kDa, formed by the association of IGF-I or -II, IGF-binding protein (IGFBP)-3 and a 85-kDa glycoprotein, acid-labile subunit (ALS) (3). The ternary complexes do not cross the capillary barrier and are considered to be a storage pool for the circulating IGFs. The remainder of the circulating IGF is carried in 50-kDa binary complexes that consist of IGFs bound to IGFBPs, mainly IGFBPs-1, -2 and -4. These complexes cross the vascular boundary, thus transporting IGFs to the target tissues. Limited proteolysis of IGFBP-3 is believed to be a key mechanism to increase the bioavailability of IGFs in several physiological and pathological situations. It results in decreased affinity of IGFBP-3 for IGFs in the ternary complexes, and leads to a shift in distribution of IGFs from the ternary towards the binary complexes (3–9). However, both the origin and the mechanism of regulation of IGFBP-3 protease(s) remain unclear.
In EPTH, oversecretion of big-IGF-II in serum leads to an impaired formation of the 150-kDa ternary complexes resulting in a shift of IGFBP-3 and tumor-secreted big-IGF-II into the 50-kDa complexes (10). These latter complexes can further leave the vascular compartment and thus provoke an increased bioavailability of big-IGF-II for insulin target tissues, which sustains hypoglycemia. We now report a study of two patients with EPTH caused by benign pleural fibroma. In order to analyse big-IGF-II and IGFBPs in serum before and after tumoral resection, we used simple Western blotting methods to characterize and semi-quantitatively multiple big-IGF-II forms, IGFBPs and IGFBP-3 proteolytic activity.

Study participants and methods

Patients and control

Patient 1, a 52-year-old man, was admitted to hospital in October 1994 because of hypoglycemic coma (plasma glucose 1.7 mmol/l). He had been previously treated by glipizide for non-insulin-dependent diabetes mellitus (NIDDM), but this treatment had been interrupted 1 month earlier, because of a first hypoglycemic episode (plasma glucose 2.2 mmol/l). On physical examination, the patient was breathless. A chest radiogram revealed a large mass in the right thoracic area, which was confirmed by a computed tomography scan. Surgical removal of the mass revealed a tumor of pleural origin, without invasion of the lung. Histological analysis demonstrated a benign pleural fibroma. No more hypoglycemic episodes were noted after surgery, and plasma glucose progressively increased to diabetic values.

Patient 2, a 58-year-old man, was admitted to hospital because of severe respiratory deficiency. His medical history revealed untreated NIDDM and coronary heart disease. Five years previously, the patient underwent exploration for a left thoracic tumor, which was erroneously reported as an anaplasic pulmonary carcinoma and treated by chemotherapy for a period of 2 years. On admission to hospital, the patient was severely hypoxic. He also reported difficulty in awakening in the morning during the previous few weeks, with a clinical improvement after breakfast. During his stay in hospital, low fasting plasma glucose values were noted. Decompressive surgery showed that the tumor was of pleural origin, compressing but not invading the lung. Histological analysis revealed a benign pleural fibroma. No more hypoglycemia was noted and plasma glucose concentrations progressively increased to diabetic values.

A 45-year-old healthy man was used as control. His physical examination was normal, and he showed no evidence of nutritional, endocrine or neoplastic pathology.

Experimental procedure

Blood samples were collected from the participants after an overnight fast and sera were frozen at -20°C until required for use. The serum profile of IGFBPs was determined by Western ligand blot (WLB) as previously described by Hossenlop et al. (11). The nitrocellulose membranes were probed with 2 x 10³–3 x 10³ c.p.m. / ml ¹²⁵I-IGF-II (Amersham, Aylesbury, UK), and IGFBPs were visualized by autoradiography.

IGFBP-3 and in vivo IGFBP-3 proteolysis were analyzed by Western immunoblot (WIB). The nitrocellulose membranes were probed with a 1/1000 dilution of rabbit polyclonal antibody against IGFBP-3 (Upstate Biotechnology, Lake Placid, NY, USA), followed by incubation with anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase. Immunoreactive proteins were visualized using the Amersham enhanced chemiluminescence (ECL) System.

 Autoradiographs were quantitated by scanning densitometry with a Sharp JX-325 laser densitometer and absorbance curves were integrated using the Image Master Software (Pharmacia Biotech, St Quentin-Yvelines, France). Densitometric analyses were performed on gels with different exposure times and those that gave linear absorbance curves were used to obtain semiquantitative data. IGFBP-3 proteolysis was estimated by calculating the ratio of the absorbance of the 30-kDa IGFBP-3 proteolytic fragment to that of the total IGFBP-3 – that is, the sum of intact IGFBP-3 and 30-kDa IGFBP-3 fragment in the same lane. The results were expressed as a percentage of IGFBP-3 proteolysis in age-matched healthy control serum processed in parallel on the same gel, which was given an arbitrary value of 100%.

Both standard and big forms of IGF-II were analyzed by WIB with a mouse anti-IGF-II monoclonal antibody (IgG1) (Upstate Biotechnology) and the ECL detection system (Amersham). Briefly, serum was first desalted by ultrafiltration in Centricon-10⁰ microconcentrator (molecular weight cut-off 10 kDa; Amicon, Beverly, MA, USA), then the serum retentate was reconstituted with 1 mol/l acetic acid in order to dissociate IGFs from IGFBPs. Acidified serum was submitted to ultrafiltration in a Centricon-30⁰ microconcentrator (molecular weight cut-off 30 kDa) and the filtrate was lyophilized then reconstituted in electrophoresis sample buffer and submitted to WIB analysis.

Plasma glucose was measured by the hexokinase method (Bayer Diagnostics, Puteaux, France). Commercial radioimmunoassays kits were used to measure insulin (Sorin, Saluggia, Italy), C-peptide (DSL, Webster, TX, USA) and IGF-I (Biosource, Fleurus, Belgium).

Results

Biological results for the two patients are listed in Table 1.

Analysis of IGFBP profiles by WLB

The IGFBP molecular profiles in serum were analyzed by WLB (Fig. 1). Preoperative serum from both patients
presented a similar profile. Intact IGFBP-3 (doublet at 39–42 kDa identified by WIB, see below) was reduced to 66% and 54%, respectively, of that in age-matched control serum (Fig. 1). IGFBP-2 (the band at 34 kDa identified by WIB, not shown) was markedly increased compared with control serum, in which this band was very faint. The band at 30 kDa that corresponds at least in part to IGFBP-1 was also increased in EPTH sera, in association with the low concentrations of insulin.

During the postoperative period, we first observed a dramatic decrease in intact IGFBP-3. For patient 1, intact IGFBP-3 was undetectable on the 3rd day after operation, then it progressively increased, although remaining less than the preoperative values on day 14 (Fig. 1A). For patient 2, intact IGFBP-3 was still undetectable at day 14 after surgery. It returned to a value slightly lower than control on day 90 (Fig. 1B).

Although the IGFBP-2 concentration slowly decreased after surgery in patient 1, no marked change was noted in patient 2 (compare Figs 1A and 1B).

In vivo IGFBP-3 proteolysis by WIB

WIB analysis of EPTH and age-matched healthy control serum probed with an anti-IGFBP-3 antibody demonstrated the intact IGFBP-3 doublet (39–42 kDa), in addition to a major immunoreactive IGFBP-3 proteolytic fragment at 30 kDa, the latter not being detected by WLB (Fig. 2). An additional minor IGFBP-3 fragment migrating at 24 kDa was detected in EPTH sera during the early postoperative period (Fig. 2A, lane D3; Fig. 2B, lane D8). In preoperative serum, the extent of IGFBP-3 proteolysis that had already occurred in patients' tissues and sera, termed in vivo IGFBP-3 proteolysis, was markedly reduced in patient 1 (9% of control serum, Fig. 2A), whereas it was not grossly altered in patient 2 (120% of control serum, Fig. 2B). As previously reported by others (6, 7), we observed a dramatic increase in IGFBP-3 proteolysis after surgery in both patients (211% at day 3 in patient 1 and 437% at day 8 in patient 2, compared with control serum). IGFBP-3 proteolytic activity then progressively decreased in patient 1 (52% of control serum, Fig. 2A), whereas it remained at a high level in patient 2 (448% of control serum, Fig. 2B) at day 14 after surgery. Three months after the operation, the rate of in vivo IGFBP-3 proteolysis in patient 2 was still greater than in the preoperative state (236% of control serum, Fig. 2B).

Analysis of big-IGF-II by WIB

WIB analysis (Fig. 3) demonstrated several immunoreactive IGF-II bands in preoperative serum from both patients and in serum from an age-matched healthy control. The band at 7.5 kDa corresponded to mature IGF-II, whereas bands with a greater molecular weight (range 10–20 kDa) were precursor IGF-II forms, termed big-IGF-II. Densitometric analyses indicated that big-IGF-II was dramatically increased in EPTH serum. It represented 65% and 57% of total IGF-II immunoreactivity in

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**Table 1** Fasting serum concentrations of glucose, insulin, C-peptide and IGF-I in two patients with EPTH before (Preop.) and 14 days after (Postop.) surgical removal of the tumor. A, patient 1; B, patient 2.

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<tr>
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<th>Preop.</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>3.8–6.6</td>
<td>2.8</td>
</tr>
<tr>
<td>Insulin (μU/l)</td>
<td>5–15</td>
<td>&lt;5</td>
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<tr>
<td>C-peptide (nmol/l)</td>
<td>0.4–0.8</td>
<td>&lt;0.05</td>
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<tr>
<td>IGF-I (ng/l)</td>
<td>107–265</td>
<td>36</td>
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**Figure 1** Western ligand blot analysis (WLB) of serum IGFBPs in two patients with EPTH before and after surgical removal of the tumor. Serum samples were submitted to WLB as described in Study participants and methods. IGFBPs were probed with [125I]-IGF-II and radioactive bands were visualized by autoradiography. Positions and sizes of molecular weight markers are shown at the right. A, patient 1; B, patient 2. Lanes C, healthy control sera; lane D0, preoperative serum. Remaining lanes, postoperative serum at 6 h (H6) and days 3 (D3), 8 (D8), 14 (D14) and 90 (D90) after surgical removal of the tumor.
in patient 1 and in patient 2, respectively, compared with 25% in control serum. In addition, preoperative serum from both patients contained molecular forms of big-IGF-II that migrated at 10, 12, 15 and 18 kDa, whereas only bands at 10 and 12 kDa were detected in control serum (Fig. 3). Accordingly, 10- and 12-kDa forms were named ‘standard big-IGF-II’, as they were present in both control and EPTH sera, whereas 15- and 18-kDa forms detected only in EPTH sera were called ‘big big-IGF-II’. The amount of big-big-IGF-II differed between the two patients: in patient 1, it represented 48% of total IGF-II and 72% of total big-IGF-II, but in patient 2 it was present in lower amounts, representing 31% of total IGF-II and 55% of total big-IGF-II. Big-IGF-II almost completely disappeared from sera, as early as 6 h after surgery in patient 2 (Fig. 3B). Neither big-big-IGF-II nor standard big-IGF-II were detectable in serum on day 3 (patient 1) or day 8 (patient 2) after surgery (Fig. 3).

Discussion
In this study, we have evaluated the efficacy of using a combination of Western blotting experiments for clinical diagnosis and follow-up in two patients with EPTH. This was achieved by qualitative and semi-quantitative determination of big-IGF-II, IGFBPs and IGFBP-3 proteolysis in the serum of these patients before and after tumoral resection. Most of the tumors associated with EPTH syndrome are known to secrete increased amounts of high-molecular-weight forms of IGF-II (10–15 kDa) that represent partially processed IGF-II precursors, termed big-IGF-II (2, 12). Big-IGF-II is believed to have a primary role in the pathophysiology of hypoglycemia (1, 12). In the literature, detection of big IGF-II in serum is generally reported to have been performed by chromatographic methods (1, 2, 12, 13). We successfully used a different strategy: large forms of IGF-II were isolated from acidified serum by ultrafiltration on membranes (molecular weight cut-off 30 kDa), then the abundance and size heterogenicity of IGF-II were analyzed by WIB. In addition to the mature 7.5-kDa IGF-II, greater molecular weight IGF-II-immunoreactive bands (10, 12, 15 and 18 kDa) were detected in the preoperative sera of patients with EPTH. As these bands disappeared completely after removal of the tumor, they probably represented big-IGF-II secreted...
by the tumoral tissue. Healthy control serum also contained big IGF-II; however, it was present in smaller amounts than in EPTH sera and was, in addition, characterized by a molecular profile constituted by 10- and 12-kDa forms only. High-molecular-weight forms of IGF-II have been previously reported in normal serum, in which they represent up to 15% of total IGF-II (14). These forms correspond to partially processed pro-IGF-II still containing an E domain extension of 21 amino acids. It should be emphasized that, in the present study, the 15- and 18-kDa forms, named big big-IGF-II, were detected in EPTH sera only. These molecular forms may correspond to pro-IGF-II, in which only the initial step in processing has been made, at a cleavage site of the E domain located beyond the 21 amino acids extension (14). Alternatively, they may correspond to various glycosylated forms of big-IGF-II. O-Glycosylation of the E domain has, indeed, been suggested to have a critical role during the intracellular processing of pro-IGF-II (15). Daughaday et al. (16) reported that the tumor-produced big-IGF-II in serum from EPTH was almost entirely lacking O-glycosylation, and suggested that this could explain the observed defective processing of big-IGF-II in these tumors. However, in NIH-3T3 cells transfected with plasmids that express either glycosylated or unglycosylated forms of pro-IGF-II, Yang et al. (17) observed a similar processing of pro-IGF-II, thus indicating that it was unrelated to the presence or absence of oligosaccharide chains in the E domain. In our two EPTH patients, we also observed a decrease in molecular weight of big-IGF-II after enzymatic treatment with neuraminidase and O-glycosidase, suggesting that tumor-secreted big-IGF-II was O-glycosylated (not shown).

In both patients, we observed a similar preoperative IGFBP profile, characterized by a decrease in intact IGFBP-3 associated with an increase in IGFBP-2 compared with age-matched control serum. In EPTH, low concentrations of intact IGFBP-3 associated with high concentrations of IGFBP-2 have also been reported previously by Zapf (13), and interpreted as resulting from a negative feed-back on the somatotropic axis. Such a pattern is also observed in various physiological or pathological states, especially pregnancy, surgical stress and diabetes, in association with induction of IGFBP-3 proteolytic activity (4–6). However, the low preoperative concentrations of intact IGFBP-3 in EPTH patients reported here were not associated with an increase in IGFBP-3 proteolysis, but, in contrast, with either no change (patient 2) or a marked reduction (patient 1). This observation was surprising, as (i) the patients were expected to be in a catabolic state as a result of the voluminous tumoral mass, and (ii) IGFBP-3 proteolytic activity has been reported to be increased in patients with relative (NIDDM) or absolute (IDDM) insulin deficiencies, thus suggesting a possible role for insulin in the regulation of IGFBP-3 protease(s) (7, 8). Because insulin was undetectable in the patients’ preoperative sera, we expected an increase in IGFBP-3 proteolysis. To our knowledge, inhibition of IGFBP-3 proteolytic activity in EPTH has never been reported.

Impaired formation of 150-kDa ternary complexes between IGF-II (mainly big-IGF-II), IGFBP-3 and ALS is now well known to occur in patients with EPTH (10, 18), and results in almost all big-IGF-II circulating with IGFBP-3 in 50-kDa binary complexes, which increases the bioavailability of big-IGF-II. In such a context, we suggest that inhibition of IGFBP-3 proteolytic activity may represent a compensatory feed-back mechanism, albeit inadequate, by which the excessive insulin-like potential of big IGF-II can be counterregulated. However, the magnitude of this phenomenon appears to have been different in our two patients: inhibition (patient 1), compared with non-induction (patient 2), of IGFBP-3 proteolysis. Although the histological type of the tumor was the same for both patients – a benign pleural fibroma – it is of interest to note that their molecular profile of serum big-IGF-II was different. In particular, the proportion of big big-IGF-II was smaller in patient 2 than in patient 1 (55% and 72% of total big-IGF-II, respectively). Baxter et al. (18) reported that, in an 83-year-old woman with EPTH, tumoral big-IGF-II was markedly less effective than mature IGF-II in allowing radiolabeled ALS to bind human plasma IGFBP-3. Moreover, a fraction of big-IGF-II with a larger molecular weight was 10-fold less effective than a smaller big-IGF-II form in allowing ALS binding to IGFBP-3 to form storage ternary complexes. This observation is in complete agreement with our present results suggesting that the degree of impairment in the formation of the 150-kDa complexes in patient 2 was not sufficient to initiate a regulatory process leading to inhibition of IGFBP-3 specific proteolysis below the normal values. Alternatively, it could be suggested that the apparently normal level of IGFBP-3 protease activity in patient 2 in fact corresponded to an inhibited level of activity, for a patient with a previous history of NIDDM, a disease well known to be associated with increased IGFBP-3 proteolysis. This hypothesis is supported by an IGFBP-3 protease activity that remained four-fold greater in the serum obtained 3 months after surgery than in the preoperative serum.

In conclusion, this study has shown that several key details of pathophysiology in patients with EPTH can be obtained by a combination of different Western blotting methods that can be available routinely in most laboratories. In particular, it underlines the concept that the molecular pattern of high-molecular-weight pro-forms of IGF-II in serum could interfere with the regulation of IGFBP-3 proteolytic processing in vivo.
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