Characterization of Insulin-Like Growth Factor II (IGF-II) and IGF Binding Proteins in Patients with Non-Islet-Cell Tumor Hypoglycemia

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Abstract. Insulin-like growth factor II (IGF-II) in serum and tumor extracts from five patients with non-islet-cell tumor hypoglycemia (NICTH) has been characterized. These tumors contained large quantities of IGF-II (2.4–14.2 µg/g tissues). The serum IGF-II levels in four of five patients were a little high and the serum IGF-I levels in five patients were low. The serum IGF-II/IGF-I ratios in these patients ranged from 24.1 to 64.2, and the values were significantly greater than those in normal subjects (1.7–7.1). When the sera were gel-filtered on a Sephacryl S-200 column under neutral conditions, the proportion of the free form of IGF-II was not increased. However, in four of five patients, an abnormal IGF-II-IGF binding protein complex was found. When serum IGF binding proteins (IGFBPs) were analyzed by Western ligand blotting, serum IGFBP-2 increased in these patients. When the tumor extracts and sera were gel-filtered on a Biogel P-60 column under acidic conditions, the majority of IGF-II in these sera was a big form of IGF-II. As compared to authentic IGF-II, insulin receptor reactivities and IGF-II receptor reactivities of tumor extracted IGF-II increased in two of three patients. These data indicate that in patients with NICTH, heterogenous IGF-II is produced in respect of size and bioactivities, and that the characteristics of IGF binding protein are altered. Thus, to find IGF-II producing tumors among extrapancreatic tumors associated with hypoglycemia, the quality of IGF-II as well as the quantity should be studied.

Key words: IGF-II, Hypoglycemia, NICTH (non-islet-cell tumor hypoglycemia), IGFBP (insulin-like growth factor binding protein).

EXTRAPANCREATIC tumors associated with hypoglycemia (non-islet-cell tumor hypoglycemia, NICTH) is one of the major causes of fasting hypoglycemia [1]. In some patients with NICTH, insulin-like growth factor II (IGF-II) produced by and secreted from the tumor is thought to be a hypoglycemic agent [2, 3]. However, the mechanism of the hypoglycemia is still unknown, because serum IGF-II level is often not increased in the patient with NICTH [4, 5]. Daughaday et al. reported that an increase in the percentage of a large molecular weight form of IGF-II in serum and tumor extracts of patients with NICTH was found [3], suggesting that heterogenous IGF-II might be related to hypoglycemia. In addition, it has been reported that IGFBPs bind to six insulin-like growth factor binding proteins (IGFBPs) in serum and tissues, and that these IGFBPs may act as a reservoir for IGFs but also modulate bioavailabilities of IGFs [6]. In NICTH, it has been speculated...
that the altered IGFBP pattern might be also related to hypoglycemia [7–9]. Recently, we had an opportunity to characterize IGF-II in serum and tumor extracts and serum IGFBPs from five patients with NICTH, and the results are reported herein.

**Materials and Methods**

**Serum and tumor samples**

Serum samples were obtained from five patients with non-islet-cell tumor hypoglycemia [10–12]. The clinical findings for the five patients are shown in Table 1. In two patients (Cases 1 and 2), serum samples were obtained after successful tumor resection. In addition, serum samples were also obtained from 44 normal subjects, seven patients with acromegaly, seven patients with hypopituitarism and six patients with anorexia nervosa to measure IGF-I and IGF-II. Serum samples were kept at −20°C.

In four of five patients with NICTH (Cases 1–4), tumor tissues were obtained at surgery. The tumor tissues were kept at −70°C until extraction.

**Peptides**

Two preparations of recombinant human IGF-II (rhIGF-II) were used because of the limited availability of rhIGF-II. One preparation was provided by the Eli-Lilly Co. (Indianapolis, IN) and was used for iodination and as an unlabeled standard for IGF-II radioimmunoassay (RIA). The other preparation was provided by Dai-ichi Pharmaceutical Co. (Tokyo) and used for investigating receptor reactivities of IGF-II in IGF-II, IGF-I, and insulin RRA.s. These two preparations had the same potency in IGF-II RIA. Recombinant human IGF-I and porcine insulin were provided by Fujisawa Pharmaceutical Co. (Osaka) and Eli-Lilly Co. (Indianapolis, IN), respectively. IGF-I and IGF-II were iodinated by a modification of the chloramine-T method. Specific activities were 5.2–7.4 MBq/µg and 4.4–7.4 MBq/µg, respectively. 125I-insulin was purchased from Amersham Co. (Buckinghamshire, England).

**Extraction of IGFs**

Serum IGFs were extracted with acid-ethanol by a modification of the method of Daughaday et al. [13]. Tumor IGFs were extracted with acetone-formic acid by a modification of the method of Lee et al. [14]. Briefly, tumor tissues were homogenized in four volumes of 3.3 M formic acid/5% Tween 20 and centrifuged at 40,000×g for 10 min. 150 µl aliquots of the supernatants were then heated at 90°C for 30 min. The extracts were treated with 350 µl of reagent grade acetone, mixed and centrifuged for 15 min at 3,000×g. The extracts were dried in a Speed Vac-Concentrator (Savant Inst., Hickville, NY). The samples were reconstituted with assay buffer.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, Sex</th>
<th>Tumor</th>
<th>Blood Glucose (mg/dl)</th>
<th>IRI (µU/ml)</th>
<th>Serum IGF-II (ng/ml)</th>
<th>Serum IGF-I (ng/ml)</th>
<th>Tumor IGF-II (µg/g tissue)</th>
<th>Tumor IGF-I (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50, M</td>
<td>Mesotheioma</td>
<td>Before 25</td>
<td>0.6</td>
<td>Before 1124</td>
<td>41</td>
<td>14.2</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>53, F</td>
<td>Histioctoma</td>
<td>After 89</td>
<td>80</td>
<td>After 555</td>
<td>&lt;35</td>
<td>12.9</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>65, F</td>
<td>Mesotheioma</td>
<td>Before 45</td>
<td>6</td>
<td>805</td>
<td>25</td>
<td>5.6</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>62, M</td>
<td>Hepatoma</td>
<td>After 4</td>
<td>6</td>
<td>899</td>
<td>14</td>
<td>2.4</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>68, M</td>
<td>Gastric Cancer</td>
<td>21</td>
<td>4.1</td>
<td>600</td>
<td>18</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>
To investigate gel filtration profiles of IGF-II in serum, the serum sample was gel filtered at neutral pH. 200 µl serum samples were gel filtered on a Sephacryl S-200 column (1 x 48 cm) equilibrated in and eluted with 0.1 M phosphate buffer with 0.15 M NaCl, pH 7.4, at 4°C. 0.5 ml fractions were collected. Each fraction was extracted with acid-ethanol, and the extract was assayed by IGF-II RIA.

To investigate the size heterogeneity of IGF-II in the serum and tumor, the serum samples and tumor extracts were gel filtered at acidic pH. Serum samples (250–500 µl) were incubated with the same volume of 0.5 M acetic acid/0.15 M NaCl, pH 2.3 for one h at 4°C, and then the acidified samples were gel filtered on Biogel P-60 (1.5 x 86.5 cm) equilibrated in and eluted with 0.5 M acetic acid/0.15 M NaCl, pH 2.3, at 4°C. 0.98 ml fractions were collected and lyophilized. Each fraction was reconstituted with RIA buffer, and measured by IGF-II RIA. 200 µl tumor extracts were also analyzed by gel filtration on a Biogel P-60 column.

**RIA and RRA**

IGF-I and IGF-II were measured by RIA as previously reported [15, 16]. The normal values for serum IGF-I and IGF-II of adults are from 88 to 240 ng/ml, and from 374 to 804 ng/ml, respectively.

Receptor reactivities of tumor extracted IGF-II for IGF-II, IGF-I, and insulin receptors were measured by RRA with placental membrane preparations [17].

**Western ligand blotting of IGFBP**

 Serum IGFBPs were analyzed by Western ligand blotting according to the method of Hossenlopp et al. [18]. Briefly, serum (2 µl) was electrophoresed on 12% SDS/PAGE acrylamide gel under nonreducing conditions. The size fractionated proteins were electroblotted onto nitrocellulose sheet. The nitrocellulose sheet was treated with Nonidet P-40, BSA and Tween 20, and incubated with a mixture of 125I-IGF-I and 125I-IGF-II (1 x 10^6 cpm each) for 2 days. After extensive washing of the nitrocellulose, the IGFBPs were detected by autoradiography.

### Results

**IGF-I and IGF-II values in serum and tumor extracts**

Serum IGF-I and IGF-II values in five patients with NICTH are shown in Table 1. Serum IGF-II was slightly increased in four of five patients, but in one patient (Case 5) serum IGF-II was within the normal range. In two patients (Cases 1 and 2), serum IGF-II was normalized after tumor resection when the hypoglycemia disappeared. Serum IGF-I was low in all patients, and the values were normalized after successful tumor resection.

Serum IGF-II and IGF-I values in 44 normal subjects, seven patients with acromegaly, seven patients with hypopituitarism, and six patients with anorexia nervosa were 588 ± 17 and 191 ± 7 (Mean ± SEM), 1736 ± 31 and 871 ± 135, 371 ± 41 and 39 ± 12, and 286 ± 34 and 26 ± 5 ng/ml, respectively. Serum IGF-II/IGF-I ratios in normal subjects and patients with acromegaly, with hypopituitarism and with anorexia nervosa ranged from 1.7 to 7.1 with a mean of 3.3 ± 0.2, from 0.3 to 2.5 with a mean of 1.1 ± 0.3, 5.6 to 17.9 with a mean of

![Fig. 1. Serum IGF-II/I ratio in normal adults and patients with acromegaly, hypopituitarism, anorexia nervosa and NICTH (IGF-IIoma).](image-url)
Fig. 2. A: Elution profiles of serum IGF-II on Biogel P-60 (pH 2.3) in 4 patients with NICTH (cases 1, 2, 4 and 5) and 2 normal adults. B: Elution profiles of serum IGF-II on Biogel P-60 (pH 2.3) in 2 patients with NICTH (cases 1 and 2) after tumor resection. Serum samples (250–500 µl) were acidified and gel filtered on a Biogel P-60 (1.5×86.5 cm) equilibrated in and eluted with 0.5 M acetic acid/0.15 M NaCl at 4°C. Arrows represent void volume, chymotrypsinogen A (25k), ribonuclease A (13.7k) and authentic IGF-II, respectively.
12.8±1.9, and from 7.5 to 19.2 with a mean of 12.2±1.9, respectively. In the patients with NICTH, serum IGF-II/IGF-I ratios ranged from 24.1 to 64.2, with a mean of 36.2±7.2 (±SEM). Thus, the serum IGF-II/IGF-I ratios in patients with NICTH were extremely high. In two patients with NICTH (Cases 1 and 2), the ratios were decreased after tumor resection to 3.9 and 4.4, respectively.

The tumor IGF-I and IGF-II contents were measured in four patients (Cases 1-4) with NICTH (Table 1). The tumor contained large quantities of IGF-II.

Size heterogeneity of IGF-II

Serum samples and tumor extracts were gel filtered at acidic pH to investigate the size heterogeneity of IGF-II. Sera from four patients with NICTH and two normal subjects were gel filtered on a Biogel P-60 column at pH 2.3 (Fig. 2A). In normal subjects, more than 80% of immunoreactive IGF-II eluted in the same fraction as did rhIGF-II (7.5 K). However, in the patients with NICTH (Cases 1, 2, 4, 5), 14, 15, 25, and 21% of immunoreactive IGF-II eluted in the same fraction as did rhIGF-II, and the remainder was a high molecular weight form (13.7 K–25 K). In two patients (Cases 1 and 2), the proportion of authen-

Fig. 3. Elution profiles of extracted tumor IGF-II on Biogel P-60 (pH 2.3) in patients with NICTH. Two hundred µl tumor extracts were acidified and gel filtered on a Biogel P-60 (1.5×86.5 cm) equilibrated in and eluted with 0.5 M acetic acid/0.15 M NaCl at 4°C. Arrows mean the same as in Fig. 2.
The IGF-II receptor activity increased and that of the high molecular weight form of IGF-II decreased after tumor resection (Fig. 2B).

Tumor extracts from four patients with NICTH (Cases 1–4) were also analyzed by gel filtration on a Biogel P-60 column at pH 2.3. In the tumor extracts, most of the IGF-II immunoreactivity was high molecular weight forms (Fig. 3).

Table 2. IGF-I, IGF-II, and insulin receptor reactivities of tumor extracts of IGF-II

<table>
<thead>
<tr>
<th>Case</th>
<th>IGF-II receptor activity (%)</th>
<th>IGF-I receptor activity (%)</th>
<th>Insulin receptor activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>145</td>
<td>42</td>
<td>45</td>
</tr>
<tr>
<td>Case 2</td>
<td>286</td>
<td>100</td>
<td>206</td>
</tr>
<tr>
<td>Case 3</td>
<td>98</td>
<td>90</td>
<td>294</td>
</tr>
<tr>
<td>rhIGF-II</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 4. Elution profiles of serum IGF-II on Sephacryl S-200 (pH 7.4) in 5 patients with NICTH and a normal adult. Two hundred µl serum samples were gel filtered on a Sephacryl S-200 (1×48 cm) column equilibrated in and eluted with 0.1 M phosphate buffer with 0.15 M NaCl at 4°C. Arrows represent void volume, bovine gamma globulin (158k), chicken ovalbumin (44k) and authentic IGF-II, respectively.
Receptor reactivity of tumor extract

Tumor extracts (Cases 1–3) were measured by RIA and RRA for IGF-II with the same IGF-II standard. As compared to immunoreactive IGF-II, receptor reactive IGF-II increased in two of three patients (Table 2: Cases 1 and 2). Tumor extracts (Cases 1–3) and rhIGF-II were measured by RRAs for IGF-I and insulin. In the RRAs for IGF-I and insulin, the relative potencies of rhIGF-II for displacing $^{125}$I-IGF-I binding and $^{125}$I-insulin were 38% of IGF-I and 16% of insulin, respectively. IGF-I and insulin receptor reactivities of rhIGF-II in these RRAs are defined as 100%, and the relative potencies of these receptor reactivities of tumor extracted IGF-II were calculated on the basis of the weight of IGF-II measured by RIA (Table 2). Insulin receptor reactivities increased in two of three patients (Cases 2 and 3).

Gel filtration profiles of IGF-II in serum and serum IGFBPs

Sera were gel filtered on a Sephacryl S-200 column at neutral pH to investigate gel filtration profiles of IGF-II in serum (Fig. 4). When normal serum was gel filtered on Sephacryl S-200, 150kD and 40kD complexes of IGF-II with IGFBPs were found. However, in sera from four of five patients with NICTH, the 150kD and 40kD complexes were not found, but a single IGF-II binding complex eluted between 150kD and 40kD was found.

The proportion of free form IGF-II was not increased in these patients.

Serum IGFBPs were analyzed by Western ligand blotting. Sera from normal subjects revealed four IGFBPs with mol wts of 41/38, 34, 30 and 24kD, identified as IGFBP-3, -2, -1 and -4, respectively (Fig. 5). In the patients with NICTH, serum IGFBP-2 increased. In two patients with NICTH, the serum IGFBP-2 decreased and serum IGFBP-3 increased after successful tumor resection (Fig. 5).

Discussion

In Japan, it has been reported that NICTH is the second cause of spontaneous hypoglycemia [1]. Thus, NICTH is a common cause of hypoglycemia, but the mechanism of hypoglycemia was not elucidated. Recently, it has been reported that some tumors in NICTH produced and secreted IGF-II [3, 19]. Since IGF-II has a hypoglycemic effect in vivo [20], IGF-II secreted from a tumor with NICTH is thought to be a hypoglycemic agent. Serum IGF-II levels in patients with IGF-II producing NICTH were not always increased. In the present study, we observed that serum IGF-II levels in four of five patients with NICTH were above the normal range, but were not extremely high and were in the same range as those in patients with acromegaly who did not have hypoglycemia. Therefore, the measurement of serum IGF-II is unsatisfactory for diagnosing IGF-II producing NICTH.

We found that serum IGF-I was markedly suppressed in all cases as reported previously [21]. Although we did not study on the GH secretion status in these patients, the decreased IGF-I might be due to suppression of GH secretion [19] acting through IGF-II secreted from the tumor [2]. Thus, serum IGF-I levels were inappropriately low for serum IGF-II levels in cases of IGF-II producing NICTH. In our assay system, if the serum
IGF-II/IGF-I ratio is more than 20, IGF-II producing NICTH might be considered.

Although increased IGF-II and GH suppression might be related to hypoglycemia, the mechanism of hypoglycemia in NICTH remains to be clarified. A possible mechanism of hypoglycemia in NICTH is an increase in the free form of IGF-II. In the present study, we could not find an increase in the free form of IGF-II in the five patients by the gel filtration method. However, taking the sensitivity of the gel filtration method into consideration, this possibility could not be ruled out.

Since the hypoglycemic effect of IGF-II acts mainly through the insulin and/or IGF-I receptor but not the IGF-II receptor [22], increased receptor reactivities of tumor extracted IGF-II for insulin or IGF-I could be related to hypoglycemia in NICTH. As compared to authentic IGF-II, receptor reactivities for insulin in tumor extracted IGF-II increased in two of three patients, but those for IGF-I did not change.

A large molecular weight forms of IGF-II (big IGF-II) might be related to hypoglycemia. As reported previously [3], we found that most of the IGF-II in serum and tumors were big IGF-II in all five patients. After successful tumor removal, the proportion of big IGF-II in total IGF-II of serum was decreased. It has been suggested that the big IGF-II has low affinity for IGFBPs [23], so that big IGF-II exists in a free form in serum. As mentioned above, we could not find an increase in the amount of the free form of IGF-II. For this reason better resolution might be required. Furthermore, Daughaday et al. reported that less big IGF-II was extracted with acid-ethanol than authentic IGF-II [3], and less immunoreactive IGF-II was obtained by acid-ethanol extraction than actual IGF-II.

It has been suggested that the altered IGF-II-IGFBP formation might play a role in hypoglycemia [7–9]. When sera from patients with NICTH were gel filtered on Sephacryl S-200 under neutral conditions, single IGF-II-IGFBP complex eluted between 150kD and 40kD, but no 150kD complex of IGF-II with IGFBP was found in four of five patients. In Western ligand blotting, serum IGFBP-2 increased in these patients as reported previously [7]. After successful removal of the tumor, serum IGFBP-2 decreased and IGFBP-3 increased. The complex of IGF-II with IGFBP-2 more easily passes through the capillary membrane and would have increased the affinity with target tissues [24]. In cases of NICTH, there is a marked reduction in the binding of IGF-IGFBP-3 to the alpha-subunit, and the binary complexes might readily pass from the circulation to the extracellular space, and access to their target cells [9].

In the present study, we demonstrate that heterogeneous IGF-II is produced in respect of size and bioactivity, and IGF binding proteins are altered in patients with NICTH. These results suggest that to find IGF-II producing tumors among extrapancreatic tumors associated with hypoglycemia, the quality of IGF-II as well as the quantity should be investigated.

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References


