Antibodies Directed against the E Region of Pro-Insulin-like Growth Factor-II Used to Evaluate Non-Islet Cell Tumor-induced Hypoglycemia

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Background: Detection of incompletely processed pre-cursor forms of insulin-like growth factor-II ("big" IGF-II) in plasma is essential for both the diagnosis and follow-up of non-islet cell tumor-induced hypoglycemia (NICTH) and may be relevant to other diseases as well. RIA using an antibody raised against a synthetic peptide consisting of the first 21 amino acids of the E domain [E(68–88)] of human pro-IGF-II cannot distinguish between E-peptide-containing big IGF-II and cleaved E domain or fragments. We therefore developed and validated an ELISA that specifically detects big IGF-II in plasma.

Methods: The ELISA used a solid-phase antibody to E(68–88) and a liquid-phase monoclonal hIGF-II antibody. Pro-IGF-II purified from normal human plasma was used as a calibrator. Acid Sep-Pak C18 extracts of plasma from NICTH patients were analyzed, and the results were compared with those obtained for plasma samples from healthy individuals. In addition, blood specimens derived from dialyzed patients with chronic renal failure, which contained relatively high concentrations of cleaved E domain or fragments, were studied. The results were validated by acid Sephadex G-50 gel filtration.

Results: Results from this ELISA indicated that the concentration of big IGF-II in NICTH plasma was higher (mean ± SD, 22.6 ± 9.4 nmol/L) than in normal plasma (3.8 nmol/L). Conversely, the concentrations in pooled CRF plasma (2.0 ± 0.8 nmol/L) were low. Antibodies directed against either E(68–88) or E(13–134) of pro-IGF-II could be used to detect these peptides in tumor tissue by immunohistochemistry.

Conclusions: The possibility of quantifying pro-IGF-II by ELISA in plasma represents a potentially useful tool for the diagnosis and follow-up of NICTH and should facilitate further in vitro and in vivo studies on its regulation and function in humans.

The gene for human insulin-like growth factor-II (IGF-II)4 consists of nine exons with four promoters, and its expression is tissue specific and developmentally regulated (1), which leads to multiple transcripts that all encode the same monomeric IGF-II precursor. The primary IGF-II translation product (pre-pro-IGF-II) contains 180 amino acids, including an N-terminal signal peptide of 24 amino acid residues, the 67-amino acid-long mature IGF-II (7.5 kDa), and an 89-residue extension at the COOH terminus (2, 3). The latter has been designated the E domain (4). Posttranslational processing of pre-pro-IGF-II involves removal of the N-terminal leader sequence and the addition of sialic acid-containing O-linked oligosaccharides on Thr275 (and possibly other sites) of the E domain, followed by sequential proteolytic cleavage of the latter extension to the mature protein (5). Apparently, the processing within the E domain of pro-IGF-II is not necessarily coupled to secretion. IGF-II with a 21-amino acid E extension has been identified and purified from serum (6, 7). The molecular masses of these preparations varied

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4 Nonstandard abbreviations: IGF, insulin-like growth factor; NICTH, non-islet cell tumor-induced hypoglycemia; IGFBP, IGF-binding protein; IMR, immunoreactivity; CRF, chronic renal failure; KLH, keyhole limpet hemocyanin; HRP, horseradish peroxidase; and BSA, bovine serum albumin.
between 10 and 15 kDa, which is probably attributable to variable sialated O-glycosylation on Thr²⁵ (7–9). High-molecular-mass IGF-II (commonly designated as "big" IGF-II) accounts for ~10–15% of the total IGF-II in the serum of healthy adults (10). Substantial amounts of big IGF-II have also been found in cerebrospinal fluid, extracts of the brain and pituitary gland, and in the conditioned medium of several cell types (7).

Many tumors highly express the IGF-II gene compared with healthy tissue (11). Overexpression of IGF-II in tumors has been attributed to the loss of imprinting and mutations in tumor suppressor genes (11). IGF-II has been shown to be required for tumor progression in a mouse model for oncogene-induced tumorigenesis (12). It seems likely that many neoplastic cells are not capable of adequate processing of the relatively high amounts of pro-IGF-II produced. The syndrome of non-islet cell tumor-induced hypoglycemia (NICTH) may represent extreme cases of excessive production of high-molecular-mass IGF-II by an often large tumor. Sera of these patients usually contain relatively high concentrations of big IGF-II, associated with a considerably enhanced direct insulin action on various tissues, ultimately leading to episodes of hypoglycemia (13, 14). Although the exact mechanism is still unknown, big IGF-II seems to possess properties that do not allow the proper formation of a 150-kDa complex with IGF-binding protein-3 (IGFBP-3) and the acid-labile subunit (14). As a consequence, tumor-derived high-molecular-mass IGF-II forms smaller binary complexes with IGFBPs. These smaller complexes have a greater capillary permeability and thus are thought to increase IGF bioavailability to the tissues, leading to hypoglycemia through action on the insulin receptor.

Initially, the detection of serum big IGF-II in the diagnosis of NICTH required size separation by, e.g., Bio-Gel P-60 acid gel filtration (13). Although this method generally provides good separation of the big IGF-II and 7.5-kDa IGF-II, the procedure is rather cumbersome and time-consuming. An advance in this respect was the development of a RIA with an antibody raised against the pro-IGF-II ELISA. Creatinine concentrations in these four pools. These preparations were used for validation of the pro-IGF-II ELISA. Creatinine concentrations in these four pools were 939–972 μmol/L. All participants in this study gave informed consent for use of their blood samples and/or tumor-derived material for investigation.

**Materials and Methods**

**Patients and samples**

Plasma samples were obtained from nine patients (age range, 26–81 years) suffering from NICTH (four females, one each with lung carcinoma, hemeangiopericytoma, leiomyosarcoma, or colon carcinoma; five males, two with hemeangiopericytoma and one each with schwannoma, fibrosarcoma, or leiomyosarcoma). In addition, a large hepatic metastasis was resected from one of the patients with a hemeangiopericytoma, and cyst fluid was aspirated. Tumor tissue from a NICTH patient with a leiomyosarcoma, derived from a metastasis adjacent to the spleen, was also available for analysis. Pooled normal plasma consisted of blood samples collected from healthy volunteers (10 females and 8 males; age range, 22–55 years) working in our department. In addition, a plasma pool was prepared from blood specimens of elderly healthy individuals (four males; age range, 65–68 years) that better matched the age range of the NICTH patients investigated. Spare blood specimens obtained from various dialyzed adult patients with CRF (24 females and 33 males; age range, 19–80 years) admitted to our hospital for routine investigations were randomly divided into four pools. These preparations were used for validation of the pro-IGF-II ELISA. Creatinine concentrations in these four pools were 939–972 μmol/L. All participants involved in this study gave informed consent for use of their blood samples and/or tumor-derived material for investigation.

**Peptides**

A synthetic peptide consisting of the first 21 amino acids of the predicted hIGF-II E-domain region [E(68–88); 2355 Da] was prepared by Sigma Genosys Biotechnologies, Inc. In addition, another peptide, comprising a tyrosine (included to allow labeling with I²¹) preceding amino acids 113–134 of hpro-IGF-II ([Tyr]⁰-E(113–134); 2904 Da) was
synthesized. Both peptides were determined to be >80% pure by HPLC. A portion of each synthetic peptide was coupled to keyhole limpet hemocyanin (KLH), by the carbodiimide method, for immunization purposes. Mature 7.5-kDa hIGF-II and a preparation consisting of a mixture of two glycosylated forms (mean molecular mass, 9700 Da) of pro-IGF-II, containing the first 20 amino acids of the E domain [pro-IGF-II E(68–87)], were obtained from Cohn fraction IV of human plasma and purified as described elsewhere (8, 19). Recombinant (Escherichia coli) nonglycosylated high-molecular-mass (16–17 kDa, according to the manufacturer) pro-hIGF-II (named rhpro-IGF-II), containing both the full-length (156 amino acids) and some lower molecular mass forms, was purchased from GroPep Pty., Ltd.

**ANTIserA**

New Zealand White rabbits were immunized with 120 μg of either KLH-E(68–88) or KLH-[Tyr]E(113–134) in complete Freund’s adjuvant administered by multiple subcutaneous injections along the back and proximal limbs. Subsequent boosts (100 μg of KLH-coupled peptide in Freund’s incomplete adjuvant) were given subcutaneously every 2 weeks. After five boosts, plasmapheresis was performed. Antisera directed specifically against E(68–88) (WKZ6279) and E(113–134) (WKZ6281) were obtained and used throughout the study.

Mouse anti-human IGF-II monoclonal antibody was used as secondary antibody for the pro-IGF-II ELISA and was purchased from Research Diagnostics Inc. Another anti-IGF-II monoclonal antibody (as used in our hIGF-II RIA) was obtained from Amano Pharmaceutical Co.

Horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-mouse IgG + IgM (H + L) was obtained from Jackson ImmunoResearch Laboratories Inc.

**pReASSAY extrACTION**

Before application in the pro-IGF-II ELISA, 0.5-mL aliquots of EDTA plasma or serum were acidified by the addition of 2 mL of 0.5 mol/L HCl containing 5 mmol/L CaCl₂ and were incubated at room temperature for 1 h. Subsequently, the IGFs were separated from IGFBPs by chromatography using Sep-Pak C₁₈ Vac 3-mL (500 mg) columns (Waters Millipore Corporation) (20). Dried extracts were dissolved in assay buffer [0.05 mol/L sodium phosphate buffer, pH 7.4, containing 2 g/L bovine serum albumin (BSA), 0.5 g/L Tween 20, and 0.01 mol/L EDTA].

E(68–88) RIA

E(68–88) RIA was radioiodinated via the chloramine-T method, by reacting 1 μg of peptide with 0.5 mCi of Na¹²⁵I (Amersham International plc). Free¹²⁵I was removed from¹²⁵I-labeled E(68–88) by use of a Sep-Pak C₁₈ cartridge. After a wash with 20 mL of 1 mL/L trifluoroacetic acid, the radiolabeled peptide was eluted in 2 mL of a mixture containing acetonitrile and 1 mL/L trifluoroacetic acid (1:1 by volume) and stored at 4°C.¹²⁵I-labeled hIGF-II was separated from free¹²⁵I on a PD-10 (Phadex G-25) column (Pharmacia Biotech). Specific activities varied between 50 and 70 μCi/μg of protein.

For the E(68–88) RIA, the assay buffer was composed of 50 mmol/L sodium phosphate (pH 7.4), 10 nmol/L EDTA, 0.5 g/L Tween 20, 2 g/L BSA, and 0.2 g/L NaN₃. E(68–88) synthetic peptide was used as a calibrator (range, 0.01–3.40 pmol/tube). The incubation mixture consisted of 200 μL of calibrator or diluted sample, 50 μL of WKZ6279 E(68–88) antiserum (final dilution in assay buffer, 1:5400), and 50 μL of ¹²⁵I-E(68–88) tracer (~10 000 cpm). After equilibrium incubation for 17 h at 4°C in polystyrene tubes, 100 μL of Sac-Cel solid-phase antirabbit IgG-coated cellulose suspension (Immunodiagnostics Systems) was added. Complex formation was complete after 30 min at room temperature, and 0.5 mL of distilled water was added to the samples, which were subsequently centrifuged at 10 000g for 4 min. Pellets were counted in a gamma counter (Packard Instruments, Inc.). In the E(68–88) RIA, 27.2% ± 2.1% (mean ± SD; n = 11) of the ¹²⁵I-E(68–88) tracer was bound specifically, whereas the nonspecific binding of the tracer measured in the absence of primary antibody was 3.9% ± 1.3%. Addition of increasing concentrations of unlabeled E(68–88) inhibited the binding of ¹²⁵I-E(68–88) to the antiserum in a dose-dependent manner. Half-maximal displacement (ED₅₀) of tracer occurred at 0.37 pmol (0.87 ng) of E(68–88)/tube. Neither hIGF-II nor IGF-I at concentrations up to 17 pmol/tube cross-reacted in the E(68–88) RIA. Intraassay variations (eight replicates) were 7.0% and 9.5% at mean plasma concentrations of 6.5 and 23.2 nmol/L E(68–88) IMR, respectively. The respective interassay variations (n = 10) at these concentrations were 9.2% and 10%. The lowest detectable dose was 0.13 nmol/L (absolute concentration). Because IGFBPs did not interfere in the RIA, E(68–88) IMR could be assayed in both Sep-Pak C₁₈-extracted and unextracted plasma. E(68–88) RIA’s with either exogenous E(68–88) peptide or pro-IGF-II E(68–88), ranging from 91 to 127 nmol/L, added to plasma revealed that >80% of these proteins was recovered after Sep-Pak C₁₈ extraction.

**PRO-IGF-II E-PEPTIDE ELISA (OPTIMIZED PROTOCOL)**

An IgG fraction of the WKZ6279 antiserum to E(68–88) was isolated on a column (12 × 50 mm) of protein A-Sepharose CL4B (Sigma). The IgG-containing eluate (in 1 mol/L acetic acid) was neutralized with 2 mol/L Tris base, concentrated on a Centricon Y-10 filter (Waters Millipore Corporation), and readjusted with PBS to the original volume of crude antiserum applied on the column (i.e., 1.5 mL). Microfilter plates (96 wells; ELISA/RIA strip plate 92592; Costar) were coated with 100 μL per well of capture anti-E(68–88) IgG (diluted 50-fold with 10 mmol/L Tris buffer, pH 8.3, containing 0.1 mol/L NaCl) and incubated for 8 h at room temperature. The plates were washed once with 200 μL of PBS per well. The remaining protein binding sites on the plates were
blocked by incubation overnight at 4 °C with 200 µL/well of blocking buffer (0.1 mol/L NH₄HCO₃ containing 20 g/L BSA and 0.5 g/L NaN₃). The wells were washed three times with PBS, followed by the addition of 100 µL of the calibrators [i.e., pro-IGF-II E(68–87); range, 0.008–1.05 pmol/well], quality controls, or unknown samples. Appropriate dilutions of calibrators and reconstituted Sep-Pak C₁₈ extracts were made in assay buffer. The plate was incubated for 6 h at room temperature. After the wells were washed three times, 10 ng/well of mouse anti-human IGF-II monoclonal detection antibody, diluted in assay buffer (see above) was added and incubated overnight at 4 °C. The wells were washed three times, and then 100 µL/well of HRP-conjugated goat anti-mouse IgG + IgM (H + L), diluted 1:3000 in assay buffer, was added for 45 min at 37 °C. After three subsequent washes, the wells were incubated with 50 µL of substrate for 10 min at room temperature. The substrate consisted of 1 g/L o-phenylenediamine dihydrochloride (Sigma) dissolved in 0.1 mol/L phosphate-citric acid buffer, pH 4, containing 0.15 mL/L H₂O₂. Finally, 50 µL of 2 mol/L sulfuric acid stop solution was added to each well. The plates were then read at 490 nm on the Bio-Rad Model 550 Microplate reader (Bio-Rad Laboratories). The absorbance (A) at 490 nm was plotted against the log of the pro-hIGF-II concentrations in the calibrators, and for the linear part of the curve, a straight line was computer-fitted by least-squares regression analysis. Application of the four-parameter logistic function (MultiCalc™, Wallac Oy) to all data of the calibration curves yielded similar results. Dose–response curves were assessed for both quality controls and unknown samples, and their concentrations were computed by interpolation from the calibration curves.

DETERMINATION OF IGF-I, TOTAL IGF-II, AND IGFBP-3

The specific RIAs used to determine circulating concentrations of IGF-I, total IGF-II, and IGFBP-3 in plasma have been described elsewhere (21–23). Recently, the IGF-II RIA, which is performed with Sep-Pak C₁₈-extracted samples, was calibrated against the WHO reference preparation of recombinant rhIGF-II (24). IGF-II RIAs with exogenous IGF-II or pro-IGF-II E(68–87) added to plasma samples revealed that 80–90% of the IMR was recovered.

CHROMATOGRAPHIC METHODS

Sephadex G-50 exclusion chromatography was carried out on Sep-Pak C₁₈ extracts, using a 1.5 × 90-cm column according to Daughaday and Trivedi (3). Acetic acid (0.1 mol/L) was passed in a descending direction at a rate of 4.8 mL/h, and ~3-mL fractions were collected. The column was calibrated with lactalbumin (14.5 kDa), hIGF-II (7.5 kDa), and insulin-B (3.5 kDa).

In addition, several samples were also analyzed by acid Bio-Gel P-60 chromatography, as described previously (10).

WESTERN IMMUNOBLOTTING

From the various plasma samples, aliquots extracted by Sep-Pak C₁₈ or 2 mol/L HCl–ethanol [12.5:87.5 by volume (25)] were prepared. Dried extracts were reconstituted with electrophoresis sample buffer. The various plasma extracts, tumor cyst fluid, and preparations of pro-IGF-II, IGF-II, and E peptides were subjected to electrophoresis on 10–20% gradient sodium dodecyl sulfate–polyacrylamide gels under nonreducing conditions. The size-fractionated proteins were electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and detected (after the membrane was blocked with 20 g/L BSA) by use of an antibody directed against IGF-II (Amano; dilution 1:15,000), E(68–88) (dilution 1:5000), or [Tyr]⁰-E(113–134) (dilution 1:4000). HRP-conjugated secondary antibodies (Amersham) to mouse and rabbit immunoglobulin were used at 1:10,000 and 1:7500 dilutions, respectively. The bands were visualized by use of the SuperSignal chemiluminescent substrate (Pierce) and Hyperfilm ECL film (Amersham).

HISTOLOGIC EXAMINATIONS

Tumor tissues were fixed for 24 h in PBS-buffered formalin (3.8 mL/L) at 4 °C. After dehydration through a series of ethanol solutions and incubation in xylol, tissues were embedded in paraffin. Sections (7–10 μm) were cut and mounted onto glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma) and used directly for in situ hybridization. The methods used for in situ hybridization with digoxigenin-labeled hIGF-II complementary RNA probes have been described previously (26). Sections were counterstained with nuclear fast red, dehydrated through a series of ethanol solutions, and mounted in Euparal. Negative controls for in situ hybridization were prepared using sense probes.

Before immunohistochemistry, paraffin sections of tumor tissues were dewaxed, rehydrated, and pretreated with 3 mL/L H₂O₂ to block endogenous peroxidase activity. To enhance the accessibility, sections were incubated for 3 min in distilled water in the microwave at 1400 W, followed by 15 min at 450 W, and a 15-min cooling period. Sections were incubated for 1 h at room temperature with rabbit anti-hIGF-II antiserum (no. C65; generously provided by Dr. B.H. Breier, Auckland, New Zealand) (27), WKZ6279 E(68–88) antiserum, or WKZ6281 E(113–134) antiserum diluted (in PBS) 1:100, 1:50, and 1:100, respectively. After several washes in PBS, the sections were incubated with biotinylated second antibody (goat anti-rabbit; 1:200 dilution). The bound immunocomplex was visualized with HRP-avidin-biotin complex using nickel-enhanced 3,3-diaminobenzidine as chromogenic substrate. Sections were counterstained with nuclear fast red and mounted with DEPEX. Control experiments were performed with preimmune primary antisera, diluted 1:50.
Concentrations (nmol/L) of IGF-I, total IGF-II, and IGFBP-3 in plasma were calculated as means ± SD unless stated otherwise. Circulating concentrations of these analytes in NICHTH patients were also expressed as SD scores to correct for age and gender, using reference curves based on the respective normative data, which were published previously (23, 28). Data were analyzed with SPSS 9.0 software, using standard statistical methods.

Results

Characterization of E(68–88) and E(113–134) Antibodies by Western Immunoblotting

The E(68–88) antibody used in the present study detects, in addition to the E(68–88) peptide, several molecular mass forms of both glycosylated and nonglycosylated pro-IGF-II, as demonstrated by Western ligand blotting of purified human pro-IGF-II E(68–87) (two different degrees of glycosylation) and rhpro-IGF-II (Fig. 1). The latter recombinant preparation appeared to consist of several isoforms of pro-IGF-II, which according to the manufacturer correspond to the full-length 156-amino acid protein and smaller species with C-terminal truncations of ~5–15 residues, resulting from the purification process. In addition, a substantial amount of E(68–88) IMR was noted at ~45 kDa, possibly representing dimers. Similar results were obtained with the Amano antibody to IGF-II. Furthermore, acid-ethanol extracts of plasma from NICHTH patients (two examples are shown in Fig. 1) contained several forms of IGF-II and E(68–88) IMR at 9–14 kDa, whereas in extracts of normal plasma (Fig. 1) and CRF plasma (results not shown), 7.5-kDa IGF-II predominated. The E(113–134) antibody appeared to recognize full-length recombinant pro-IGF-II and one of its truncated isoforms (Fig. 1). Obviously, pro-IGF-II E(68–87) could not be detected with this antibody. No E(113–134) IMR was visualized after Western immunoblotting of tumor cyst fluid and either acid-ethanol or Sep-Pak C18 extracts (i.e., up to 400 µL of plasma equivalents) of plasma from healthy individuals and patients. Both the E(68–88) and
E(113–134) antibodies could be used in immunohistochemistry. Only the former antibody appeared to be useful for application in the quantitative determination of IMR in plasma by RIA or ELISA (see below).

IGF-I, TOTAL IGF-II, AND IGFBP-3 CONCENTRATIONS IN PLASMA AND TUMOR CYST FLUID
On admission to hospital, all NICHTH patients included in this study had very low plasma concentrations of insulin and growth hormone. NICHTH patients (n = 9) characteristically exhibited markedly decreased concentrations of both IGF-I (mean ± SD, 5.2 ± 2.5 nmol/L; SD score, −4.2 ± 1.9; P < 0.0001) and IGFBP-3 (22.0 ± 4.0 nmol/L; SD score, −4.4 ± 0.9; P < 0.0001) in their circulation. An aliquot of tumor cyst fluid appeared to contain a low amount of IGFBP-3 (11.0 nmol/L), whereas IGF-I (1.1 nmol/L) was hardly detectable.

Total concentrations of IGF-II IMR (i.e., 7.5-kDa IGF-II plus higher molecular mass forms of pro-IGF-II) were determined in Sep-Pak C18 extracts of the various plasma samples by RIA using the Amano anti-rat IGF-II monoclonal antibody. On a molar basis, pro-IGF-II E(68–87) displaced the 125I-IGF-II tracer with a lower ED50 than the rhIGF-II WHO standard preparation (Table 1). Serial dilutions of this preparation of pro-IGF-II paralleled the IGF-II calibration curve (data not shown). Results of the measurements of total IGF-II are shown in Table 2. Values for individual NICHT patients varied considerably. For only one of these patients was the plasma IGF-II concentration higher than ±2 SD scores. Two NICHT patients exhibited concentrations below ±2 SD scores. As expected, cyst fluid aspirated from a large tumor in the liver of a patient with a metastatic hemangiopericytoma contained a relatively large amount of IGF-II IMR. Increased IGF-II was also found in pooled plasma from CRF patients.

DETERMINATION OF TOTAL E(68–88) IMR
In the E(68–88) RIA, both our purified human pro-IGF-II (E(68–87)) preparation and rhpro-IGF-II were capable of displacing the tracer, albeit with different potencies (Table 1 and Fig. 2). Total E(68–88) concentrations were determined in Sep-Pak C18 extracts of the various plasma samples and tumor cyst fluid to allow for comparison with data obtained by measurements by pro-IGF-II ELISA (see below). Reconstituted Sep-Pak C18 extracts of normal human plasma, pooled CRF plasma, and several individual samples from NICHTH patients gave dose–response curves that paralleled those of E(68–88), pro-IGF-II (Fig. 2), and samples assayed directly (data not shown). As can

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**Table 1. Cross-reactivity of the various pro-IGF-II and E-domain preparations on a molar basis in the hIGF-II RIA, E(68–88) RIA, and hpro-IGF-II ELISA, as calculated from dose–response curves relative to the particular calibrators used in the assays.**

<table>
<thead>
<tr>
<th></th>
<th>hIGF-II RIA</th>
<th>E (68–88) RIA</th>
<th>hpro-IGF-II E(68–88) ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Relative potency, %</td>
<td>n</td>
</tr>
<tr>
<td>rhIGF-II (WHO preparation)</td>
<td>5</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>E(68–88) peptide</td>
<td>5</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Native 7.5-kDa hIGF-II</td>
<td>5</td>
<td>59 (16)b</td>
<td>5</td>
</tr>
<tr>
<td>rhpro-IGF-II</td>
<td>3</td>
<td>8 (3)b</td>
<td>7</td>
</tr>
<tr>
<td>hpro-IGF-II E(68–87)</td>
<td>4</td>
<td>66 (18)b</td>
<td>8</td>
</tr>
</tbody>
</table>

a) Calibrators used were rhIGF-II, E(68–88) peptide, and hpro-IGF-II E(68–87), respectively.
b) Mean (SD).

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**Table 2. Total IGF-II, total E(68–88), and pro-IGF-II E(68–88) IMR as determined by RIAs and ELISA, respectively, in Sep-Pak C18 extracts of tumor cyst fluid, and plasma samples derived from patients with NICHT or CRF, and healthy controls.**

<table>
<thead>
<tr>
<th></th>
<th>Total IGF-II, nmol/L</th>
<th>Total E(68–88), nmol/L</th>
<th>pro-IGF-II E(68–88), nmol/L</th>
<th>pro-IGF-II E(68–88)/total E(68–88) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NICHTH plasma (n = 9)</td>
<td>102.9 (32.4)</td>
<td>45.3 (15.9)a</td>
<td>22.6 (9.4)b</td>
<td>0.53 (0.24)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>42.3–103.1</td>
<td>24.7–70.0</td>
<td>9.6–31.2</td>
<td>0.17–0.88</td>
</tr>
<tr>
<td>NICHTH tumor fluid</td>
<td>143.5</td>
<td>234.0</td>
<td>70.1</td>
<td>0.30</td>
</tr>
<tr>
<td>CRF plasma, pooled (n = 4)</td>
<td>93.7 (41.1)</td>
<td>26.8 (6.5)c</td>
<td>2.0 (0.8)</td>
<td>0.08 (0.04)b</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>62.5–153.7</td>
<td>20.2–35.6</td>
<td>1.2–2.9</td>
<td>0.05–0.14</td>
</tr>
<tr>
<td>Normal plasma, pooled</td>
<td>51.5</td>
<td>8.4</td>
<td>3.8</td>
<td>0.66</td>
</tr>
<tr>
<td>Normal plasma, pooled (elderly)</td>
<td>61.7</td>
<td>7.6</td>
<td>1.9</td>
<td>0.25</td>
</tr>
</tbody>
</table>

a–c Versus pooled normal plasma; a P < 0.0001; b P < 0.001; c P < 0.05.
be deduced from Table 2, plasma samples from NICHT patients, tumor cyst fluid, and the plasma pools prepared from dialyzed CRF patients contained markedly higher amounts of E(68–88) IMR than normal plasma.

**PRO-IGF-II ELISA**

Obviously, determination of E(68–88) IMR in the circulation or other body fluids does not discriminate between E-domain-containing pro-IGF-II forms (pro-IGF-II E) and free fragments of the E domain. Hence, a two-site ELISA was developed for quantitative measurement of pro-IGF-II E peptide. In this assay the pro-IGF-II E peptide to be detected was sandwiched between the WKZ6279 antibody (i.e., IgG fraction) and the polyclonal IGF-II antibody from Research Diagnostics. The latter antibody was used because the quantity of monoclonal IGF-II Amano antibody available was limited and in- tended exclusively for use in routine RIA measurements. Essentially, when applied in a RIA, the characteristics of the Research Diagnostics antibody were similar to those of the Amano monoclonal antibody, with only its affinity for rhpro-IGF-II being 2.7-fold higher. Standard titrations of pro-IGF-II E(68–88) and rhpro-IGF-II in the ELISA are shown in Fig. 3. The detection efficiency (on a molar basis) for rhpro-IGF-II was ~5.7-fold lower than for pro-IGF-II E(68–87). Both IGF-II E(68–88) peptide and IGF-II, up to concentrations of 80 pmol/well, did not cross-react in the assay. When the ELISA was applied directly to either control or patient plasma, the dilution–response curves did not parallel those of pro-IGF-II E(68–87) or rhpro-IGF-II (Fig. 3). Moreover, the addition of known amounts of purified pro-IGF-II E(68–87) (see also below) to plasma led to a poor recovery (i.e., <20%) of immunoreactive material when unextracted samples were assayed directly by ELISA. Addition of an excess of IGF-I (81 pmol/well) to plasma before assaying did not improve this situation. However, acid Sep-Pak C18 extraction before measurement of pro-IGF-II E peptide solved this problem (Fig. 3). The apparent requirement for preassay extraction of IGFs from plasma was presumably attributable to (as yet unknown) matrix effects but not to interference of IGFBPs in the ELISA because addition of an excess of IGFBP-3 (13 pmol/well) did not influence the capture of pro-IGF-II E(68–87).

Intraassay variation (10 replicates) was 9.1% and 10% at mean plasma concentrations of 3.19 (normal pooled plasma) and 23.4 nmol/L (plasma from a NICHT patient), respectively. The interassay variation at these concentrations (n = 10) was 13% and 14%, respectively. The lowest detectable dose, defined as 1.5 times the background value (i.e., the mean absorbance of at least four wells containing all reagents except the sample) was 0.04 nmol/L (absolute concentration).

Above the highest dose of pro-IGF-II(68–87) used in the calibration curve (1.05 pmol/well, or 10.5 nmol/L), a high-dose hook effect emerged. ELISAs with exogenous pro-IGF-II E(68–87) concentrations of 1.3, 3.8, and 11.4 nmol/L added to aliquots of pooled normal plasma revealed that >80% of this protein was recovered (including extraction). Repeated cycles (up to four times) of freezing and thawing of samples (either plasma or serum) did not influence the results significantly.

Plasma samples from healthy individuals and CRF and NICHT patients were subsequently analyzed, and the results are listed in Table 2. When compared with normal plasma, samples from all NICHT patients included in the study exhibited increased plasma concentrations of pro-IGF-II E(68–88), albeit to a variable extent. For one patient, suffering from NICHT attributable to a metastatic hemeangioepitocytoma, a postoperative plasma sample (i.e., 2 weeks after partial removal of a large tumor in the liver, which temporarily abolished hypoglycemia) was available for determination of pro-IGF-II E(68–88) IMR by ELISA. This postoperative sample contained 11.5 nmol/L
IGF-II E(68–87) IMR, which is substantially lower than the value found before reduction of the tumor load (30.5 nmol/L). This tendency was confirmed by acid Bio-Gel P60 chromatographic analysis of these two samples (data not shown). In contrast to plasma from NICTH patients, plasma pools derived from CRF patients tended to contain relatively low amounts of IGF-II E(68–87) IMR. This latter finding, together with the high E(68–88) IMR values measured by the RIA, would indicate a relative abundance of E-domain-derived fragments in the circulation of CRF patients.

Validation of the pro-IGF-II ELISA by acid gel filtration

For the different categories of plasma samples and tumor cyst fluid, the results of pro-IGF-II E-peptide measurements by ELISA were verified by characterizing the elution profiles of IGF-II and E(68–88) IMR after acid gel filtration. For this purpose, 0.5-mL aliquots of plasma were acidified and subjected to Sep-Pak C18 extraction. Peptides in the dried extracts were dissolved in 0.1 mol/L acetic acid, and either 2 mL of plasma equivalents or 0.4 mL of tumor cyst fluid was applied to a Sephadex G-50 column. Shown in Fig. 4A is a typical result for normal plasma. Two IGF-II peaks, as detected by RIA (using the Amano monoclonal antibody) were observed. The smaller of these peaks represented pro-IGF-II, as revealed by E(68–88) measurements. The second peak, comprising the major part of the total IGF-II IMR (i.e., 79%) eluted from the column, corresponded to mature IGF-II. The elution pattern of E(68–88) IMR also showed two major peaks. The first peak coincided with that of the IGF-II IMR, contributing more than two-thirds of the total E(68–88) IMR collected. The remainder of the E(68–88) IMR was fractionated at a substantially lower molecular mass, i.e., between 7.5 and 3.5 kDa. Peptide(s) in this peak virtually lacked immunoepitopes for the IGF-II monoclonal antibody (and also for the IGF-II polyclonal antibody from Research Diagnostics used in the pro-IGF-II ELISA; data not shown) and therefore most likely represented cleaved E domain or fragments.

When plasma extracts of patients with NICTH were
subjected to acid gel filtration, IGF-II IMR was distributed among the same major molecular size fractions as found for normal plasma. An example is shown in Fig. 4B. For the three NICTH patients investigated, most of the IGF-II IMR in plasma (65–78%) was associated with big IGF-II, whereas free E-domain fragments were hardly detectable. Gel filtration of tumor cyst fluid from the same patient (Fig. 4C) revealed that both IGF-II and E(68–88) IMR could be exclusively attributed to big IGF-II. The E(68–88) elution pattern of plasma extracts from CRF patients behaved strikingly different from those of the other samples investigated (Fig. 4D). In this kind of plasma, low-molecular-mass E-domain-related peptides appeared to predominate profoundly (~79% of the total E(68–88) IMR). Unfortunately, we were not able to visualize these peptides by Western immunoblotting. Qualitatively, the results were in good agreement with those derived from direct measurements with ELISA, indicating the validity of this sandwich detection method. When several samples of acidified whole plasma and tumor cyst fluid were subjected to Bio-Gel P-60 column chromatography, the IGF-II IMR was distributed similarly as described above. The same applied for IGF-II E(68–88) IMR associated with big IGF-II. However, low-molecular-mass IGF-II E(68–88) IMR eluted in a relatively large number of fractions, hampering adequate quantification.

IGF-II expression in NICTH tumor tissue

From two NICTH patients included in this study, tumor tissues were available for detection of (pro)-IGF-II mRNA and protein by in situ hybridization and immunohistochemistry, respectively. In Fig. 5, the results are shown for a tumor adjacent to the spleen obtained from a patient with a metastatic leiomyosarcoma. The vital tumor tissue consisted of cells with spindle-shaped nuclei and flattened ends or cells exhibiting enlarged round, sometimes irregular, nuclei, in part surrounded by vacuolated cytoplasm. Occasionally, the cells resembled smooth muscle tissue, although this could not be confirmed by specific staining for actin. IGF-II transcripts were abundant in the cytoplasm of tumor cells, but were absent in the endothelial cells of diverging vessels and in necrotic areas (Fig. 5A). When hybridization was performed with the sense IGF-II probe, no detectable signal was observed in the tissue sections (Fig. 5D). Immunohistochemistry with anti-IGF-II antibody (Fig. 5B) revealed diffuse staining of the majority of tumor cells. In contrast, dense granular cytoplasmic staining of the tumor cells was observed when E(68–88) or E(113–134) antibodies were used (Fig. 5, C and F). No significant signals were encountered when we replaced the primary antibodies with the corresponding preimmune sera (Fig. 5E). With the same methods, tumor tissue derived from the liver of a patient with a metastatic hemaangiopericytoma also demonstrated a
high expression of (pro)-IGF-II at both the mRNA and protein level (data not shown).

**Discussion**

The present study demonstrates that tumors associated with NICTH may exhibit excessive concentrations of IGF-II mRNA, which is in accordance with previous reports (10, 29). Using specific antibodies directed against the E region of pro-IGF-II, immunohistochemical analysis of metastatic tumor sections from two different NICTH patients clearly revealed the abundant presence of both E(68–88) and E(113–134) immunoreactive peptides in the cytoplasm of tumor cells. These results were more pronounced than immunostaining of IGF-II(1–67) with an anti-IGF-II antibody. Although the extent to which these E(68–88)- or E(113–134)-containing peptides represent precursor forms of IGF-II or E-domain-derived fragments is not known, they may be considered suitable histochemical markers in the pathologic evaluation of IGF-II producing tumors. As outlined in the Introduction, part of the partially processed tumor-derived pro-IGF-II may leak into the circulation, leading to a cascade of events ultimately giving rise to NICTH. Individuals suffering from this syndrome often show markedly decreased secretion of growth hormone. Presumably, the increased bioavailability of IGFs exerts substantial feedback inhibition of pituitary growth hormone production, which overrules the effect of hypoglycemia (74). As a consequence, this results in low circulating concentrations of IGF-I and IGFBP-3. All NICTH individuals included in our study showed this pattern. Presumably, the actual concentration of IGFBP-3, under typical circumstances the most abundant IGFBP in the circulation, heavily contributes to the half-life and thus the steady-state concentration of IGF-II in plasma. This would explain, at least in part, the lack of increase in circulating total IGF-II [as determined by RIA with an antibody to IGF-II(1–67)] in NICTH patients, in spite of the high expression of the IGF-II gene and secretion of big IGF-II by these tumors.

We found markedly increased concentrations of total E(68–88) IMR in the plasma of NICTH patients. However, diazylated CRF patients appeared to accumulate E(68–88) IMR in their plasma as well. Although the literature on this subject is scarce, others (16–18) have reported similar results for these two categories of patients. In addition, Liu et al. (17) found increased plasma E(69–84) in acromegalic individuals. Relatively high concentrations also occur in the amniotic and seminal fluid of healthy individuals. Thus, increased plasma concentrations of E-domain IMR are not restricted to NICTH patients only. In accordance with results from a previous study on this subject (3), we demonstrated that E(68–88) IMR in human plasma is present in at least two different apparent molecular mass classes after acid gel filtration. For normal and NICTH plasma, as well as the sample of tumor cyst fluid, the major proportion of E(68–88) IMR eluted as big IGF-II because this peak also contained IGF-II(1–67) IMR.

The low-molecular-mass peak of E(68–88) IMR contained peptides that lacked determinants for the monoclonal Amano IGF-II antibody and therefore likely represented breakdown products of free E domain. In contrast to the situation for normal and NICTH plasma (and tumor cyst fluid), these peptides were excessively present in blood specimens from CRF patients, contributing most of the E(68–88) IMR. Western ligand blotting experiments with CRF plasma confirmed the abundant existence of low-molecular-mass E(68–88) epitope-bearing peptides. Presumably, a disturbed renal clearance contributes to the accumulation of both E-domain-derived fragments and the various IGFBPs in the circulation of CRF patients.

On the basis of the foregoing considerations, one can conclude that the determination of total E(68–88) concentrations in blood specimens or other body fluids may be informative. However, especially when increased values are found, further analysis (e.g., by column chromatographic methods or Western immunoblotting) of the samples is still necessary to demonstrate that prohormone is being detected. This problem is circumvented by application of the ELISA we developed for direct detection of E(68–88)-containing partially processed forms of pro-IGF-II. To our knowledge, this is the first report on this subject. Confirmatory to semiquantitative data obtained by acid gel filtration, in CRF plasma the pro-IGF-II E(68–88)/total E(68–88) IMR ratio, derived directly from measurements by ELISA and RIA, respectively, was low compared with values found for either normal or NICTH plasma. Similarly, the differences found between the various samples with respect to their pro-IGF-II E(68–88)/total IGF-II ratios determined by conventional column chromatographic analysis and those calculated from direct determination of pro-IGF-II E(68–88) by ELISA showed the same tendencies. These observations point to the validity of the ELISA in the unequivocal detection of pro-IGF-II E(68–88). However, the absolute values for the ratios between pro-IGF-II E(68–88) and total E(68–88) or total IGF-II calculated from the elution patterns of acid gel filtration of the various samples tested were consequently higher than those obtained from determinations by ELISA. Presumably, this problem could be solved when the molecular masses of the calibrators used in the various assays are accurately known and the assays could be mutually calibrated.

Analogous to the pro-IGF-II E(68–88) ELISA, using the antibody to [Tyr]-E(113–134) we developed a sensitive ELISA for detection of pro-IGF-II E(113–134) (unpublished results). This ELISA could readily detect recombinant pro-IGF-II from GroPep and had a lower detection limit of 0.4 nmol/L rhpro-IGF-II IMR. However, when we investigated the various plasma samples and tumor cyst fluid directly, as Sep-Pak C_{18} extracts, or as neutralized acid-ethanol extracts, in all cases pro-IGF-II E(113–134) IMR appeared to be below the detection limit. Western immunoblotting experiments confirmed the lack of IGF-II E(113–134) IMR in these samples. These results would
suggest that E(113–134)-containing precursor forms of IGF-II are not stable after being secreted by the tumor. Likewise, Fukuda et al. (30) could not detect pro-IGF-II E(138–156) IMR in sera from various NICTH patients by Western immunoblotting with a antibody specific for E(138–156).

As indicated by the present results, the quantitative detection of pro-IGF-II by ELISA can be applied in the definitive diagnosis and follow-up of NICTH. Other than in NICTH, relatively little is known about the natural occurrence of big IGF-II in plasma and other biological fluids in health and disease. The pro-IGF-II ELISA would be a convenient tool to establish normative ranges of big IGF-II and to evaluate whether there are other disease entities in which abnormal processing of pro-IGF-II plays a role in the etiology and/or pathophysiology. For example, Daughaday et al. (31) reported that individuals who have immunologic markers of hepatitis B virus infection may exhibit an increased proportion of partially processed pro-IGF-II in their circulation, as assessed by the acid gel filtration method. Indeed, a study by Lee et al. (32) suggests that the human hepatitis B virus transactivator X gene product induces the (over)expression of hepatic IGF-II. Hepatitis B-positive patients exhibiting increased plasma big IGF-II might be at increased risk for the development of hepatocellular carcinoma. The possibility of quantifying pro-IGF-II by specific ELISA should facilitate further investigations on this and other subjects.

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