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Research Article

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Can "Big" Insulin-like Growth Factor II in Serum of Tumor Patients Account for the Development of Extrapancreatic Tumor Hypoglycemia?

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Abstract

The pathogenesis of extrapancreatic tumor hypoglycemia has been related to the secretion of big insulin-like growth factor (IGF) II by the tumor. In 25 of 28 patients with this type of hypoglycemia we found 1.5–8-fold elevated serum levels of immunoreactive big (15–25 kD), but decreased levels of normal IGF II. After removal of the tumor, big IGF II disappeared and normal IGF II increased. Tumors contained elevated levels of IGF II, 65–80% in the big form. The insulin-like bioactivity of big IGF II and its affinity towards IGF-binding proteins (IGFBP)-2 and -3 are similar to those of normal IGF II, but two- to threefold higher on a molar basis. Big IGF II is mainly bound to the 50-kD IGFBP complex. The latter contains ~ 10 times more of this peptide than in normal serum and displays three- to fourfold increased insulin-like bioactivity. The formation of the 150-kD IGFBP complex with ^{125}I–recombinant human IGFBP-3 is impaired in tumor serum. This results in sequestration of IGFBP-3 and predominant association of big IGF II with IGFBP-2 and -3 in the 50-kD complex. Increased bioavailability of big IGF II in this complex due to unrestricted capillary passage and enhanced insulin bioactivity of this big IGF II pool provide a continuous increased insulin-like potential available to insulin and type 1 IGF receptors of insulin-sensitive tissues and thus may lead to sustained hypoglycemia. (*J. Clin. Invest.* 1992. 90:2574–2584.) Key words: insulin-like growth factor II • tumors • hypoglycemia • insulin-like activity • insulin-like growth factor–binding proteins

Introduction

Extrapancreatic tumor hypoglycemia (EPTH),[1] i.e., hypoglycemia associated with nonislet cell tumors, has for many years remained one of the "most baffling of the unsolved para-endocrine puzzles" (1). Tumors of mesodermal, epithelial, and hematopoietic origin have been described to be associated with hypoglycemia (2). The tumors are usually large and slowly growing. Hypoglycemia is a fasting hypoglycemia (2) with symptoms resulting from impaired supply of glucose to the brain, like headache, tiredness, confusion, amnesia, or even epileptic seizures. EPTH is characterized by three prominent metabolic features (3–5): a block of hepatic glucose production due to inhibition of glycogenolysis and gluconeogenesis (2, 4); a block of lipolysis in adipose tissue resulting in low serum free fatty acid levels; and increased glucose assimilation, which may be partly due to increased glucose use by the tumor itself, but mainly by skeletal muscle (5).

The pathogenesis of EPTH has long been a matter of debate. In 1974, Megyesi et al. (6) reported elevated serum levels of nonsuppressible insulin-like activity (NSILA-S) in five of seven patients with nonislet cell tumors. In an extended study of 52 patients with EPTH, 37% of the patients had elevated NSILA-S levels (7). Daughaday et al. (8) found elevated insulin-like growth factor (IGF) II in 10 of 14 sera from patients with tumor-related hypoglycemia. Thus, increased serum concentrations of an insulin-like substance, most likely IGF II, appeared to be responsible for the development of hypoglycemia in more than one-third of the patients. However, in contrast to these reports, we have never been able to detect significantly elevated serum levels of immunoreactive (ir) or receptorreactive (ir) IGF II in such patients nor did we find a decrease of IGF II after successful removal of the tumors (9, 10). An important step to the solution of the puzzle was made in 1988: Daughaday et al. (11) described a patient with a leiomyosarcoma and recurrent hypoglycemia whose serum IGF II was normal both by IGF II RIA and radioreceptorassay, but in whose serum 70% of the total IGF II occurred as a higher molecular weight form, termed big IGF II. In addition, the tumor itself contained high concentrations of IGF II mRNA and of IGF II peptide. 77% of the IGF II in the tumor was present as a large molecule of ~ 12–15 kD (11). The authors showed that after successful removal of the tumor the serum content of big IGF II had dropped to nearly zero. They concluded that the tumor was the main source of big IGF II in the serum of their patient and that the cause of the patient's recurrent hypoglycemia was IGF II produced by the tumor.

We have analyzed sera from 28 patients with EPTH for big IGF II and found elevated levels in 25 of these patients. Total irIGF II was not increased when determined by our standard method (9). After successful operation, big IGF II disappeared from the serum, but total irIGF II did not decrease significantly.

We, therefore, addressed the question whether or not big IGF II can account for hypoglycemia in the presence of normal total IGF II levels. In a first approach we standardized the procedure for determination of big IGF II in sera and tumor extracts. We then tried to find out what distinguishes big from normal IGF II to explain its hypoglycemic action. In this context, we asked (*a*) Does big IGF II exert enhanced insulin-like biological effects? (*b*) Is its affinity towards IGF-binding proteins (IGFBPs) altered? (*c*) Does it occur in serum bound to

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1. *Abbreviations used in this paper:* EPTH, extrapancreatic tumor hypoglycemia; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; ir, immunoreactive; NSILA-S, nonsuppressible insulin-like activity.

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IGFBPs or rather in the free form? (*d*) Is bound big IGF II biologically active?

Methods

Tumor sera and tumors. Sera from patients with EPTH were obtained from referring physicians from several European countries. All sera had been stored at −20°C until used for analysis. The tumors had been classified as follows: three fibrosarcomas, two benign fibromas, two mesenchymal fibrous tumors, three mesotheliomas, four leiomyosarcomas, one histiocytoma, one hemangioendothelioma, one hemangiopericytoma, eight carcinomas (three carcinomas of the colon, one of the sigma, one adenocarcinoma of the stomach, one undifferentiated squamous carcinoma of the thorax, one undifferentiated carcinoma of the lung, one medullary thyroid carcinoma), and one gastrointestinal lymphoma. For two tumors we did not obtain a histological diagnosis.

Biogel P-60 Chromatography at acid pH. 0.5–1.0 ml of serum was gel-filtered on a Biogel P-60 column (Bio-Rad Laboratories, Richmond, CA; 2.2 × 70 cm, flow rate 12 ml/h) in 1 M acetic acid containing 20 mg/100 ml of HSA (Swiss Red Cross, Bern, Switzerland). The column had been calibrated with ^{125}I-rhIGF I and ^{125}I-rhIGF II and with unlabeled rhIGF II (rhIGF I and II were kindly provided by Drs. K. Müller and W. Märki, Ciba-Geigy AG, Basel, Switzerland). Four fractions each between fractions 44 and 119 were pooled, lyophilized, taken up in 2 ml 0.1 M NH_4HCO_3, and relyophilized. They were dissolved in 1 ml PBS, pH 7.4, containing 0.2 g/100 ml of HSA (RIA buffer) and assayed at two different dilutions in the IGF I and IGF II RIA (9). Fractions 48–119 did not contain measurable amounts of IGFBPs as tested by Western ligand blotting with ^{125}I-rhIGF II (12).

For the determination of biological activity, 1.0–1.5 ml of normal or tumor serum was passed over the acidic Biogel P-60 column. 10 fractions each starting from fraction 25 were pooled, lyophilized, neutralized with 2 ml 0.1 M NH_4HCO_3, and relyophilized. Pools were dissolved in 1.0 ml of Krebs–Ringer–Hepes buffer, pH 7.4, and tested at two to three different dilutions (with the same buffer containing 0.2 g/100 ml of HSA) in the presence of 0.3 μl of insulin antiserum (neutralizing capacity 0.4 mU) in the rat fat-cell assay (13) using porcine insulin and rhIGF II as standards. In another set of experiments, eight fractions each starting from fraction 40 were pooled, processed as above, dissolved in 0.7 ml of Krebs–Ringer–Hepes buffer, and tested in the fat-cell assay. IrIGF II- (9) and ^{125}I-IGF II–displacing potencies using rhIGFBP-3 (reference 16; see below) were determined in the same pools.

Biogel P-60 chromatography at neutral pH. A Biogel P-60 column (ϕ 2.2 × 70 cm, flow rate 10 ml/h) was equilibrated in 0.1 M NH_4HCO_3 containing 20 mg/100 ml of HSA and 20 mg/100 ml of NaN_3 and was calibrated with different molecular weight markers (as indicated in Fig. 2) and with ^{125}I-rhIGF I and ^{125}I-rhIGF II.

For the determination of the apparent molecular mass of big IGF II, fractions 44–67 obtained by acidic Biogel P-60 chromatography of 1 ml of tumor serum (Fig. 1, peak *I*) were pooled, lyophilized, dissolved in 1 ml 0.1 M NH_4HCO_3, and passed over the neutral Biogel P-60 column. Similarly, pooled fractions 68–95 and 96–115 (Fig. 1, peaks *II* and *III*) from the acidic Biogel P-60 column were rechromatographed at neutral pH. Every five fractions between fractions 40 and 85 were pooled, lyophilized, dissolved in 1 ml of RIA buffer, and tested at two different dilutions in the IGF II RIA (9).

For determination of biological activity in neutral Biogel P-60 pools, 2 ml of normal or tumor serum was passed over the neutral Biogel P-60 column. Fractions 32–40, and then every five following fractions, were pooled, dialyzed against 0.1 M NH_4HCO_3 in dialysis tubing (Spectrapor; Spectrum Medical Industries, Inc., Los Angeles, CA; molecular weight cut-off, 3,500), lyophilized, dissolved in 1 ml 0.1 M NH_4HCO_3 containing 6 g/100 ml of HSA, and tested at two or three different dilutions in the rat fat-cell assay (13) against insulin as standard. Insulin dilutions were made in 0.1 M NH_4HCO_3 containing 6 g/100 ml of HSA. The lyophilized pool of fractions 32–40 was dissolved in 2 ml 0.1 M NH_4HCO_3 and tested at 1:2 and 1:4 dilutions.

Northern blots of IGF II mRNA. Total RNA was isolated according to a standard protocol (14). 20 μg of total RNA was separated on a 1% agarose gel containing 2 M formaldehyde and transferred to a nylon filter (Hybond-N; Amersham International, Amersham, UK). Filters were prehybridized at 42°C for 2 h in a solution containing 50% formamide, 5× Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrollidone), 5× SSPE (20× = 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7), 0.2% SDS, and 100 μg/ml of heat-denatured salmon sperm DNA. RNA was hybridized with a human IGF II cDNA (kindly provided by Dr. G. I. Bell, Chicago, IL) that had been labeled (15) to a specific activity of 2×10^9 cpm/μg DNA. The hybridization solution (same as above) contained 10 ng (2×10^7 cpm) of labeled cDNA/filter. After 48 h at 42°C, the filters were washed twice in 2× SSPE, 0.1% SDS for 15 min at room temperature and three times in 0.1× SSC, 0.1% SDS for 20 min at 55°C.

Extraction of IGF II peptide from tumor tissue. For the extraction of IGF II peptide, tumor tissue was homogenized (3 × 60 s) with a homogenizer (Polytron) in 5 vol of 1 M acetic acid containing 0.02% HSA, 1 μM pepstatin-A, 0.6 TI U/ml aprotinin, 10 μM leupeptin, and 1 mM phenylmethylsulfonylfluoride (all protease inhibitors were from Sigma Chemical Co., St. Louis, MO). The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C. 1 ml of the supernate was chromatographed on the acidic Biogel P-60 column (see above).

Binding of partially purified big and normal IGF II to rhIGFBP-2 and rhIGFBP-3. Fractions 44–67 and 96–119 obtained by acidic Biogel P-60 chromatography of 10 × 0.5 ml of different tumor sera were pooled, lyophilized, dissolved in 1 ml H_2O, and rechromatographed on the acidic Biogel P-60 column. Fractions 48–67 containing big IGF II and fractions 96–119 containing normal IGF II were pooled, lyophilized, dissolved in RIA buffer, and standardized in the IGF II RIA (9). The two preparations did not contain measurable amounts of IGFBPs or insulin. Their potencies to compete with ^{125}I-rhIGF II for binding to rhIGFBP-2 and rhIGFBP-3 were compared with that of rhIGF II in the protein-binding assay (16). 0.4 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.8 mg of HSA and 5 ng of rhIGFBP-2 (gift of Dr. J. Shuster, Chiron Corp., Emeryville CA) or 4.5 ng of rhIGFBP-3 (glycosylated, kindly provided by Drs. A. Sommer and C. A. Maack, BioGrowth, Richmond, CA), 50,000 cpm (~ 0.1 ng) ^{125}I-rhIGF II, and increasing amounts of unlabeled rhIGF II or of the two IGF II preparations, was incubated for 2 h at room temperature. 20 min after the addition of 0.5 ml of a charcoal suspension (containing 2 g/100 ml of charcoal and 2 g/100 ml of HSA) at 4°C, the mixture was centrifuged (4°C, 15 min, 2,000 *g*), and 0.5 ml of the supernate was counted in a γ counter.

The ^{125}I-IGF II displacing potencies of pooled fractions obtained by acidic Biogel P-60 chromatography of 1.5 ml of normal or tumor sera (see above) were determined by the same assay using rhIGFBP-3.

Distribution of IGF I and II between the 150- and 50-kD IGFBP complexes. 2 ml of normal or tumor serum was passed over a Sephadex G-200 (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) column (2.2 × 70 cm, flow rate 10 ml/h) in Dulbecco's buffer, pH 7.4, containing 20 mg/100 ml of NaN_3. Fractions 51–64, 65–71, and 72–84 corresponding to the elution ranges of the 150- and 50-kD IGFBP complexes (as determined from the radiochromatographic pattern of normal and tumor serum preequilibrated with ^{125}I-rhIGF II and gel filtered over the same Sephadex G-200 column) and fractions eluting before (35–50) and after (85–95 and 96–115) the two complexes (2 mg of HSA was added to fractions 80–115 to prevent adsorption of IGF to the tubes) were pooled, dialysed against 0.1 M NH_4HCO_3 in Spectrapor dialysis tubing (molecular weight cut-off, 3,500), lyophilized, and dissolved in 2 ml Krebs–Ringer–Hepes buffer, pH 7.4. The biological activity in each pool was determined in the presence of 0.3 μl of insulin antiserum (neutralizing capacity 0.4 mU) in the rat fat-cell assay (13). 1 ml of each pool was also passed over the acidic Biogel P-60 column described above. Fractions were pooled starting from fraction 44, pro-

cessed as described above, and tested at two different dilutions in the IGF I and IGF II RIAs (9).

Radiochromatography of sera with rhIGFBP-3. ^{125}I-rhIGFBP-3 (glycosylated, specific radioactivity ~ 160 μCi/μg) was obtained from Anawa (Dübendorf, Switzerland). 0.2 ml serum was diluted with 0.3 ml Dulbecco's buffer, pH 7.4, and incubated with 3×10^5 cpm of ^{125}I-rhIGFBP-3 and 25 μl Trasylol (Bayer AG, Leverkusen, Germany) for 24 h at 4°C. The incubation mixtures were passed over the neutral Sephadex G-200 column described above and fractions were counted in a γ counter.

Results

Elution profiles of IGF I and II on Biogel P-60 at acidic and neutral pH. Fig. 1 *A* shows the elution profile of ^{125}I-IGF II and unlabeled rhIGF II (1 μg) compared with the elution profile of irIGF I and irIGF II in normal human serum. Whereas ^{125}I-rhIGF I elutes as a single radioactive peak (not shown), ^{125}I-rhIGF II yields two radioactive peaks (peaks II and III). Like ^{125}I-rhIGF II, unlabeled rhIGF II elutes in two immunoreactive peaks as determined by IGF II RIA. The two peaks coincide with those of the radiolabeled IGF II. The recovery of unlabeled rhIGF II by this method was 106%. The peak of irIGF I obtained in serum appears at the same position as radiolabeled rhIGF I. In contrast to IGF I, the IGF II profile of serum consists of three immunoreactive peaks: a small peak (peak I) precedes peaks II and III. When peak II and III material is rechromatographed on a neutral Biogel P-60 column and the fractions are assayed by IGF II RIA, irIGF II of both peaks elutes at the same position as ^{125}I-rhIGF II (Fig. 2). Apparently, IGF II forms aggregates (dimers) at acidic pH. Peak III obviously corresponds to the 7.5-kD IGF II monomer. The radioactivity of peaks II and III obtained after acidic chromatography of the IGF II tracer binds identically and is identically displaced by unlabeled rhIGF II in the IGF II RIA (not shown).

When serum from a hypoglycemic patient with a leiomyosarcoma was gel filtered on the acidic Biogel P-60 column, peak I (big IGF II) became most prominent (Fig. 1 *B*). It eluted earlier than peak I of normal serum, indicating that its irIGF II has a somewhat higher molecular mass than in normal serum. Rechromatography of peak I from the tumor serum on a neutral Biogel P-60 column yields an apparent molecular mass of 20 kD (range, between 15 and 25 kD) (Fig. 2).

After acidic Biogel P-60 chromatography, pooled fractions of the tumor serum were tested in the rat fat-cell assay for insulin-like bioactivity. Insulin and rhIGF II were used as stan-

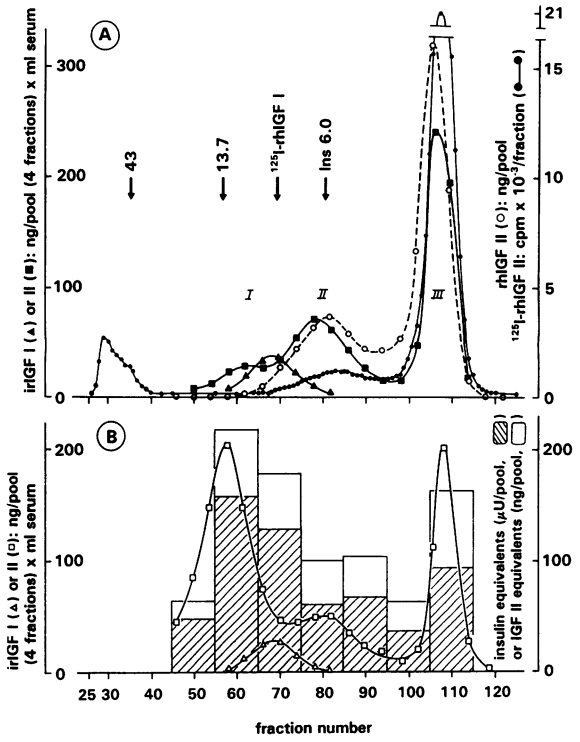

Figure 1. (*A*) Acidic gel filtration on Biogel P-60 of ^{125}I-rhIGF II (●), unlabeled rhIGF II (○), and of normal human serum. ^{125}I-rhIGF II (3×10^5 cpm, ~ 0.5 ng), 1 μg of unlabeled rhIGF II in 0.5 ml Dulbecco's buffer/5.4% HSA, pH 7.4, or 1 ml of normal human serum were eluted in 1 M acetic acid containing 20 mg/100 ml of HSA. Radioactive fractions were counted in a γ counter. For unlabeled rhIGF II and serum, four fractions each were pooled, processed as described in Methods, and assayed for irIGF I (▲) and II (■). (*B*) Acidic gel filtration on Biogel P-60 of serum (0.5 ml) from a patient with a leiomyosarcoma causing hypoglycemia. Four fractions each were pooled, processed as described in Methods, and assayed for irIGF I (△) and II (□). For determination of biological activity, 1 ml serum of the same patient was chromatographed. 10 fractions each from 45 to 115 were pooled, processed as described in Methods, and tested in the rat fat-cell assay in the presence of insulin antiserum (0.3 μl, neutralizing capacity 0.4 mU). The height of the whole column represents rhIGF II RIA ng equivalents/pool × ml serum; hatched columns represent insulin equivalents in μU/pool × ml serum.

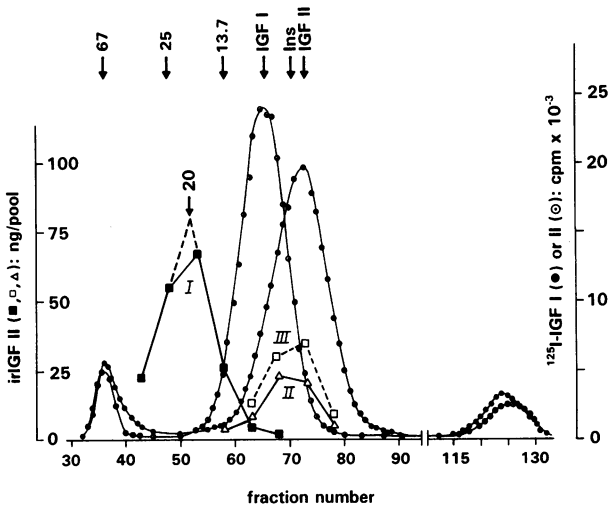

Figure 2. Chromatography of ^{125}I-rhIGF I (⊙) and ^{125}I-rhIGF II (●) and rechromatography of the three IGF II pools of experiment 1B on Biogel P-60 at neutral pH. ^{125}I-rhIGF I (3.5×10^5 cpm, ~ 0.6 ng) or ^{125}I-rhIGF II (3.5×10^5 cpm, ~ 0.6 ng) in 0.5 ml Dulbecco's buffer/5.4% HSA, pH 7.4, were eluted in 0.1 M NH_4HCO_3 containing 20 mg/100 ml of HSA and 20 mg/100 ml of NaN_3, and fractions were counted in a γ counter. Fractions 48–69 (pool I), 70–90 (pool II), and 99–119 (pool III) from the gel filtration experiment shown in Fig. 1 *B* were pooled, lyophilized, and rechromatographed. Five fractions each (from 41–85) were pooled, lyophilized, and dissolved in 1 ml of RIA buffer for IGF II RIA (■, △, □: irIGF II from pool I, II, and III, respectively). The arrows with numbers give the molecular masses of markers in kilodaltons. The arrow and number over I indicates the estimated molecular mass of the peak I material (big IGF II).

dards. The bioactivity profile more or less parallels the profile of IGF II immunoreactivity (Fig. 1 *B*). When expressed in milliunits of insulin equivalents per milligrams of irIGF II, the pooled fractions 55–65 (Fig. 1 *B*) corresponding to the peak of big IGF II display a similar specific insulin-like activity (~ 360 mU/mg) as pooled fractions 105–115 (Fig. 1 *B*) corresponding to 7.5-kD IGF II (~ 300 mU/mg). Thus, on a molar basis (with an estimated molecular mass of 20 kD for big IGF II) and assuming 100% cross-reactivity in the IGF II RIA, the insulin-like bioactivity of big IGF II would be enhanced approximately threefold compared with that of normal (7.5-kD) IGF II. In three other tumor sera the specific insulin-like bioactivity of pooled fractions 56–63 and 64–71 was enhanced two to threefold compared with pooled fractions 104–111, when calculated on a molar basis (not shown).

Big IGF II in tumor sera and in tumors of patients with EPTH. The acidic Biogel P-60 elution profiles of irIGF II in two tumor sera obtained before and after successful removal of the tumor show that the increased amounts of big IGF II disappeared after operation (Fig. 3). Big IGF II of tumor serum and of tumor extract elute in the same range (Fig. 3). In the tumor extracts an even greater portion (65–80%) of total IGF II than in serum (see below) was due to big IGF II. Total IGF II in the two tumors was 1.98 and 2.69 μg/g wet weight, big IGF II, 1.59 and 1.76 μg/g, respectively. The relatively high content of IGF II peptide in the tumors compared with normal tissues (17) is also reflected by the huge tissue level of IGF II mRNA compared with that of adult normal liver or adipose tissue (Fig. 4). The latter tissue is among those with the highest IGF II mRNA levels of all adult normal human tissues tested so far (Peter, M. and J. Zapf, unpublished results). The Northern blot pattern of the tumors shows at least six different bands at approximately 7, 6, 4.5, 3.4 (3.0), 2, and 1.6 kb.

On the basis of the elution profile of irIGF II on Biogel P-60 at acidic pH (Figs. 1 and 3), we have analyzed 28 sera from patients with EPTH for big IGF II, IGF II dimer, and 7.5-kD IGF II (Fig. 5). According to this profile, irIGF II in fractions 44–67 has been defined as big IGF II, that in fractions 68–95 as IGF II dimer, and that in fractions 96–115 as 7.5-kD IGF II. 25 of the 28 patients had 1.5–8-fold elevated big IGF II levels. 52.6±11.8% of total IGF II was accounted for by big IGF II, whereas in normal serum 10% occurs as a bigger form (Figs. 1 and 3). The three patients in whom big IGF II was not elevated had a gastrointestinal lymphoma, a hemangioendothelioma, and an adenocarcinoma of the colon. The percentage of big IGF II in their sera was < 10% of total. Their total IGF II levels determined by acidic Biogel P-60 chromatography were 844, 554, and 551 ng/ml compared with 920 ng/ml in normal serum. Only 4 of the 25 patients with elevated big IGF II had total IGF II levels > 1,000 ng/ml (1,062, 1,255, 1,328 and 1,369 ng/ml). In contrast to the elevation of the big form of IGF II, the IGF II dimer and the 7.5-kD IGF II were decreased in the 25 tumor sera compared with normal serum (Fig. 5). This explains why total IGF II in most of these patients was within the normal range. In postoperative sera from four patients, big IGF II that had been elevated before removal of the tumor had dropped to or below normal, whereas the IGF II dimer and 7.5-kD IGF II had increased (Fig. 5, *right*).

The affinities of big IGF II from tumor patients towards IGFBPs. The affinities for rhIGFBP-2 and rhIGFBP-3 of big and normal (7.5-kD) IGF II partially purified from 10 different

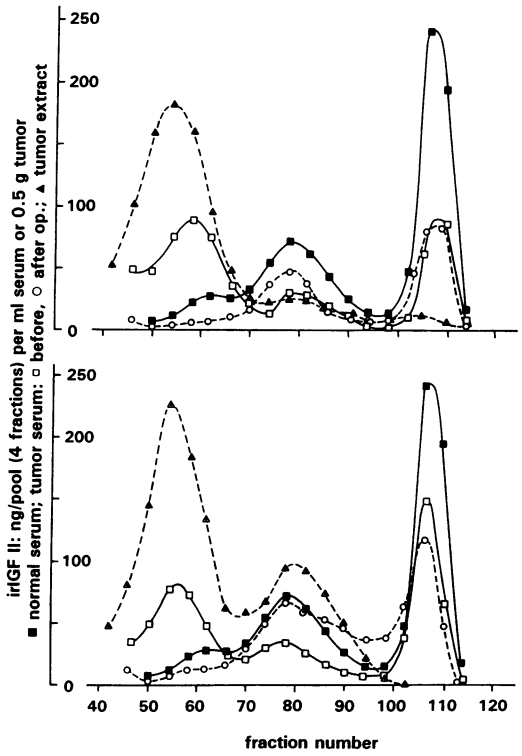

Figure 3. Acidic Biogel P-60 chromatography of pre- (□) and postoperative (○) serum and of tumor extract (△) from two patients with hypoglycemia caused by a mesothelioma (*top*) and mesenchymal fibrous tumor of the pleura (*bottom*). 0.5 ml of serum or extract from 0.5 g of tumor were passed over the column. Four fractions each (from 44 to 115) were pooled, processed as described in Methods, and assayed in the IGF II RIA.

tumor sera and standardized in the IGF II RIA were determined in a protein-binding assay (16). The competing potencies for rhIGFBP-2 and rhIGFBP-3 of the two IGF II species were nearly the same as that of rhIGF II (Fig. 6). Furthermore, pooled fractions 56–63 and 64–71 obtained by acidic Biogel P-60 chromatography of three individual tumor sera displayed similar ^{125}I-IGF II–displacing potencies in the rhIGFBP-3–binding assay as pooled fractions 104–111 (not shown). Thus, on a molar basis, the affinity of big IGF II for rhIGFBP-2 and -3 appears approximately threefold increased compared with that of normal IGF II. Fig. 6 also shows that the

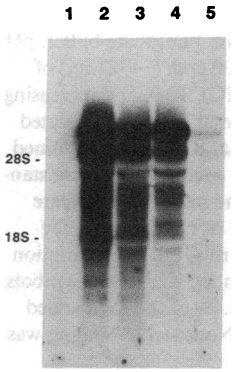

Figure 4. IGF II mRNA levels in three tumors (lanes *2–4*: mesothelioma, mesenchymal fibrous tumor, leiomyosarcoma) causing hypoglycemia compared with normal liver (lane *1*) and adipose tissue (lane *5*). 20 μg of total RNA was hybridized with an IGF II cDNA probe as described in Methods. The membrane was exposed to the film for 5 h.

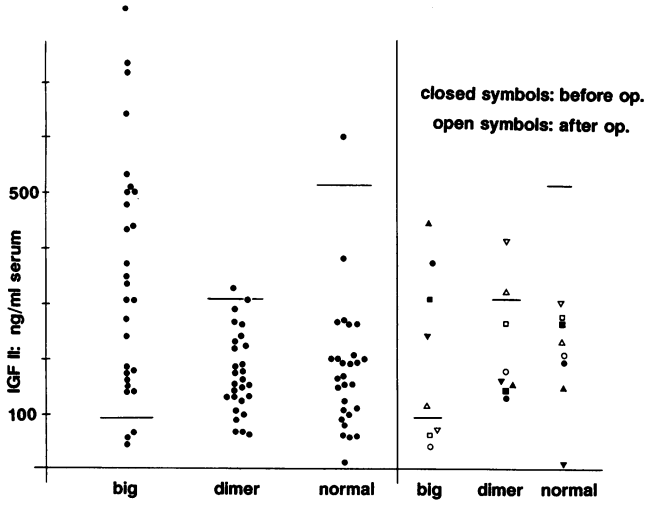

Figure 5. Levels of big IGF II, IGF II dimer, and 7.5-kD IGF II in 28 patients with tumor-associated hypoglycemia (*left*) and in four pre- and postoperative tumor sera (*right*). 0.5 ml of the sera was chromatographed on the acidic Biogel P-60 column. Four fractions each were pooled, processed as described in Methods, and IGF II was determined by RIA. According to the IGF II profile of Figs. 1 and 3, big IGF II, IGF II dimer, and 7.5-kD IGF II were calculated from the IGF II RIA values of fractions 44–67, 68–95, and 95–115, respectively. The horizontal lines indicate the values for big IGF II, IGF II dimer, and 7.5-kD IGF II determined for a normal serum pool (mean value of two determinations from two different chromatographies). On the right, one and the same symbol is used for the same patient, filled symbols representing preoperative and open symbols postoperative values.

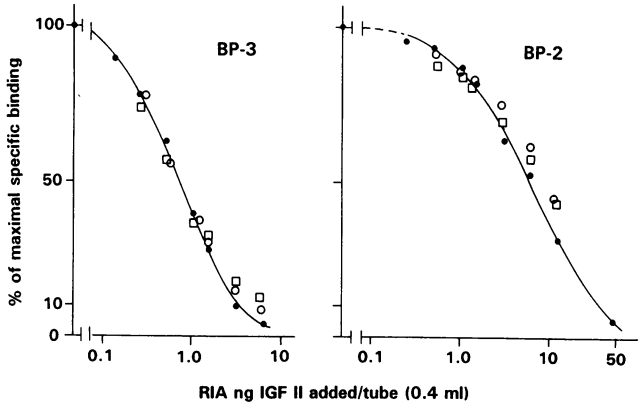

Figure 6. Competitive inhibition of ^{125}I-IGF II binding to rhIGFBP-3 (glycosylated) and rhIGFBP-2 by rhIGF II (●), partially purified big IGF II (□), and partially purified normal IGF II (○). The binding assay was performed in 0.4 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 200 mg/100 ml of HSA, 50,000 cpm (~ 0.1 ng) of ^{125}I-rhIGF II, 5 or 4.5 ng of rhIGFBP-2 or rhIGFBP-3, and increasing concentrations of rhIGF II, big IGF II, or normal IGF II as indicated in the Figure. Partially purified big and normal IGF II were obtained from a pool of 10 tumor sera as described in Methods and were standardized in the IGF II RIA. All points are the means of duplicate determinations and are expressed as percent of maximal specific binding in the presence of 100 ng/0.4 ml of rhIGF II. The variation between duplicate determinations was within the size of the symbols. Total binding of ^{125}I-IGF II was 15.4 and 22.4% of the total added counts for rhIGFBP-2 and -3, respectively. Nonspecific binding was 4.1 and 8.7%, respectively.

affinity of big and normal IGF II towards rhIGFBP-3 is ~ 10 times higher than for rhIGFBP-2.

Distribution of big IGF II between the 150- and 50-kD IGFBP complexes of normal and tumor sera. To test whether or not big IGF II is bound to IGFBPs or occurs in the free form in native serum, we performed the experiments shown in Fig. 7 and Table I. After neutral Sephadex G-200 gel permeation chromatography of normal or tumor serum (leiomyosarcoma), pooled fractions were rechromatographed on the acidic Biogel P-60 column for analysis of big and normal (dimer and 7.5-kD) IGF II. The same experiment was carried out with serum

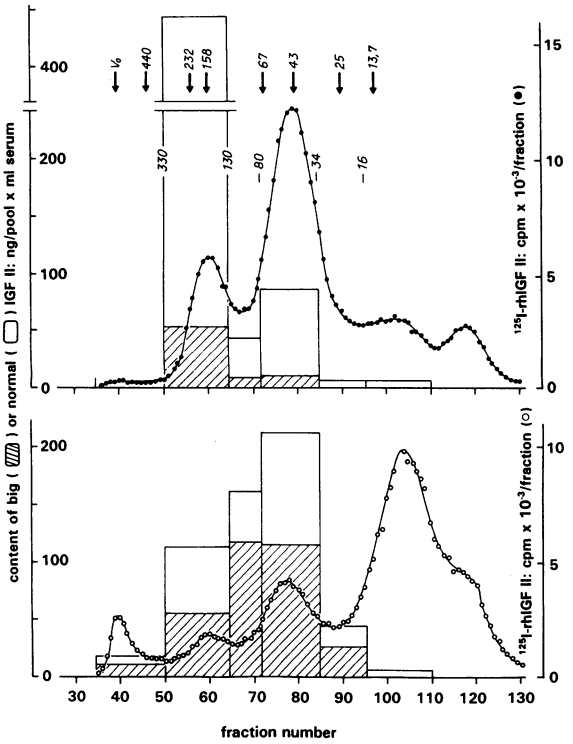

Figure 7. Molecular size distribution of big and normal (dimer and 7.5-kD) IGF II in normal serum and in serum of a patient with a leiomyosarcoma (same serum as in Fig. 1 *B*). 2 ml of a normal serum pool or tumor serum was gel filtered on Sephadex G-200 at neutral pH (Dulbecco's buffer containing 20 mg/100 ml of NaN_3). Fractions 35–50, 51–64, 65–71, 72–84, 85–95, and 96–115 were pooled, dialyzed in Spectrapor tubing (molecular weight cut-off, 3,500) against 0.1 M NH_4HCO_3, lyophilized, and dissolved in 2 ml of Krebs–Ringer–Hepes buffer, pH 7.4. 1 ml of each pool was subsequently chromatographed on the acidic Biogel P-60 column. Four fractions each (starting from fraction 44) were pooled, processed as described in Fig. 1, and assayed for IGF II in the IGF II RIA. According to the IGF II profile of Fig. 1, the IGF II RIA values of fractions 44–67, 68–95, and 96–115, respectively, were added up to calculate big IGF II, IGF II dimer, and 7.5-kD IGF II. Values for the IGF II dimer and for 7.5-kD IGF II were counted together as normal IGF II. The radiochromatographic pattern of normal or tumor serum preequilibrated with ^{125}I-IGF II (4×10^5 cpm, ~ 0.8 ng) shows the location of the 150- and 50-kD IGFBP complexes. Arrows with numbers on the top of the figure indicate molecular mass markers. Open and hatched columns represent the values for normal and big IGF II, respectively. The total height of the column represents total IGF II. Values are given in ng/pool × ml serum. The numbers in the middle of the figure give the estimated molecular mass ranges of the pooled serum fractions. V_0, void volume.

Table I. Molecular Size Distribution of Big and Normal IGF II before and 5 mo after Successful Removal of a Mesenchymal Fibrous Tumor of the Pleura That Had Caused Hypoglycemia

Pooled fractions	irIGF II				irIGF I	
	Big	Normal	Total			
	ng/pool × ml serum			%	*ng pool × ml serum*	%
Before operation						
51–64	40	40	80	16.7	27	42.2
65–71	160	40	200	41.8	23	35.9
72–84	85	105	190	39.8	14	21.9
85–95	3	5	8	1.7	—	
After operation						
51–64	20	260	280	58.6	94	64.0
65–71	4	46	50	10.5	12	8.2
72–84	12	133	145	30.3	41	27.9
85–95	—	3	3	0.6	—	

1 ml of pre- or postoperative serum was first gel filtered on Sephadex G-200 at pH 7.4, pooled, and processed as described in Fig. 7. Pools were dissolved in 1 ml of Krebs–Ringer–Hepes buffer, pH 7.4, and chromatographed on the acidic Biogel P-60 column. Fractions were pooled and processed as in Fig. 1 and were assayed in the IGF I and II RIA. Big and normal (IGF II dimer and 7.5-kD IGF II) were calculated as described in Figs. 5 and 7. IGF I gave only one single peak of immunoreactivity, which eluted between fractions 60 and 80 (see Fig. 1, *A* and *B*).

Table II. Nonsuppressible Insulin-like Activities (NSILA) of the Pooled Serum Fractions in Fig. 7 Obtained by Neutral Sephadex G-200 Chromatography

Fractions	Normal serum	Tumor serum
	μU/pool × ml serum	
35–50	145±55	96±25
51–64	115±19	142±22
65–71	61±7	98±12
72–84	34±1	107±16
85–95	25±5	42±3

$U^{14}C$-glucose incorporation into rat fat cells was determined at three different concentrations. The biological activities were read from an insulin-standard curve and are expressed as μU of insulin equivalents/pool × ml serum measured in the presence of insulin antiserum (0.3 μl, neutralizing capacity 0.4 mU). Mean values±SD obtained at the three different dilutions are given for each pool.

from a patient with a mesenchymal fibrous tumor of the pleura before and after operation (Table I). Fig. 7 *A* shows the typical distribution of IGF II between the two IGFBP complexes in normal serum: 67% (440 ng) of the total irIGF II is found associated with the 150-kD binding protein complex. This complex also contains 72% (54 ng) of the total big IGF II. The 150-kD complex of the tumor serum contains only one-fourth of the IGF II of normal serum, but the same amount of big IGF II. In contrast to normal serum, 70% of the total IGF II in the tumor serum appears in the range of the 50-kD complex. More importantly, the latter contains 10–12 times more big IGF II than normal serum. Some big IGF II in the tumor serum is also found in the pool corresponding to the expected elution volume of free big IGF II (15–25 kD). However, this may also have partly resulted from "trailing" of some of the 50-kD complex into the range of free big IGF II during Sephadex chromatography.

The molecular size distribution of total and big IGF II in the postoperative tumor serum (Table I) is similar to that of normal serum. After operation, big IGF II nearly disappeared from the 50-kD complex. The distribution of irIGF I between the two IGFBP complexes more or less parallels the distribution of irIGF II. Total IGF I is decreased in the preoperative serum and, like IGF II, is shifted towards the 50-kD complex.

Normal and tumor serum also differ with respect to their insulin-like bioactivities measured in the Sephadex G-200 pools of Fig. 7: nonsuppressible insulin-like activity of the pool corresponding to the 50-kD binding protein complex of the tumor serum (fractions 72–84) is threefold higher than in normal serum (Table II). The same results are obtained after neutral Biogel P-60 chromatography (Fig. 8). These differences in biological activity are not due to differences in the content of large molecular weight NSILA, a still unidentified serum activity (18); after separation of IGF by acidic Biogel P-60 gel filtration the remaining large molecular weight NSILA is decreased rather than increased in the tumor serum pools (not shown). Thus, the increase of irIGF II in the native 50-kD binding protein complex of the tumor serum, which is exclusively ac-

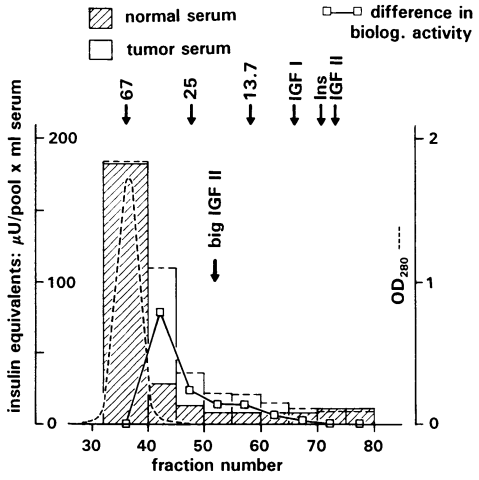

Figure 8. Nonsuppressible insulin-like activities of serum fractions obtained by neutral gel filtration on Biogel P-60. 2 ml of normal or tumor serum (same patient as in Figs. 1 *B* and 7) was chromatographed on the neutral Biogel P-60 column described in Fig. 2. Fractions 35–45, and then every five following fractions, were pooled, dialyzed in Spectrapor tubing (molecular weight cut-off, 3,500) against 0.1 M NH_4HCO_3, lyophilized, and dissolved in 1 ml of Krebs–Ringer–Hepes buffer, pH 7.4. Pools were assayed at two or three different dilutions in the rat fat-cell assay. The columns represent biological activities in μU of insulin equivalents/pool × ml serum. Hatched columns represent biological activities in normal serum pools, open columns represent those of the tumor serum pools. Arrows indicate the location of molecular mass markers. Open squares give the differences of biological activities between the tumor and normal serum.

counted for by big IGF II (Fig. 7 and Table I), goes along with an increased insulin-like bioactivity.

A possible explanation for the overall increase of IGF II in the 50-kD complex of the tumor serum is provided by Fig. 9. IGFBP-3 is sequestered from the 150- into the 50-kD binding protein complex. At the same time, IGFBP-2 in the 50-kD complex is increased. The reason for the sequestration of IGFBP-3 into the 50-kD binding protein complex is shown in Fig. 10. When normal serum is preequilibrated with ^{125}I-rhIGFBP-3 and chromatographed on the neutral Sephadex G-200 column, a major radioactive peak appears at the position of the 150-kD complex. In tumor serum this peak is lacking, and the radioactivity elutes at the position of "free" ^{125}I-rhIGFBP-3. As estimated from the apparent molecular mass of 90 kD, ^{125}I-rhIGFBP-3 obviously elutes as a dimer. Successful removal of the tumor completely restores the normal binding pattern (Fig. 10, *middle* and *bottom*). The peak at fraction number 118 corresponds to 100% bed volume and probably represents free ^{125}I- or ^{125}I-labeled tyrosine resulting from degradation.

Discussion

Big IGF II, the key to the solution of the riddle of extrapancreatic tumor hypoglycemia. The results of this study confirm and extend findings originally reported by Daughaday et al. (11) on the presence of big IGF II in serum and tumor extract of a patient with hypoglycemia caused by a leiomyosarcoma. A big form of IGF II with an apparent molecular mass of 9 kD had been demonstrated in normal human serum and cerebrospinal fluid by Haselbacher and Humbel (19) in 1982 using acidic Biogel P-100 gel filtration. At acidic pH, Biogel P-60 does not only separate big from normal IGF II, but completely separates IGF I from 7.5-kD IGF II (reference 11; Fig. 1 *A*) despite their nearly identical molecular masses. Therefore, molecular mass estimations under these conditions can be misleading. A third IGF II species (Fig. 1, peak *II*) is detected with the acidic Biogel P-60 column. Most likely, it represents IGF II dimer that forms under acidic conditions because at neutral pH it elutes at the same position as unlabeled and radiolabeled 7.5-kD IGF II (Fig. 2).

The finding that big IGF II disappears from the patients' sera after successful removal of the tumor (Figs. 3 and 5), as previously also reported by Daughaday et al. (11), suggests that it is secreted by the tumor into the circulation. (We have recently followed the time course of disappearance of big IGF II from serum after successful removal of a mesothelioma of the thorax that had caused severe hypoglycemia: big IGF II disappeared with a half-life of < 1 h. In the same patient, normal IGF II, which was decreased by 74% before the operation, rose with a half-life of ~ 48 h.) The elevated IGF II mRNA levels in the three tumors (Fig. 4) and their increased content of IGF II, which is mainly accounted for by the big form (Fig. 3), support this conclusion. Enhanced levels of IGF II mRNA in tumors associated with hypoglycemia as well as increased tissue levels of IGF II peptide with a high percentage of big IGF II have also been reported by others (11, 20–22). The nucleotide sequences of IGF II cDNAs cloned from the cDNA library of a mesotheli-

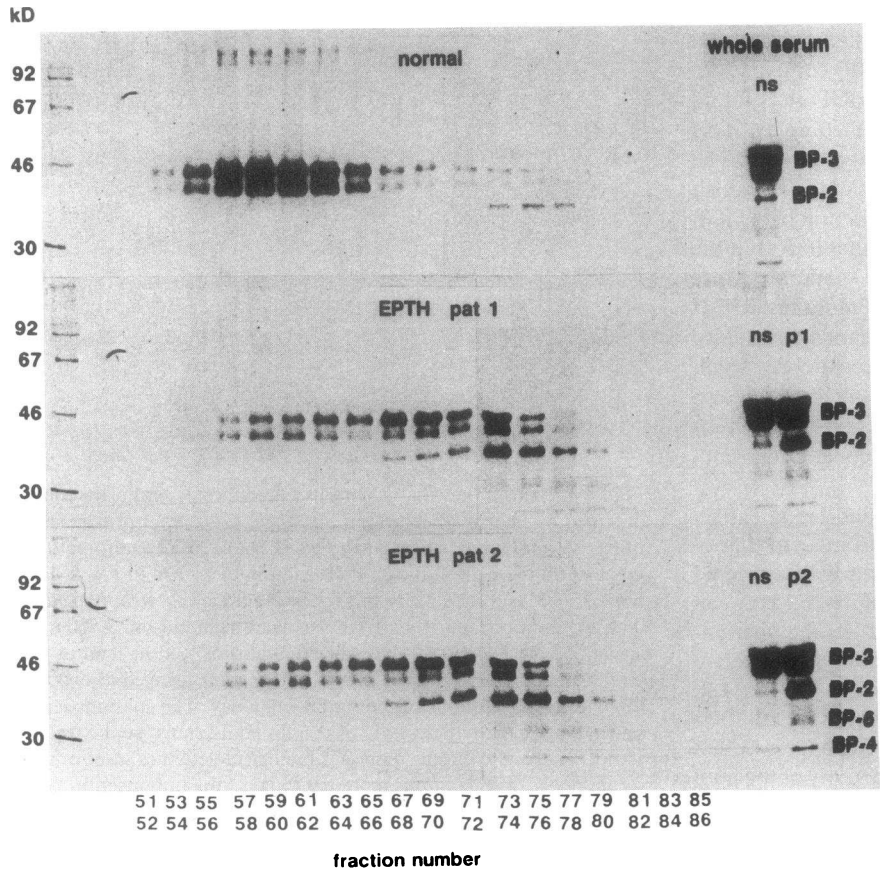

Figure 9. ^{125}I-IGF II ligand blots of fractions obtained by neutral Sephadex G-200 gel permeation chromatography of 1 ml of normal serum and of sera from two patients with EPTH. (Patient 1, mesothelioma of the pleura; patient 2, lung tumor of unknown histology). Two fractions each were pooled, dialyzed against 0.1 M NH_4HCO_3, lyophilized, and dissolved in 1 ml H_2O. 20 μl was subjected to electrophoresis and blotted as described in reference 12. Fractions 51–64 and 72–84 correspond to the ranges of the 150- and 50-kD IGFBP complexes, fractions 65–71 correspond to an overlapping range between the two complexes. The ligand blots to the right of the panel were performed with 3 μl of whole normal serum (*ns*) or of tumor serum (*p1* and *p2*).

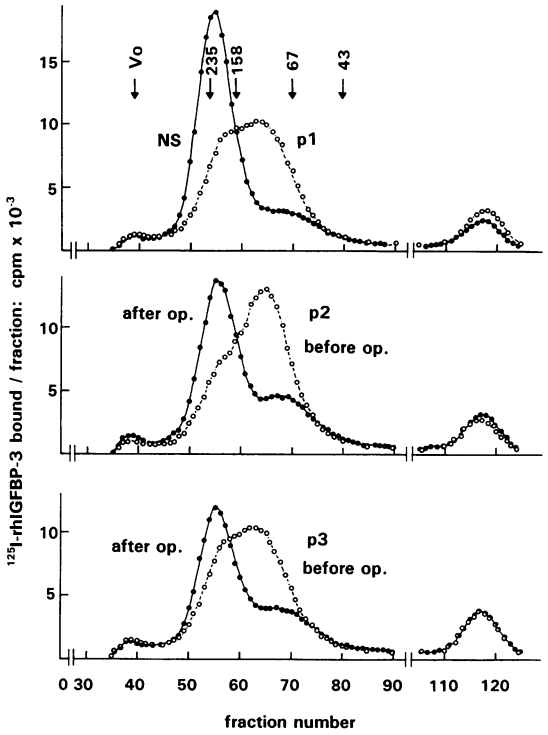

Figure 10. Radiochromatographic patterns with ^{125}I-rhIGFBP-3 (glycosylated) of normal serum (●, NS) and of tumor sera before (○) and after successful removal of the tumor (●). 0.2 ml serum was preequilibrated for 24 h at 4°C with 0.3 ml Dulbecco's buffer, pH 7.4, containing 0.02 g/100 ml of NaN_3, and with ^{125}I-rhIGFBP-3 (3×10^5 cpm, ~ 160 μCi/μg), and passed over the neutral Sephadex G-200 column (see Methods); fractions were counted in a γ counter. The peak at fraction 118 represents ^{125}I-iodine or ^{125}I-labeled tyrosine at 100% bed volume. Patient 1 (*p1*), leiomyosarcoma of the small bowel, same as in Fig. 1 *B;* patient 2 (*p2*), mesenchymal tumor of the thorax; patient 3 (*p3*), retroperitoneal leiomyosarcoma.

oma that had caused hypoglycemia were identical (Kiefer, M. C., and J. Zapf, unpublished results) to the cDNAs of normal IGF II and the IGF II variant cloned from a human liver cDNA library (23). The various bands detected by Northern blot analysis of tumor RNA may correspond to the six mRNA species described by Sussenbach et al. (24). These transcripts of 6, 5.3, 5, 4.8, 2.2, and 1.8 kb result from four different promoters in the human IGF II gene. It appears that all six species were transcribed in the three tumors analyzed by us.

The analysis of 28 sera from patients with EPTH does not only show that big IGF II is elevated in nearly all of these patients but also explains why we do not find elevated levels of total IGF II in most of the sera (Fig. 5). The total content of IGF II in normal serum as determined by acidic Biogel P-60 chromatography was 920 ng/ml. In only 4 of the 28 analyzed sera was total IGF II above 1,000 ng/ml (1,062–1,369 ng/ml). The elevation of big IGF II in the tumor sera is compensated by a decrease of "normal" IGF II (dimer and 7.5-kD IGF II) (Fig. 5). On the other hand, when big IGF II drops after removal of the tumor, normal IGF II rises (Fig. 5). This inverse relationship between big and normal IGF II may be explained in analogy to the situation where recombinant IGF I is infused in normal subjects (reference 25; see below).

The exact nature of the large molecular mass immunoreactive IGF II in tumor sera and tumor extracts has not yet been defined. As suggested by Daughaday et al. (11), it probably consists of incompletely processed or unprocessed pro-IGF II. In normal serum, a big form of IGF II with a molecular mass of 15 kD has been purified from human Cohn fraction IV (26). Its affinities for the type 2 IGF receptor of rat placental membranes and for an IGFBP isolated from the medium of cultured Buffalo rat liver cells (most likely rat IGFBP-2) were similar to those of 7.5-kD IGF II, but it was 50% more potent in stimulating [^{3}H]thymidine incorporation into human skin fibroblasts. Recently, the same group showed that the 15-kD peptide is a precursor form of IGF II that is posttranslationally *O*-glycosylated at Thr^{75} and contains ~ 10-kD of protein and 4–5 kD of an oligosaccharide (27). The 10-kD protein core of the 15-kD peptide comprises the 67 amino acids of the mature IGF II and a 21-amino acid COOH-terminal extension (27). According to the nucleotide sequence of the IGF II cDNA (23), unprocessed pro-IGF II contains 156 amino acids, which would account for a molecular mass of ~ 17 kD. In the presence of 4 to 5 kD of oligosaccharide the apparent molecular mass of the unprocessed molecule would be 21–22 kD and thus correspond to the apparent molecular mass estimated on the neutral Biogel P-60 column (Fig. 2). However, it is possible that big IGF II in tumors and tumor sera also contains partially processed pro-IGF II and displays oligosaccharide microheterogeneity similar to the 15-kD form in normal serum (27).

Biological activity and affinities of partially purified big IGF II for serum IGFBPs. In view of the normal serum IGF II levels found by RIA and radioreceptorassay in nearly all of our patients with EPTH (9, 10) and in more than half of the patients reported by Gorden et al. (7), the question arises whether the elevation of big IGF II in the presence of a normal total IGF II serum level can account for hypoglycemia. Partially purified big IGF II stimulates glucose metabolism in rat fat cells (Fig. 1 *B*). Calculated on a molar basis, its biological insulin-like activity is two- to threefold higher than that of normal IGF II. Since IGFs stimulate glucose metabolism in rat fat cells via the insulin receptor (28, 29), big IGF II may activate the insulin receptor more efficiently than normal IGF II. However, this is not yet sufficient to explain hypoglycemia in tumor patients: in normal serum > 98% of total IGF II is bound to IGFBPs (30), and 70–80% is associated with a 150-kD IGFBP complex (31). The bioavailability of this latter IGF pool is largely restricted by the capillary barrier (31, 32). To cause hypoglycemia the bioavailability of big IGF II has to be enhanced. This can be achieved either by an elevation of circulating free big IGF II or/and an altered distribution of big IGF II between the 150- and 50-kD IGFBP complexes.

Increased levels of free big IGF II would result if its affinity towards serum IGFBPs were reduced. Shapiro et al. (21) reported that the highest molecular mass fraction of IGF II-like material extracted from hepatocellular carcinomas that had caused hypoglycemia barely competed for binding of ^{125}I-IGF II to serum IGFBPs. The authors, therefore, suggested that low affinity of serum IGFBPs for big IGF II might be associated with elevated levels of the free peptide. However, they have not excluded the presence of IGFBPs in this fraction, which would account for the lack of competition. We used rhIGFBP-2 and rhIGFBP-3, the predominant IGFBPs of the 50-kD complex in tumor sera (12), to compare the competing potencies of pooled fractions from tumor sera containing big or 7.5-kD IGF

II standardized in the IGF II RIA. RIA nanogram equivalents of big and 7.5-kD IGF II competed for both recombinant binding proteins with similar potencies as rhIGF II. Thus, on a molar basis, the competing potency of big IGF II is even enhanced. It is, therefore, unlikely that the affinity of big IGF II towards the main IGFBPs of the 50-kD complex is altered such as to cause an essential rise of free big IGF II in tumor serum.

The distribution of big IGF II between the 150- and 50-kD IGFBP complexes and its bioavailability. Besides the IGF pool associated with the 150-kD complex, two other IGF pools exist in serum; 20–30% of the total IGF of normal serum resides in a 50-kD IGFBP complex (31). This IGF pool disappears from the circulation with a half-life of 20 to 30 min (30) and, therefore, has a high turnover rate. The 50-kD binding protein complex of normal serum contains mainly IGFBP-2 and relatively little IGFBP-3 (12, 33). This complex can cross the capillary barrier (32) and may thus render its IGF bioavailable to tissues. A third IGF pool consists of free IGF in equilibrium with the two IGFBP complexes. Free IGF is readily bioavailable (34), but < 2% of total IGF II in normal serum occurs in the free form (30). Increased bioavailability of big IGF II would be achieved by its predominant association with the 50-kD binding protein complex. This would lead to increased delivery of big IGF II to tissue receptors.

Previous studies by Daughaday and Kapadia (35) and by our own group (12) have shown that the IGF II content of the 50-kD complex of tumor sera is increased. In addition, the 50-kD complex of tumor sera contains increased amounts of IGFBP-2 and IGFBP-3 (12), whereas the IGFBP-3 content of the 150-kD complex is reduced (reference 12, and Fig. 9). The three patients in Daughaday's and Kapadia's study (35) showed a lack of the 150-kD binding protein complex, which was reversed after operation. We have so far not seen a complete lack of the 150-kD complex in tumor sera, but usually find it reduced (Fig. 7 *B*), probably because of decreased production of the acid-labile subunit (see below). As a consequence, IGFBP-3 is sequestered into the 50-kD complex (Fig. 9). The latter also contains increased amounts of IGFBP-2 (reference 12; Fig. 9). These changes result in an apparent shift of IGF II from the 150- into the 50-kD complex with a two- to fourfold increase of its IGF II content (reference 12; Fig. 7, Table I) and of its insulin-like activity (Fig. 8, Table II). The increase of IGF II in the 50-kD complex is exclusively accounted for by big IGF II (Fig. 7, Table I). Hence, the turnover and bioavailability of big IGF II is considerably enhanced.

The differences between the radiochromatographic patterns of normal and tumor serum (Fig. 7, *A* and *B*) are compatible with the changes of the IGF and IGFBP contents in the 150- and 50-kD binding protein complexes. The decreased binding of IGF II tracer to the 150-kD complex is mainly due to the reduction of its binding protein-3 content, whereas decreased binding to the 50-kD complex of the tumor serum is accounted for by its increased content of IGF II. The overall decreased binding capacity of tumor serum explains the higher peak of nonbound IGF II tracer.

The development of EPTH. The predominant association of big IGF II with the 50-kD binding protein complex in tumor serum, which enhances its bioavailability to tissues, raises the question about the reason(s) underlying the abnormal distribution of big IGF II and IGFBPs in this disorder. As shown in Fig. 10 with ^{125}I-rhIGFBP-3, the 150-kD complex does not appropriately form in tumor serum. Similar data have been obtained recently by Baxter and Daughaday (36). They demonstrated that the formation activity of the 150-kD binding protein complex in preoperative sera of patients with EPTH was 75–85% below normal. Although total levels of the acid-labile subunit of the 150-kD complex were significantly decreased in these patients, Baxter and Daughaday concluded from their data that this is not the reason for impaired complex formation. They suggested that big IGF II bound to IGFBP-3 is unable to form the 150-kD binding protein complex. Several of our findings do not support this suggestion: (*a*) the 150-kD binding protein complex of tumor sera does contain big IGF II (Fig. 7 and Table I). (*b*) When normal serum is preequilibrated with ^{125}I-rhIGF II in the presence of partially purified big IGF II, the latter competes with the same efficiency for binding to the 150-kD and to the 50-kD complex (Zapf, J., unpublished data). (*c*) Although most tumor sera contain 30–50% normal IGF II (Figs. 5 and 7, and Table I), formation of the 150-kD complex is largely impaired (Fig. 10).

Summarizing others' and our own data on EPTH, we suggest the following sequence of events for the development of this disorder (Fig. 11): oversecretion of big IGF II by the tumor first leads to an elevation of total and thus of free serum IGF II, a stage that is probably transient and may be associated with first symptoms of hypoglycemia. Oversecretion of big IGF II may be compared with the experimental situation where IGF I is infused into normal subjects (25). If we assume that the actions of oversecreted big IGF II are similar to those of infused exogenous IGF I, we would expect suppression of growth hormone and insulin secretion (25), a finding typical of EPTH

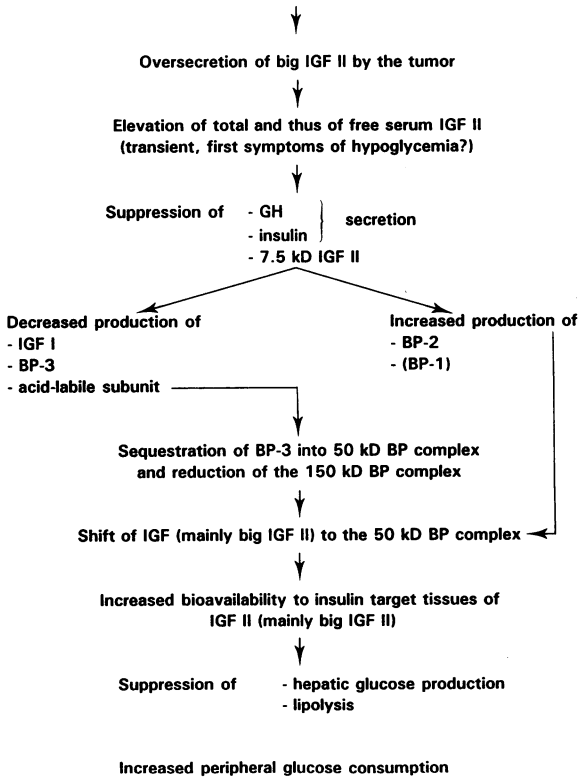

Figure 11. Suggested mechanism of the development of extrapancreatic tumor hypoglycemia.

(11, 20, 21, 37–39), and, in the case of big IGF II excess, a decrease of endogenous IGF I and of normal IGF II. The latter two changes probably result from the displacement of IGF I and IGF II from IGFBPs by big IGF II as well as from the suppression of growth hormone secretion. Although IGF II is less dependent on growth hormone than IGF I, its level is decreased in growth hormone–deficient states (9). Suppression of growth hormone secretion leads to a diminished production of IGFBP-3 (33) and of the acid-labile subunit of the 150-kD binding protein complex (36). Baxter and Daughaday (36) found low normal RIA-IGFBP-3 levels in patients with EPTH. It is possible that the RIA also recognizes degraded IGFBP-3, which might result from increased protease activity in the sera of tumor patients. However, we have not detected significant amounts of truncated binding protein-3 as an indicator of increased protease activity in our patients' sera. As tested by Western ligand blotting, the amount of binding protein-3 in our patients was variable ranging from near normal to significantly decreased levels.

Decreased acid-labile subunit levels probably account for the reduction or disappearance of the 150-kD complex and thus for the sequestration of IGFBP-3 into the 50-kD complex. Its increased content of IGFBP-2 due to the suppression of insulin and growth hormone secretion (12) and of IGFBP-3 goes along with the predominant association with big IGF II. Because of the short half-life of the 50-kD IGF pool and its high turnover rate (30), increased amounts of big IGF II reach the interstitial space and become bioavailable to insulin target tissues. This then leads to stimulation of glucose consumption in skeletal muscle via the insulin and/or the type 1 IGF receptor and to suppression of hepatic glucose production and of lipolysis in adipose tissue via the insulin receptor and results in sustained hypoglycemia. Low serum free fatty acid levels resulting from inhibition of lipolysis may contribute to enhanced peripheral glucose consumption via the Randle cycle (40). In addition, upregulation of insulin receptors may occur in the presence of low serum insulin levels and thus cause enhanced insulin sensitivity. Indeed, Stuart et al. (41) found a three- to fivefold increase in the insulin receptor number on mononuclear cells and on liver and muscle plasma membranes of a patient with hypoglycemia associated with a colon carcinoma metastatic to the liver. However, big IGF II in the serum and the tumor of this patient was not reported. Within the same context, Barzilai et al. (42) described a diabetic patient with a hepatoma subsequent to hemochromatosis and cirrhosis who lost dependence on insulin and experienced episodes of hypoglycemia. The insulin dose–response curve in this patient as determined by euglycemic clamp technique was shifted to the left, and steady state glucose consumption was 50% higher than in normal control subjects. Total IGF I and IGF II in the patient's serum were not elevated, but again, big IGF II was not assessed.

Finally, it is easily conceivable that big IGF II secreted by the tumor stimulates glucose uptake by the tumor in an auto/paracrine fashion. Since the tumor mass not infrequently reaches $\geq$ 2–3 kg, the latter mechanism may come into play although it has been calculated that it would not be sufficient to fully account for hypoglycemia in this syndrome (for review see reference 1).

The picture of the puzzling disorder of EPTH has started to become clearer. Nevertheless, it remains unexplained why three of our patients with EPTH (an adenocarcinoma of the cecum, a hemangioendothelioma, and a gastrointestinal lymphoma) developed hypoglycemia without having elevated IGF II nor increased big IGF II in their serum. Detailed metabolic and hormonal studies in such patients are necessary to find out whether still other causes besides oversecretion of big IGF II by the tumor may lead to hypoglycemia.

Acknowledgments

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