

Significance of abnormal serum binding of insulin-like growth factor II in the development of hypoglycemia in patients with non-islet-cell tumors

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Contributed by William H. Daughaday, June 12, 1989

ABSTRACT We reported that serum and tumor from a hypoglycemic patient with a fibrosarcoma contained insulin-like growth factor II (IGF-II), mostly in a large molecular form designated "big IGF-II." We now describe two additional patients with non-islet-cell tumor with hypoglycemia (NICTH) whose sera contained big IGF-II. Removal of the tumor eliminated most of the big IGF-II from the sera of two patients. Because specific IGF-binding proteins modify the bioactivity of IGFs, the sizes of the endogenous IGF-binding protein complexes were determined after neutral gel filtration through Sephadex G-200. Normally about 75% of IGFs are carried as a ternary complex of 150 kDa consisting of IGF, a growth hormone (GH)-dependent IGF-binding protein, and an acid-labile complexing component. The three patients with NICTH completely lacked the 150-kDa complex. IGF-II was present as a 60-kDa complex with variable contributions of smaller complexes. In the immediate postoperative period, a 110-kDa complex appeared rather than the expected 150-kDa complex. Abnormal IGF-II binding may be important in NICTH because the 150-kDa complexes cross the capillary membrane poorly. The smaller complexes present in our patients' sera would be expected to enter interstitial fluid readily, and a 4- to 5-fold increase in the fraction of IGFs reaching the target cells would result.

Many mesenchymal tumors express the insulin-like growth factor II (IGF-II) gene (1–7). Increased concentrations of IGF-II have been detected in some of these tumors (3, 6, 7), and explants of one tumor have released IGF-II into the medium (7). It has been suspected that secretion of IGF-II by certain large mesenchymal tumors might be related to the syndrome of non-islet-cell tumor hypoglycemia (NICTH), although attempts to detect such peptides in serum with relatively nonspecific insulin bioassays have given conflicting results (8, 9). Increased concentrations of serum IGF-II-like peptides were found by radioreceptor assay in some cases of NICTH (10, 11). Even when elevated, the increases in IGF-II concentrations were often small and could not be confirmed with a number of assay methods by Widmer *et al.* (12). Recently, Daughaday *et al.* (6) described a case of a woman with a fibrosarcoma (originally identified as a leiomyosarcoma) with hypoglycemia, whose tumor contained greatly increased IGF-II mRNA and IGF-II peptide. After acid gel filtration, most of the serum and tumor IGF-II was present as big IGF-II.

Because the severity of the metabolic symptoms of these patients with NICTH is not easily explained by the modest degree of increase of IGF-II, we undertook studies of the association of endogenous IGF-II to serum binding proteins in three cases of mesenchymal tumors with clinically severe hypoglycemia.

METHODS

Source of Sera. *Case 1.* Sera from a 67-year-old woman with severe symptomatic hypoglycemia and a large recurrent sarcoma of her thorax were kindly provided by Mary Ann Emanuele of Loyola University School of Medicine, Chicago. The tumor was originally considered to be a leiomyosarcoma, but subsequently the diagnosis was changed to a fibrosarcoma. A second serum sample was obtained weeks postoperatively, at which time her hypoglycemia was in remission. A report describing this patient has been published (6).

Case 2. Serum from a 19-year-old young man with severe hypoglycemia and a hemangiopericytoma. A second serum was obtained after removal of the tumor while the patient was in remission from his hypoglycemia. These sera were collected by Allen Eisman of University of Kansas School of Medicine and kindly provided by Kenneth Polonsky of the University of Chicago.

Case 3. Serum was obtained from a 71-year-old woman with severe symptomatic hypoglycemia and a large leiomyosarcoma. A second serum was obtained a week after operation while the patient was euglycemic. These sera were kindly provided by Mark H. Greene of Peoria, AZ.

Chromatographic Methods. Acid gel filtration was used to determine IGF-II size heterogeneity. Two milliliters of serum was acidified with 0.2 ml of 4 M acetic acid and passed through a 2.5×98 cm column of Bio-Gel P-60 (Bio-Rad) in 0.5 M acetic acid/0.075 M NaCl at room temperature. The flow rate was 0.31 ml/min, and 5-ml fractions were collected. This column is able to separate 7.5-kDa IGF-I and 7.5-kDa IGF-II nearly completely from each other and from the serum binding proteins (6).

Neutral gel filtration was used to determine the pattern of serum binding of IGF-II. Serum (1 ml) was passed through a 44×1.4 cm column of Sephadex G-200 in 0.05 M sodium diphosphate/0.15 M NaCl/0.02% NaN₂, pH 7.4, at room temperature. The flow rate was 0.32 ml/min and 1.6-ml fractions were collected.

Assay Methods. Serum levels of IGF-I and -II were determined after acidic ethanol extraction by RIA (13). IGF-I was measured with an antibody provided by J. J. Van Wyk and L. E. Underwood of Chapel Hill, NC, and distributed by the National Pituitary Program, Baltimore, MD. Separation of bound and unbound ¹²⁵I-labeled IGF-I was accomplished by the double-antibody method. Recombinant human [Thr⁵⁹]-IGF-I (Amgen, Thousand Oaks, CA) was used for iodination and standards. In some cases IGF-I was measured by an immunoradiometric assay as described by Scott *et al.* (14). The correlation between the two assays had a value of 0.96. IGF-II was measured by double-antibody RIA with a monoclonal antibody that is highly specific for IGF-II (Amano International Enzyme, Troy, VA) (15). Recombinant human IGF-II (provided by Michael W. Draper of Lilly Research

Laboratories, Indianapolis) was iodinated. Homogenous IGF-II isolated from bovine serum was used as the standard in the RIA.

Fractions obtained from the Bio-Gel P-60 acid gel column were assayed directly. All fractions from the neutral Sephadex G-200 column were subjected to acidic ethanol extraction before assay to avoid interference from serum binding proteins.

RESULTS

The concentration of IGF-I in sera of these patients after acidic ethanol extraction was markedly suppressed. After an operation in cases 1 and 3, there was a 3- to 4-fold rise in IGF-I. In case 3, where considerable tumor was evidently left behind, the rise in IGF-I was less than 2-fold.

Table 1 presents the results of IGF-II assays of the sera of these patients. Assays performed on acidic ethanol extracts of serum were normal in all cases. When these sera were subjected to Bio-Gel P-60 acid gel filtration and the sum of the IGF-II concentrations was determined, it was noted that there was a substantial increment in IGF-II, suggesting that acidic ethanol did not recover all the IGF-II in the large molecular form ("big IGF-II").

With P-60 acid gel filtration of normal serum, 7.5-kDa IGF-II was eluted slightly after the column volume shown as the dashed line in Fig. 1. Cytochrome *c* (13 kDa) on this column was eluted with a peak at about tube 74. The preoperative sera from cases 1, 2, and 3 had most (70–93%) of the immunoreactive IGF-II as a broad irregular peak whose estimated size was from 15 to 7.5 kDa (Fig. 1). After the operation, 93% of the IGF-II from case 1 serum and 67% of the IGF-II from case 2 serum was of the 7.5-kDa size. There was an increase in the 7.5-kDa IGF-II in the serum of case 3 after the operation and a persistence of big IGF-II with a much more restricted size heterogeneity than before the operation.

The results of Sephadex G-200 neutral gel filtration of a serum from a normal young woman are shown in Fig. 2. The optical density curve has the expected triple peaks at 280- μ m absorbance representing macroglobulin, gamma globulin (160 kDa), and serum albumin (67 kDa). These proteins provide useful internal calibrations of the columns. The major IGF-containing complex of normal serum was eluted slightly after the IgG peak and will be referred to as the 150-kDa peak. Not seen in this normal serum but present in some of the sera from patients with NICTH is a peak eluted in a position intermediate between the IgG and albumin peaks. This will be referred to as the 110-kDa peak. The second most prominent portion of IGF-II complexes in normal serum was eluted slightly later than albumin and is referred to as the 60-kDa peak. The normal serum and the sera from patients with

NICTH had even smaller complexes, which are referred to as the 20- to 40-kDa complexes.

The results of Sephadex G-200 neutral gel filtration of the sera of patients with NICTH are shown in Fig. 2. Most of the IGF-II in these sera was present as the 60-kDa complex with smaller amounts as the 20- to 40-kDa complex and a minimal contribution from the 150-kDa complex. Postoperatively, in cases 2 and 3, there was the appearance of the 110-kDa complex with a decrease of the 60-kDa complex and little change in the contribution of the 20- to 40-kDa complexes.

DISCUSSION

The results of measurements of IGF-II after Bio-Gel P-60 acid gel filtration confirm our previous findings of case 1—that the major fraction of IGF-II in NICTH is present as big IGF-II. We now present further evidence that big IGF-II was derived from the tumors of these patients. In case 1 serum, big IGF-II virtually disappeared from the serum after operation, and 7.5-kDa IGF-II increased markedly. In cases 2 and 3, big IGF-II decreased after operation but did not disappear. This suggests to us that the tumors were not completely removed. Serum IGF-I concentrations were markedly suppressed in cases 1, 2, and 3 before the operation and rose after the operation, as has been reported in other cases of NICTH (6, 7, 11, 12).

There has been little attention to IGF binding proteins in NICTH. Megyesi *et al.* (16) in 1975 compared normal serum with a serum from a patient with NICTH by gel filtration through Sephadex G-50 in neutral buffer. Both types of serum had 93% of the non-suppressible insulin-like activity (IGFs) detectable by radioreceptor assay in the excluded volume. The gel filtration technique used by Megyesi *et al.* (16) would not distinguish the 150- and 60-kDa complexes. Widmer *et al.* (12) measured 125 I-labeled IGF-I binding in the pooled 0–50% bed volume fractions after acid filtrations through Sephadex G-50. There was no difference in binding between sera from normal adults and sera from 16 patients with NICTH.

In our studies of the serum transport of IGF-II in NICTH, we measured endogenous IGF-II fractions after neutral gel filtration with subsequent removal of the binding proteins in each of these fractions by acidic ethanol extraction. Most other studies are done by incubating serum with 125 I-labeled IGF with or without crosslinking reagents. This does not give an accurate pattern of the binding of endogenous IGFs because the labeled IGFs equilibrate poorly with IGFs occupying sites in the 150-kDa complex (13). The alternative procedure of removal of endogenous IGFs from binding proteins by acid gel filtration used by others is also unsatisfactory because it destroys the 150-kDa complex by denaturing the acid-labile complexing component.

One of us (W.H.D.) has previously used the Sephadex G-200 neutral gel filtration method to characterize the transport of IGF-I in normal sera (17). In seven normal sera $72 \pm 2.5\%$ of IGF-I was present as the 150-kDa complex. Most of the remainder was present as smaller complexes. We find that the transport of IGF-II in normal adult sera is similar to our earlier result with IGF-I.

The most notable abnormality in IGF-II binding in these cases of NICTH was the virtual absence of the 150-kDa complex with most of the IGF-II present in serum as 60-kDa complexes and the remainder present as smaller complexes of <40 kDa. After the operation, cases 2 and 3 had not reconstituted the normal 150-kDa complex, but there was the new appearance of a 110-kDa complex and an increase in the fraction of small complexes.

Recent progress in our understanding of the IGF-II binding proteins makes possible an interpretation of these results. There is now general agreement that there are at least two major forms of the binding protein. There is a glycoprotein

Table 1. Serum IGFs of patients with non-islet-cell tumors associated with hypoglycemia

Diagnosis	IGF-I AE,* μ g/liter	IGF-II, μ g/liter		"Big" IGF-II, %
		AE*	AG†	
Fibrosarcoma				
Pre-op	29	414	1149	70
Post-op	120	586	943	7
Hemangiopericytoma				
Pre-op	72	729	1215	84
Post-op	225	300	563	33
Leiomyosarcoma				
Pre-op	38	1300	1868	93
Post-op	64	700	1924	60

Pre-op, preoperative; post-op, postoperative.

*Serum extracted with acid ethanol.

†Serum gel filtered through Bio-Gel P-60 in acid.

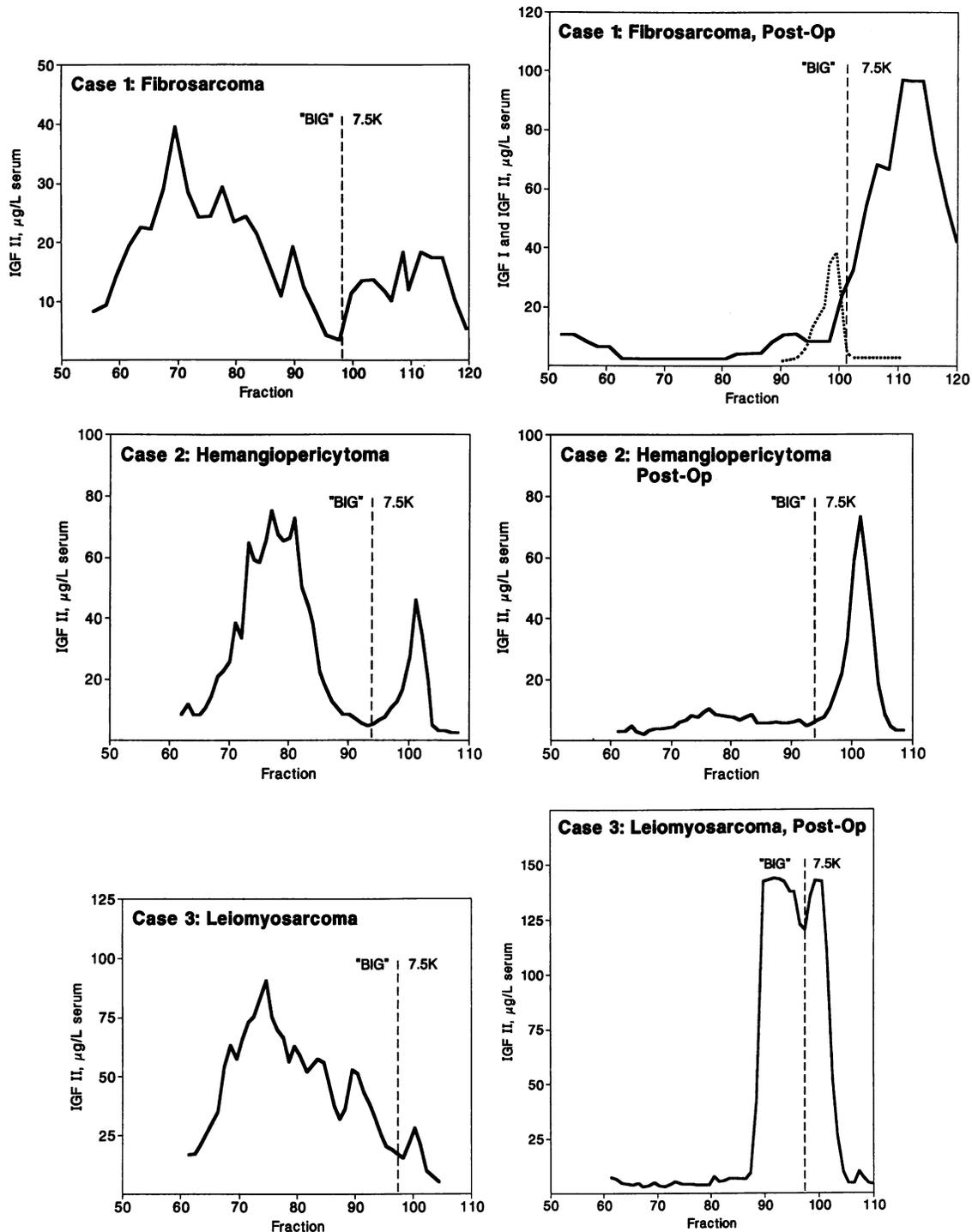


FIG. 1. Serum was gel filtered through Bio-Gel P-60 in 0.5 M HAC/0.075 M NaCl. IGF-II was measured by RIA on individual tubes in duplicate. In the postoperative (Post-Op) study on case 1 (Top Right) the dotted line indicates the elution position of IGF-I. All results are expressed as $\mu\text{g}/\text{liter}$ ($\mu\text{g}/\text{L}$) of original serum in each tube. The vertical dashed line represents the column volume used to divide big and 7.5-kDa IGF-II.

doublet with a molecular mass of 47–53 kDa that is growth hormone (GH) dependent (designated BP_{GH}) (18, 19). When this binding protein is occupied by an IGF molecule, it can complex with an acid-labile glycoprotein, designated CP, of 84–86 kDa to form the 150-kDa complex (20). Normal serum also contains a small concentration of complexes with a molecular mass of ≈ 120 kDa. Hardouin *et al.* (21) have suggested that this complex includes a deglycosylated form of BP_{GH} with a molecular mass of ≈ 30 kDa associated with IGF and CP.

The second distinct type of IGF binding protein of serum is GH independent, nonglycosylated, and unreactive with CP. This binding protein, BP_{AF} , is identical to the 28-kDa binding protein isolated from amniotic fluid (22). BP_{AF} carries little IGF in serum from normal and hypopituitary individuals (21). BP_{AF} exhibits a marked circadian pattern of concentration with highest levels reached at night and is markedly affected by changes in glucose metabolism. The concentration of this binding protein rises abruptly during hypoglycemia (23). These properties are not shared by the BP_{GH} .

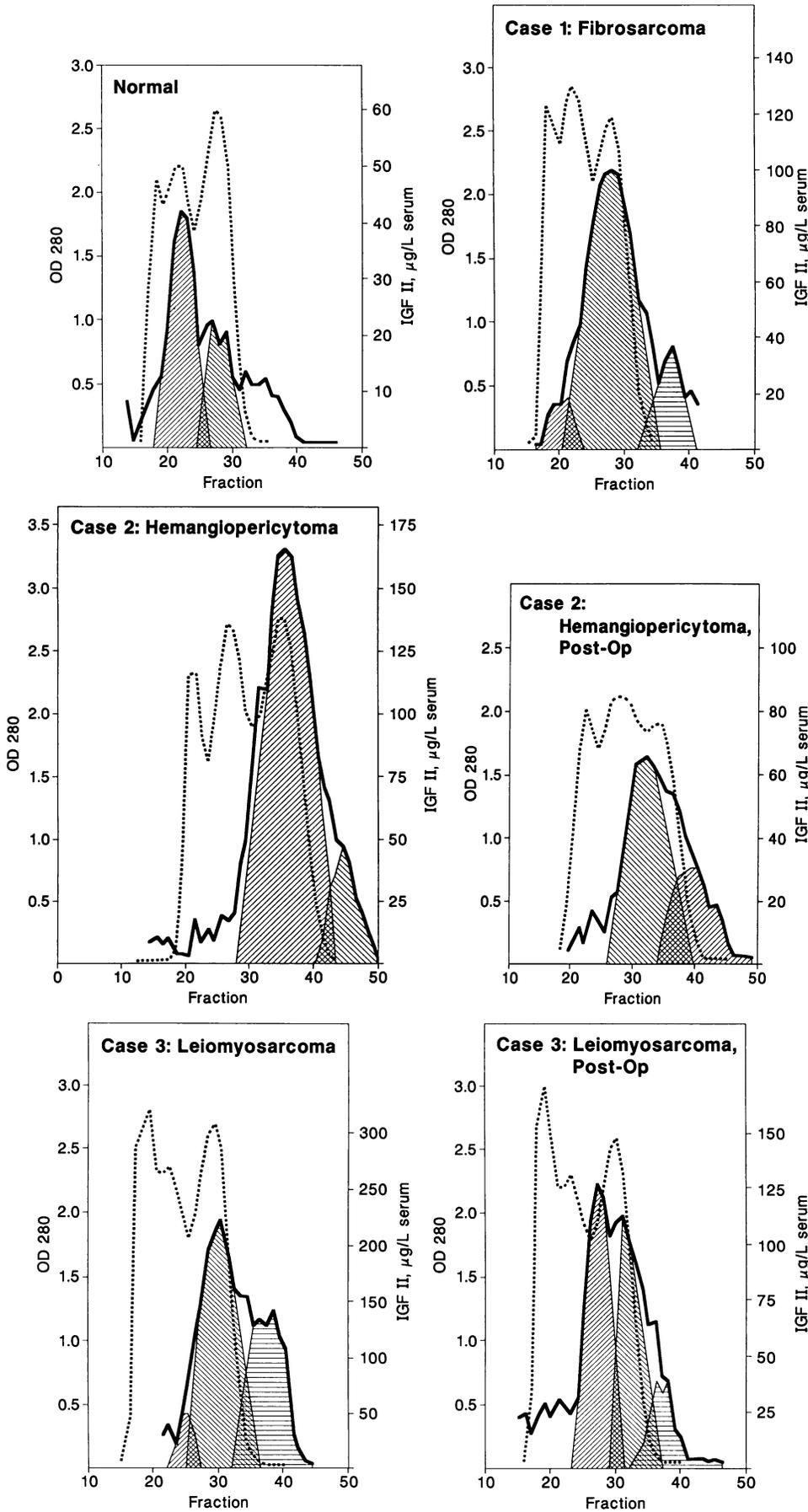


FIG. 2. Sera were passed through a Sephadex G-200 column in a neutral phosphate buffer. OD₂₈₀ is shown as the dotted line. The solid lines are the IGF-II results from RIA of each tube after acidic ethanol removal of binding proteins. The hatched areas enclosed by the fine solid lines are estimates of individual components as derived from graphic analysis, with the assumption that the major peak was symmetrical. The sera studied include normal adult serum, preoperative sera from cases 1-3, and post-operative (Post-Op) sera from cases 2 and 3. L, liter.

The dominant 60-kDa complex found in sera of patients with NICTH reported here probably consists of IGF-II and BP_{GH}.

These results suggest that the secretion of CP in these subjects is markedly suppressed. In addition to the 60-kDa complex,

IGF-II was carried to a lesser degree as smaller complexes. These could represent binding by BP_{AF} or by deglycosylated BP_{GH}. Distinguishing between these two species can only be done with specific antibodies unavailable to us.

The changes in the pattern of serum binding of IGF-II in cases 2 and 3, which occurred after surgery, were unexpected. The proportion of IGF-II present as 60-kDa complexes decreased, and complexes of roughly 110 kDa appeared that were eluted between the albumin and gamma globulin peaks. This suggests participation of CP in a complex with partial deglycosylated CP or BP_{GH}.

The pathophysiology of NICTH is complex. GH acts on target tissues to promote synthesis and secretion of IGF-I, IGF-II, GH_{BP}, and CP, primarily from the liver. In simple GH deficiency there is marked decrease in serum IGF-I, BP_{GH}, and CP with a smaller decrease in IGF-II. The tumors associated with NICTH that have been studied secrete IGF-II, and we suspect that they also secrete BP_{GH} without CP (Fig. 3). IGF-II acting on the hypothalamus and pituitary would explain the GH deficiency of these patients (6, 7), which would increase sensitivity to insulin-like peptides and explain at least in part the abnormalities in serum binding of IGF-II, which we report here.

The changes in serum binding may be important in understanding the major physiologic consequences of modest elevations of serum IGF-II in NICTH. About 75% of IGFs in normal serum are carried in the 150-kDa complexes and only 25% as small complexes. Binoux and Hossenlopp (24) found that lymph contained about 20% as much IGF-I and IGF-II as serum and that only 7% of the IGF in lymph was present as the 150-kDa complex and the remainder as small complexes. They concluded that only the small complexes can easily traverse the endothelial barrier. Because nearly all of the IGF complexes in the serum of patients with NICTH are

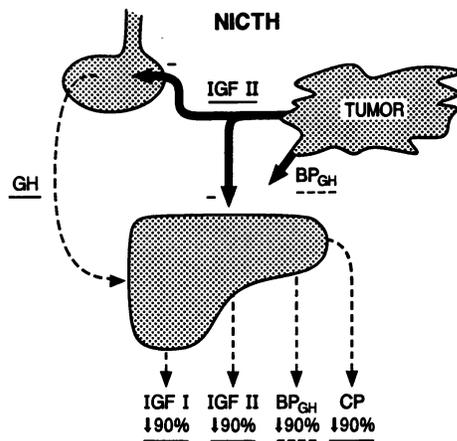


FIG. 3. A schematic diagram to show the probable pathophysiology of NICTH. The tumor is shown secreting IGF-II, which is largely big IGF-II. This acts on the pituitary (upper left) to inhibit GH secretion (broken line). Because of a GH deficiency, secretion of IGF-I, IGF-II, BP_{GH}, and CP by normal target tissues, here shown as liver, are suppressed. Tumor IGF-II may also exert negative feedback on target tissues. The tumor is shown secreting GH_{BP}, but evidence for this is indirect (indicated by dashed underline).

small, the concentration of IGF-II in lymph may be as much as 4-fold higher than would occur with normal serum binding.

We are grateful to Drs. Peter Rotwein and Sherida Tollefsen for their suggestions in preparing this manuscript. We are indebted to Ms. Dawn Engbring for expert typing of the manuscript. This work was supported in part by Program Project Grant HD20805 of the National Institute of Child Health and Human Development.

1. Reeve, A. E., Eccles, M. R., Wilkins, R. J., Bell, G. I. & Millow, L. J. (1985) *Nature (London)* **317**, 258-260.
2. Scott, J., Cowell, J., Robertson, M. E., Priestly, L. M., Wadey, R., Hopkins, B., Pritchard, J., Bell, G. I., Rall, L. B., Graham, C. F. & Knott, T. J. (1985) *Nature (London)* **317**, 260-262.
3. Haselbacher, G. K., Irminger, J. C., Zapf, J., Ziegler, W. H. & Humbel, R. E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1104-1106.
4. Tricoli, J. V., Rall, L. B., Karakousis, C. P., Herrera, L., Petrelli, N. J., Bell, G. I. & Shows, T. B. (1986) *Cancer Res.* **46**, 6169-6173.
5. Hoppener, J. W. M., Mosselman, S., Roholl, P. J. M., Lambrechts, C., Slebos, R. J. C., de Pater-Holthuisen, P., Lips, C. J. M., Jansz, H. S. & Sussenbach, J. S. (1988) *EMBO. J.* **7**, 1379-1385.
6. Daughaday, W. H., Emanuele, M. A., Brooks, M. H., Barbato, A. L., Kapadia, M. & Rotwein, P. (1988) *N. Engl. J. Med.* **319**, 1434-1440.
7. Ron, D., Powers, A. C., Pandian, M. R., Godine, J. E. & Axelrod, L. (1989) *J. Clin. Endocrinol. Metab.* **68**, 701-706.
8. August, J. T. & Hiatt, H. H. (1958) *N. Engl. J. Med.* **258**, 17-21.
9. Miller, D. R., Bolinger, R. E., Janigan, D., Crockett, J. E. & Friesen, S. R. (1959) *Ann. Surg.* **150**, 684-696.
10. Megyesi, K., Kahn, C. R., Roth, J. & Gorden, P. (1974) *J. Clin. Endocrinol. Metab.* **38**, 931-934.
11. Daughaday, W. H., Trivedi, B. & Kapadia, M. (1981) *J. Clin. Endocrinol. Metab.* **53**, 289-294.
12. Widmer, U., Zapf, J. & Froesch, E. R. (1982) *J. Clin. Endocrinol. Metab.* **55**, 833-839.
13. Daughaday, W. H., Mariz, I. K. & Blethen, S. L. (1980) *J. Clin. Endocrinol. Metab.* **51**, 781-788.
14. Scott, M. G., Cuca, G. C., Petersen, J. R., Lyle, L. R., Burleigh, B. D. & Daughaday, W. H. (1987) *Clin. Chem.* **33**, 2019-2023.
15. Tanaka, H., Asami, O., Hayano, T., Sasaki, I., Yoshitake, Y. & Nishikawa, K. (1989) *Endocrinology* **124**, 870-877.
16. Megyesi, K., Kahn, R., Roth, J. & Gorden, P. (1975) *J. Clin. Endocrinol. Metab.* **41**, 475-484.
17. Goldberg, A. C., Trivedi, B., Delmez, J. A., Harter, H. R. & Daughaday, W. H. (1982) *J. Clin. Endocrinol. Metab.* **55**, 1040-1045.
18. Martin, J. L. & Baxter, R. C. (1986) *J. Biol. Chem.* **261**, 8754-8760.
19. Baxter, R. C. & Martin, J. L. (1986) *J. Clin. Invest.* **78**, 1504-1512.
20. Baxter, R. C. (1988) *J. Clin. Endocrinol. Metab.* **67**, 265-272.
21. Hardouin, S., Hossenlopp, P., Segovia, B., Seurin, D., Portolan, G., Lassarre, C. & Binoux, M. (1987) *Eur. J. Biochem.* **170**, 121-132.
22. Pova, G., Enberg, G., Jornvall, H. & Hall, K. (1984) *Eur. J. Biochem.* **144**, 199-204.
23. Yeoh, S. I. & Baxter, R. C. (1988) *Acta Endocrinol.* **119**, 465-473.
24. Binoux, M. & Hossenlopp, P. (1988) *J. Clin. Endocrinol. Metab.* **67**, 509-514.