Erythrocyte Insulin-Like Growth Factor-I Receptor Evaluation in Normal Subjects, Acromegalics, and Growth Hormone–Deficient and Insulin-Dependent Diabetic Children

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Insulin-like growth factor-I (IGF-I) receptors are characterized in several animal and human tissues. IGF-I receptor studies performed in erythrocytes to assess IGF-I receptor status at target-cell tissues are potentially useful for clinical studies, since tissue biopsies or cultures are not required. However, validation of results is challenged by some investigators on the basis of discrepancies described in comparative studies with other cell types, probably related to populations of different cell ages affecting binding to red blood cells (RBCs). By correcting cell age for creatine, we studied IGF-I receptor status in 24 normal subjects (11 adults and 13 children, eight prepubertal and five pubertal) and 33 patients with pathologic conditions (five adult acromegalics, six children with pituitary dwarfism, and 22 type I diabetic children, 15 prepubertal and seven pubertal). Acromegalic patients with higher plasma IGF-I and insulin levels presented lower IGF-I specific binding ([B_o] mean ± SEM, 6.1% ± 0.8%) and affinity ([ED_{50}] 28.5 ± 2.2 ng/mL) than normal adults (B_o, 10.9% ± 0.7%; ED_{50}, 16.4 ± 0.9 ng/mL; $P < .001$), and growth hormone (GH)-deficient children showed higher IGF-I binding (24.6% ± 1.7%, $P < .001$) without significant affinity alterations than normal prepubertal children (B_o, 14.7% ± 1.0%). Both prepubertal and pubertal type I diabetic children with higher GH levels presented decreased IGF-I binding (11.4% ± 0.9% for prepubertal, $P < .05$; 10.0% ± 1.1% for pubertal, $P < .05$) to RBC receptors in comparison to the respective control group (14.7% ± 10% and 14.9% ± 1.3%, prepubertal and pubertal, respectively). Whereas in pubertal diabetics mean IGF-I basal levels were significantly reduced as compared with the respective controls, no difference was observed in mean IGF-I basal levels in normal and diabetic prepubertal children.

SPECIFIC RECEPTORS to insulin-like growth factor-I (IGF-I) are described in several human tissues such as fibroblasts,[1] placenta,[2] liver,[3] and ovaries,[4] and also in circulating blood cells.[5,6] Clinical studies of IGF-I binding to erythrocytes present a potential advantage since these cells are abundant in the circulation, requiring only small volumes of blood samples for receptor evaluation and avoiding uncomfortable biopsies that are necessary when other tissues are used.[7,8] Although other circulating cells are largely studied to evaluate the insulin or IGF-I receptor, only a few clinical studies have been performed in erythrocytes, since differences in binding are observed in these cells according to the cell population age, with decreasing binding in older cells.[8-10] Since it was previously reported that red blood cell (RBC) creatine could be used as a good indicator of RBC age,[11] we evaluated IGF-I binding to RBCs corrected for cell age in normal subjects and in subjects with different clinical conditions with altered growth hormone (GH) and/or IGF-I secretion such as acromegalics, children with short stature due to GH deficiency, and subjects with diabetes mellitus.

In general, children with insulin-dependent diabetes mellitus (IDDM) in poor glycemic control can have growth failure with decreased final height in comparison to normals,[12,13] but it is less certain how diabetes influences growth and adult height in children treated conventionally with insulin in whom the metabolic condition is fairly well controlled.[14,15] In diabetic patients, particularly those with poor glycemic control, higher fasting GH levels, increased pulse frequency and amplitude, and higher urinary GH excretion have been found than in normals.[12-16] However, plasma IGF-I levels were shown to be normal or inappropriately low for the higher GH levels seen in IDDM subjects,[16-18] and IGF-I response to GH infusion was impaired in direct relation to the degree of diabetic control, suggesting a degree of resistance to hepatic generation of IGF-I in response to GH.[19] Since it was shown that a strong negative relationship between IGF-I and hemoglobin $A1_c$ (HbA_{1c}) existed in puberty but no such association was apparent in prepubertal children,[20,21] it was suggested that the influence of glycemic control on the GH–IGF-I axis after the onset of puberty could be related to some other factors such as sex hormones.[21]

Regarding the IGF-I receptor, there is a report of a reduced number of binding sites per erythrocyte in several patients with acromegaly,[10] whereas in GH-deficient children and Laron-type dwarfism, a decrease in receptor concentration and gene expression was shown.[6,22,23] In streptozotocin-induced diabetes in rats, a decrease in IGF-I in plasma and in many tissues and an increase in IGF-I receptor concentration and gene expression in the kidney was shown.[22,24]

SUBJECTS AND METHODS

Patients

Fifty-seven subjects were studied: 11 adult controls (three men and eight women), eight prepubertal normal children (four boys and four girls), five pubertal normal children (three boys and two girls), six GH-deficient children with short stature (five boys and one girl), and five acromegalic men, seven prepubertal IDDM children (six boys and one girl), and 15 pubertal IDDM children (eight boys and seven girls). Anthropometric data of the subjects are listed in Table 1.

All subjects were kept on their usual diet and regular physical

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Table 1. Anthropometric Data of Study Subjects (mean ± SEM)

	Age (yr)	Weight (kg)	Height (cm)	Height (±SD)	BMI (kg/m²)
Adult controls	34.3 ± 2.3	66.3 ± 2.4	160.8 ± 2.0	—	25.6 ± 0.8
Acromegalics	40.2 ± 6.7	87.3 ± 5.9	180.9 ± 5.2	—	26.6 ± 1.0
Prepubertal					
Controls	7.8 ± 0.4	25.0 ± 1.6	125.3 ± 3.5	0.1 ± 0.3	18.8 ± 0.8
Diabetics	8.2 ± 0.6	28.0 ± 1.7	129.1 ± 4.0	0.3 ± 0.3	17.8 ± 1.0
GH-deficient	10.6 ± 1.6	20.2 ± 3.0	112.0 ± 6.4	4.6 ± 1.2	15.5 ± 0.9
Pubertal					
Controls	12.0 ± 0.7	40.3 ± 3.8	150.0 ± 2.9	0.1 ± 0.6	16.8 ± 0.7
Diabetics	14.3 ± 0.4	47.7 ± 2.2	155.5 ± 2.7	0.8 ± 1.0	19.8 ± 2.3

Abbreviations: BMI, body mass index.

activities and all drug ingestion was avoided for 30 days before blood sample collection. The IDDM patients had a known duration of disease greater than 2 years at the time of study and were treated using multiple daily injections of monocomponent pork intermediate- or rapid-acting insulin preparations. All GH-deficient patients had heights between −8.3 and −2.5 standard deviations from mean values in normal children for their ages according to Tanner's tables, and their bone ages were greater than −2 standard deviations from normal values based on the Greulich-Pyle[25] pattern for their age and sex. GH deficiency was diagnosed in each patient based on a decreased GH response (<7 ng/mL) to three different GH stimulation tests (insulin-induced hypoglycemia or clonidine and L-dopa administration after 20 minutes' exercise). None of these patients were treated previously with exogenous GH replacement.

Acromegalic subjects were all men presenting with active disease with IGF-I levels above the normal range.

Methods

Blood samples were drawn from the antecubital vein of each subject at 8 AM after an overnight fast for determination of glucose, insulin, GH, IGF-I, HbA_{1c}, and erythrocyte creatine and evaluation of RBC IGF-I receptors. Glucose was determined using an enzymatic method on an Auto-Analyzer (Technicon, Tarrytown, NY), and HbA_{1c} was determined after elution from an ion-exchange column by a modification of the method reported by Trivelli et al.[26] Insulin level was measured by radioimmunoassay using a modification of the method reported by Desbuquois and Aurbach in which a second antibody was added to polyethylene glycol to separate free and antibody-bound fractions.[27] GH level was measured using commercial radioimmunometric kits (Pharmacia, Uppsala, Sweden), and plasma IGF-I was determined by radioimmunoassay (after extraction by acid-ethanol) using a method standardized in our laboratory.[28] Creatine level in RBCs was measured by a method based on Griffiths' technique[11] with minor modifications.[29] Creatine concentration was expressed as nanograms per 4.5×10^6 RBCs.

IGF-I Receptor Evaluation

IGF-I receptor evaluation was performed according to the method reported by Hizuka et al[10] with some modifications. Erythrocytes were isolated from 10-mL heparinized blood samples using the technique described by Gambhir et al.[30] The blood was centrifuged, plasma was aspirated, and RBCs were separated from other circulating cells by centrifugation through a Ficoll-Hypaque gradient according to the technique reported by Boyum,[31] resulting in less than 1% leukocyte contamination. RBCs were washed twice with HEPES-Tris (pH 8.0), centrifuged again in Ficoll-Hypaque under the same conditions, and resuspended to a final concentration of 4.5×10^9 RBC/mL.

Cell preparations were incubated overnight for 20 hours at 4°C with 3-(^{125}I)-iodotyrosil IGF-I (0.1 to 0.2 ng/mL; specific activity, ~200 μCi/μg) purchased from Amersham (Little Chalfont, Buckinghamshire, UK) in the presence or absence of increasing concentrations (0.1 to 100 ng/mL) of unlabeled IGF-I obtained from Dr E. Martin Spencer (San Francisco, CA) in a total volume of 0.5 mL adjusted with HEPES-Tris buffer after addition of bacitracin (4 mg/mL) to every tube. After incubation, two 0.2-mL samples were transferred to microfuge tubes containing buffer (0.2 mL) and dibutylphthalate (0.2 mL) and centrifuged for 2.5 minutes in a Beckman Microfuge (Beckman Instruments, Fullerton, CA). The supernatant was aspirated, and the bottoms of tubes containing bound cells were cut to be counted in a gamma counter. Nonspecific binding was defined as the ^{125}I–IGF-I count of the RBC precipitate at the nonlabeled IGF-I concentration of 1,000 ng/mL. Specific binding is the difference between total and nonspecific binding. Data from IGF-I binding studies are presented as the percentage of total radioactivity bound specifically to the receptors at tracer concentrations ($\%B_o$). The number of receptors per cell (N) was obtained from the Scatchard plots and the affinity analysis was evaluated at the higher-affinity state at the lower level of receptor occupancy (affinity constant of the empty receptor [Ke]) and the effective IGF-I concentration corresponding to 50% total binding (IGF-I ED_{50}) in the competition-inhibition curves.

$\%B_o$ was corrected for creatine as an indicator of each patient's RBC mean age using the following formula previously proposed for RBC insulin receptors[29]:

$$\%B_o = \frac{\text{observed } B_o \text{ mean creatine concentration for correspondent control}}{\text{patient's creatine concentration}}.$$

The number of receptor sites per cell and affinity constants were calculated from the insulin binding data corrected for creatine.

Statistical Analysis

Data are expressed as the mean ± SEM. Student's ± tests for paired and unpaired samples were calculated to evaluate differences between means, with an IBM (Sao Paulo, Brazil) 386–compatible computer using the statistical package Epistat (Epitta Services, Richardson, TX).

RESULTS

Laboratory Data

Basal concentrations of GH, IGF-I, and insulin are listed in Table 2. Mean basal GH concentration was not statisti-

Table 2. Specific Binding Parameters of RBC IGF-I Receptors and Corresponding IGF-I, GH, HbA_{1c}, and Insulin Levels in Normal Subjects and Patients (mean ± SEM)

	B_o (%)	N	ED_{50} (ng/mL)	Ke ($\times 10^8$ mol/L)	Creatine (ng)*	IFG-I (ng/mL)	GH (ng/mL)	Insulin (μU/mL)	HbA_{1c} (%)
Adult controls	10.9 ± 0.7	44.2 ± 3.5	16.4 ± 0.9	3.9 ± 0.3	30.0 ± 3.0	145.3 ± 13.9	0.8 ± 0.2	9.0 ± 2.3	
Acromegalics	6.1 ± 0.8	39.7 ± 7.0	28.5 ± 2.2	2.3 ± 0.4	35.5 ± 5.2	631.9 ± 57.7	29.1 ± 0.1	24.0 ± 4.5	
Prepubertal									
Controls	14.7 ± 1.0	37.7 ± 6.1	11.0 ± 1.8	6.9 ± 0.8	30.3 ± 1.2	164.9 ± 24.5	1.1 ± 0.2	1.3 ± 0.1	6.8 ± 0.5
Diabetics	11.4 ± 0.9	37.6 ± 4.8	16.6 ± 2.4	5.0 ± 0.6	36.6 ± 3.2	184.8 ± 41.9	4.6 ± 1.5	14.2 ± 3.5	10.2 ± 0.9
GH-deficient	24.6 ± 1.7	64.5 ± 11.9	7.1 ± 2.4	10.9 ± 2.2	29.1 ± 3.2	28.9 ± 6.1	0.2 ± 0.1	1.9 ± 0.3	—
Pubertal									
Controls	14.9 ± 1.3	39.2 ± 9.3	10.7 ± 2.4	7.2 ± 1.3	33.3 ± 3.7	282.7 ± 39.0	1.1 ± 0.3	1.4 ± 0.2	6.2 ± 0.6
Diabetics	10.0 ± 1.1	34.2 ± 3.8	16.7 ± 1.2	4.5 ± 0.3	41.5 ± 3.5	224.4 ± 29.3	5.5 ± 1.1	32.9 ± 5.9	13.9 ± 0.6

*Per 4.5×10^6 RBC.

cally different between the three normal study groups. As expected, acromegalics showed significantly higher GH concentrations than corresponding adult controls ($P < .001$), and GH-deficient children presented significantly lower GH concentrations than normal prepubertal children ($P < .01$). Prepubertal and pubertal IDDM children presented significantly higher GH levels than corresponding prepubertal ($P < .001$) and pubertal ($P < .05$) normal children.

Adult IGF-I concentration (mean ± SEM) was significantly higher in acromegalics than in normal ($P < .001$), and children with GH deficiency presented lower mean IGF-I concentrations than prepubertal normal controls ($P < .001$). IDDM pubertal children showed significantly lower IGF-I values in comparison to pubertal normals ($P < .05$), but no statistical difference was observed in the prepubertal group between diabetics and normals.

Insulin concentrations showed significantly higher mean values in acromegalics versus normal adults ($P < .01$), whereas values were significantly lower in GH-deficient than in normal children ($P < .05$). Insulin concentrations in both prepubertal and pubertal diabetics were significantly higher than in respective prepubertal ($P < .0001$) and pubertal ($P < .01$) normal children.

Erythrocyte creatine content tested by ANOVA was not significantly different among the groups (Table 1). HbA_{1C} in prepubertal and pubertal IDDM children was significantly more elevated than in respective normal prepubertal and pubertal children, being significantly higher in pubertal diabetics than in prepubertal ones.

Receptor Binding Studies

^{125}I–IGF-I binding data and the derived parameters (mean ± SEM) are listed in Table 2.

Cross-reactivity of insulin to IGF-I RBC receptors was 0.55%, and of IGF-I to insulin RBC receptors, 0.04%.

Normal subjects. $\%B_o$ values in both prepubertal ($P < .01$) and pubertal ($P < .001$) normal children were significantly increased in comparison to normal adults, with no significant statistical difference between the two groups of normal children. In both prepubertal and pubertal children, an increased Ke $P < .001$; $P = .01$) and decreased ED_{50} (prepubertal, $P < .01$; pubertal, $P < .01$) were observed in comparison to normal adults, indicating an increased affinity of these receptors to IGF-I in children. However, no statistical difference in receptor concentration (N) was observed between adults and children.

Acromegalic adults and GH-deficient children. IGF-I binding to its RBC receptors (mean ± SEM) corrected for creatine (B_o) in adults was significantly reduced in acromegalic subjects in comparison to their normal controls ($P < .001$), whereas in children, those with GH deficiency showed significantly higher values than corresponding prepubertal normals ($P < .001$). Affinity was decreased in acromegalics, as indicated, respectively, by the significantly higher ED_{50} ($P < .001$) and lower Ke ($P < .001$) values than in normal adults, but no significant difference was observed between these two groups in relation to N. Children with GH deficiency showed a significantly increased N ($P < .02$) and higher Ke ($P < .05$) but no difference in ED_{50} in comparison to normal prepubertal children (Table 2).

IDDM subjects. Both prepubertal and pubertal IDDM children showed significantly lower mean B_o values in comparison to respective normal prepubertal ($P < .05$) and pubertal ($P < .05$) children (Table 2 and Fig 2). Affinity was lower in both diabetic groups in relation to respective prepubertal and pubertal normals ($P < .05$), but no difference in N was observed when diabetic and normal children were compared (Fig 2).

DISCUSSION

IGF-I initiates its action by binding to cell membrane receptors. These receptors were identified and characterized in several tissues, including circulating cells.[1-7] Thoumopoulus et al[8] in studying rats and Polychronakos et al[7] in studying humans reported that IGF-I and IGF-II could bind specifically to their receptors in erythrocytes in a manner similar to that demonstrated in other cells. Clinical studies using RBCs from relatively small blood collections to evaluate IGF-I receptor status, mirroring IGF-I in more specific target cells, could avoid inconvenient biopsies and/or fibroblast cultures. However, some criticism was directed at RBC studies,[9,10,32] since Polychronakos et al[7] reported that IGF-I binding to human erythrocytes was dependent on cell age, as is the case for insulin binding. To avoid the influence of RBC age on binding, we used a procedure previously used for insulin binding studies to

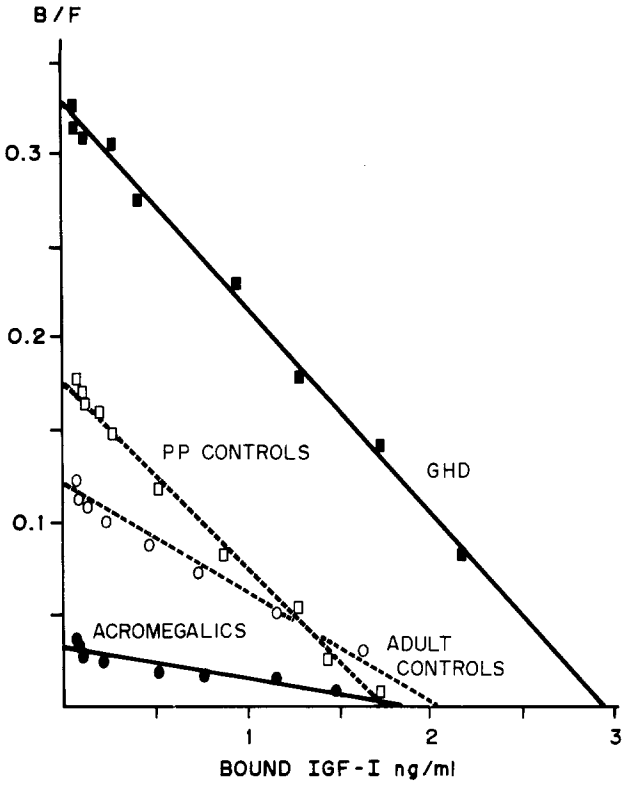

Fig 1 Scatchard analysis of mean values for IGF-I binding to human RBCs in (○) normal and (●) acromegalic adults, (□) prepubertal (PP) controls, and (■) GH-deficient (GHD) children.

correct insulin binding data on RBCs for cell age by the creatine content of erythrocytes.[29] As demonstrated by Griffiths' studies,[11] creatine content was well correlated with cell age, being a technically easier and reliable procedure comparable to other methods such as density-gradient centrifugation and intracellular enzyme determination with glucose-6-phosphate dehydrogenase or pyruvate kinase.[33] In the present study, by correcting IGF-I binding for cell age, we evaluated IGF-I receptor characteristics in normal subjects and in pathologic states separating the subjects into three groups according to age (adults and prepubertal and pubertal children), since plasma RBC IGF-I concentrations increase with age from birth to adulthood, with a further reduction from age 30 years onward.[28,34]

Our acromegalic patients with increased GH and IGF-I concentrations showed lower specific binding of the receptor to IGF-I than normal adults, indicating receptor down-regulation as also described in other cell-type studies,[1-5] whereas GH-deficient children with lower IGF-I concentrations had higher specific binding to IGF-I receptor than prepubertal children with the same developmental age, suggesting upregulation of receptors in these patients, as previously shown by Eshet et al.[6,23] In acromegalic subjects, the significantly reduced affinity to IGF-I was the predominant receptor change in response to increased IGF-I production, with no significant changes in the number of receptor binding sites in the cell (N). On the other hand, in GH-deficient children the lack of IGF-I production was associated with an increased number of receptor binding sites per cell (higher N) but no evident changes in affinity, since Ke and ED_{50} values, respectively, showed a marginal or nonsignificant difference in relation to values in normal prepubertal children. Eshet et al[6] have also shown that the high-affinity constant of RBC receptor sites for IGF-I was not significantly different from that observed in a control group. As compared with data reported by Hizuka et al[10] in human erythrocytes, our observed specific IGF-I binding to receptors in normal subjects presented similar results but our acromegalic subjects showed much lower values, explained at least in part by the correction for cell age by creatine, although the more elevated creatine concentration in acromegalics was not statistically higher than in corresponding normal controls.

IGF-I specific binding (B_o) in both prepubertal and pubertal normal children was higher than in normal adults. Since this increased specific binding in children cannot be explained as receptor regulation to IGF-I concentrations (lower in adults) or to different cell-age populations as indicated by similar erythrocyte creatine content, we could speculate that the greater receptor binding and affinity observed in children is probably a physiologic mechanism to facilitate hormone action during this phase when growth occurs. Since during childhood the IGF binding protein (IGFBF)-3 increase peaks at puberty and declines thereafter,[35] whereas IGFBP-1 levels display a pattern in child-

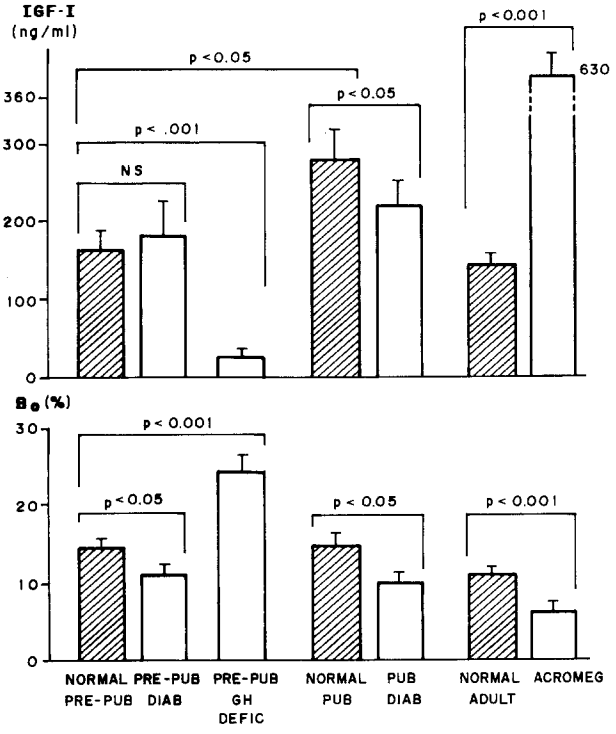

Fig 2 Mean ± SEM plasma IGF-I and IGF-I receptor binding in RBCs (B_o) from normal and diabetic (DIAB) children, GH-deficient (GH DEFIC) children, normal adults, and acromegalics (ACROMEG). PRE-PUB, prepubertal; PUB, pubertal.

hood and adolescence opposite to that of IGFBP-3,[36,37] it is possible that the increase of IGFBP-3 and its binding to IGF-I could lead to an increase in IGF-I receptor affinity as a compensatory mechanism.

As compared with corresponding normal prepubertal children, both prepubertal and pubertal IDDM children showed a lower specific IGF-I binding and affinity. These findings are not clearly explained by our data. First, the significantly increased erythrocyte creatine content in pubertal diabetic children and not in prepubertal ones was corrected in our calculation of B_o. Second, in diabetic children, increased IGF-I values known to induce inverse changes in receptor parameters were not observed. The influence of antibodies against IGF-I receptors could also be discounted, since Tappy et al[38] only observed the presence of these antibodies in one case of a total of 52 IDDM patients, and in our technique RBCs are washed three times before assay, ruling out the direct influence of antibodies.

Factors that should be taken into consideration are the role of altered circulating major IGFBPs in type I diabetics (increase in IGFBP-1 and decrease in IGFBP-3) in the regulation of IGF-I receptor binding to RBCs,[37] since the IGFBP changes are opposite to those found in normal children independently of IGF-I levels, which were normal in prepubertal diabetics[22]; and the possible effect of exogenous insulin therapy on IGF-I modulation despite the fact that in our binding studies IGF-I receptor specificity was greater than 200 times lower for insulin than for IGF-I.

However, in contrast to viewing the marked differences observed in acromegalic adults and GH-deficient children as significant in comparison to the corresponding controls, we must be cautious in interpreting the small differences in receptor binding numbers and affinities in diabetic subjects as significant, since investigators who have worked extensively with ligand binding via Scatchard plots know that there is a relatively wide range in the degree of confidence in the obtained values. This range is not necessarily corrected for by simple statistical treatment with a lower degree of significance from a limited number of samples studied in diabetic patients.

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