Receptors for Insulinlike Growth Factor I Are Defective in Fibroblasts Cultured from a Patient with Leprechaunism

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ABSTRACT We previously have demonstrated that fibroblasts from a patient with leprechaunism exhibited markedly decreased insulin binding to insulin receptors and that the ability of insulin to stimulate glucose incorporation in the patient's cells was greatly impaired. In addition, the insulinlike growth factor, multiplication-stimulating activity (MSA), also exhibited an impaired ability to stimulate glucose incorporation in the patient's fibroblasts, although in normal fibroblasts this response appears to be mediated by an insulinlike growth factor receptor. The present study examines ^{125}I-labeled insulinlike growth factor I (IGF-I) binding to patient's and control fibroblasts. ^{125}I-labeled IGF-I binds to a specific IGF-I receptor in normal fibroblasts. At steady state, binding was inhibited by unlabeled IGF-I, IGF-II, MSA III-2, MSA II, insulin, and proinsulin, in order of potency, but not by high concentrations of epidermal growth factor and human growth hormone, chemically unrelated polypeptides ^{125}I-labeled IGF-I binding to patient's cells was decreased by ~75%, whereas binding of epidermal growth factor to its cell surface receptors was unaffected. Computer curve-fitting of untransformed equilibrium binding data suggests that the decreased binding resulted from a decreased K_a for IGF-I. The ability of the patient's IGF-I receptor to recognize insulin also appears to be altered. Impaired IGF-I binding by the leprechaun patient's fibroblasts may contribute to the abnormal biological response to insulinlike growth factors observed in vitro and to the in utero growth retardation.

INTRODUCTION

Leprechaunism is a rare genetic disorder characterized by physical abnormalities, intrauterine and neonatal growth retardation, poorly developed subcutaneous fat and muscle at birth, and early death; it is frequently associated with hyperinsulinemia and insulin resistance [reviewed in (1)]. In previous studies with skin fibroblasts established in culture from a patient with typical features of the disorder, we have demonstrated a profound decrease in ^{125}I-labeled insulin binding to insulin receptors (2) and a profound impairment of insulin's ability to stimulate glucose incorporation in the patient's cells (3). In other patients with leprechaunism, insulin binding is normal and tissue resistance to insulin is thought to reflect a postreceptor defect (4, 5).

In an attempt to demonstrate the specificity of the defect in insulin binding and of the insulin biological effect in our patient, we also examined the ability of the insulinlike growth factor (IGF),[1] multiplication-stimulating activity (MSA), to stimulate glucose incorporation in patient's and control fibroblasts. The IGF are polypeptides chemically related to insulin (6, 7) that exhibit a spectrum of biological activities similar to

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[1] *Abbreviations used in this paper*: EGF, epidermal growth factor; IGF, insulinlike growth factor; MSA, multiplication-stimulating activity; NSILA, nonsuppressible insulinlike activity.

insulin, acting through IGF receptors or insulin receptors (6, 8, 9). MSA, purified from rat liver cell culture medium (10), interacts with receptors for IGF-I and IGF-II, the two IGF isolated from human plasma whose amino acid sequences have been determined (11).[2] MSA stimulated glucose incorporation in normal fibroblasts with a high potency that suggested that it acted through an IGF receptor rather than an insulin receptor (3). Surprisingly, stimulation of glucose incorporation by MSA also was impaired in the patient's fibroblasts (3). This suggested the possibility that the lesion in the leprechaun patient's cells affected both insulin receptors and IGF receptors. In the present study, we demonstrate that ^{125}I-labeled IGF-I binding to the IGF-I receptor in fibroblasts from the leprechaun patient is decreased by >70%. Studies are presented to characterize the nature of this binding defect.

METHODS

Peptides. IGF-I and IGF-II were purified from human plasma as described (15). The IGF-I preparation used for these experiments (16SPII) contained ~10% contamination by IGF-II. A partially purified preparation (1932) of IGF-I and IGF-II (designated NSILA, nonsuppressible insulinlike activity) having one-eighth the specific activity of IGF-I in stimulating glucose oxidation (36 mU/mg), and one-fifth the potency of IGF-I in inhibiting ^{125}I-labeled IGF-I binding to human fibroblasts (unpublished results) was used to determine nonspecific binding. IGF-II was from batch 9SEIV.

Epidermal growth factor (EGF) purified from the submaxillary gland of male mice according to the procedures of Savage et al. (16) was a kind gift of Wayne B. Anderson, National Cancer Institute.

MSA was purified from serum-free medium conditioned by BRL 3A cells by Dowex 50 chromatography, Sephadex G-75 gel filtration, and preparative acrylamide electrophoresis as previously described (17). The properties of Sephadex G-75 peak II MSA (mol wt 8,700) and MSA III-2 (mol wt 7,100) have been described previously (17, 18).

Monocomponent porcine insulin crystals (26.9 U/mg) (lot S8691272) and porcine proinsulin (lot no. 1670) were purchased from the Novo Research Institute (Copenhagen). Human Growth Hormone (1.8 IU/mg; radioimmunoassay grade) from the National Pituitary Agency was a gift of M. Lesniak (Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases).

Fibroblast cultures. Fibroblast cultures were established in the Department of Genetics, University of Manitoba, Winnipeg, Canada, from the leprechaun patient (skin from upper arm, day 20) and from a normal male control (foreskin, day 7) (2). After seven and six passages, respectively, cells were shipped to the National Institutes of Health for the studies to be described. They were used after an additional 3-10 passages. Subcultivation and feeding of stock cultures of patient's and control fibroblasts, and subcultivation of cells for experiments, were performed directly in parallel.

Cell cultivation. Fibroblast cultures (2, 19) were grown at 37°C in modified Eagle's minimal essential medium supplemented with 10 mM Hepes buffer, nonessential amino acids, neomycin sulfate, and 20% fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y., lot C688210). Cultures were divided 1:3 or 1:4 each week.

Patient's and control cells and media were cultured for mycoplasma (broth culture for 21 d; agar subculture for 14 d) by Microbiological Associates (Bethesda, Md.) and were negative.

IGF-I binding to fibroblasts in suspension. ^{125}I-labeled IGF-I was prepared by a modified chloramine T procedure (20). To 1 μg of IGF-I in 20 μl of Krebs-Ringer phosphate buffer, pH 7.5, was added ~2 mCi of high concentration of carrier-free ^{125}I-sodium. The reaction was initiated by addition of 333 μg of chloramine T in 10 μl of H_2O, allowed to proceed for 20 s, and quenched with 100 μl of 1 M acetic acid-1 mg/ml bovine serum albumin. The reaction mixture was fractionated immediately on Sephadex G-75 (1 cm × 50 cm, 1 M acetic acid-1 mg/ml bovine serum albumin), and the fractions corresponding to the ^{125}I-IGF-I peak stored at −20°C for 2–4 wk. For some experiments, iodinated IGF-I was further purified by gel filtration on Sephadex G-25 (PD-10) in Hepes binding buffer immediately before use. The specific radioactivity of the seven ^{125}I-labeled IGF-I preparations used for these experiments was 11–144 Ci/g.

Binding studies were performed as previously described (2, 19, 20). Fibroblasts were inoculated into 100-mm plastic petri plates and used ~7–11 d later without feeding. To detach fibroblasts from the cell monolayer for experiments, the cultures were washed with phosphate-buffered saline and incubated for 3–5 min at 37°C with trypsin (Worthington Biochemical Corp., Freehold, N. J.; 3150 BAEE U/mg; 0.1 mg/ml) and 0.5 mM EDTA. Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.; 0.2 mg/ml) was added, and the cells were sedimented, washed, and resuspended in pH 8.0 Hepes binding buffer (0.1 M Hepes, 0.12 M NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 10 mg/ml of bovine serum albumin (Sigma Chemical Co., radioimmunoassay grade).

The incubation mixture consisted of ^{125}I-labeled IGF-I (~250 pg/ml), the indicated concentration of unlabeled IGF-I, and 1–2.5 × 10^6 fibroblasts (as indicated) in a volume of 0.5 ml. Binding studies were performed under steady-state conditions. Incubation was for 3–4 h at 15°C; after which, duplicate 0.2-ml aliquots were pipetted into microfuge tubes (0.4 ml) containing 0.2 ml of iced Hepes binding buffer, the tubes were centrifuged in a microfuge B (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 min, and the supernate was aspirated and discarded. Tips containing the cell pellets were excised and assayed in a Beckman gamma 9000 spectrometer at 75% efficiency.

Nonspecific (or nonsaturable) binding was determined in the presence of a large excess of unlabeled NSILA (typically, 2–10 μg/ml).

Computer curve-fit of competitive binding data. Data from individual IGF-I competitive binding experiments were fit by least-squares analysis using the LIGAND program (21). Analysis assumed a single class of binding sites, identity of radiolabeled and unlabeled IGF-I, and 100% bindability of tracer. Data were used without transformation.

Eight paired experiments (patient/control) also were simultaneously analyzed using the LIGAND program (21). For this analysis, in addition to the assumptions stated above, the binding capacity R_0 was constrained to be the same for patient

[2] Two other polypeptides purified from human plasma, somatomedin C (12, 13) and basic somatomedin (14), are indistinguishable from IGF-I in their reactivity with antibodies and receptors, and have amino acid sequences in common with IGF-I. Somatomedin C, however, also possesses sequences not represented in IGF-I (13).

and control in a given experimental pair, but was allowed to vary between experiments.

EGF binding to fibroblasts. EGF was labeled with ^{125}I-labeled iodine by the chloramine-T procedure (22) and was purified on Sephadex G-25 (PD-10) immediately before use. Specific radioactivities were 53–68 Ci/g.

Binding assay conditions were modified from Carpenter and Cohen (22). Fibroblasts were plated at a density of 400,000–500,000 cells/well in Linbro 6-well, 35-mm cluster plates (Flow Laboratories, McLean, Va.) in growth medium. 2 d later, cell monolayers were washed with Dulbecco's phosphate-buffered saline. ^{125}I-labeled EGF (0.05–0.1 ng/ml) and the indicated concentrations of unlabeled EGF were added in incubation buffer (Hanks' balance salt solution supplemented with 1 mg/ml bovine serum albumin, 20 mM Hepes, 10 mM NH_4Cl, pH 7.5) and incubated at 37°C for the indicated times. At the end of the incubation, the medium was aspirated, the plates washed thoroughly, and cells solubilized with 0.1% sodium dodecyl sulfate-0.5 mM EDTA as previously described (2). All points were determined in duplicate or triplicate. Cell number was determined on replicate wells after trypsinization.

RESULTS

Properties of the IGF-I receptor in normal human fibroblasts. ^{125}I-labeled IGF-I bound rapidly to human fibroblasts at 15°C. Half-maximal binding was observed in ~30 min; a steady state of binding occurred from 3–5 h of incubation (results not shown). At steady state, ~85% of the total bound radioactivity was specific or saturable binding; that is, it was abolished by incubation with excess unlabeled NSILA. ^{125}I-labeled IGF-I remaining in the supernatant media after 3 h of incubation was physically intact as determined by Sephadex G-75 gel filtration in 1 M acetic acid (results not shown).

^{125}I-labeled IGF-I bound to human fibroblasts at steady state dissociated rapidly when cells were sedimented; resuspended in Hepes binding buffer, and incubated at 37°C (Fig. 1). Approximately 50% of the radioactivity dissociated in 40 min, and 85% was dissociated by 5 h. Gel filtration (Sephadex G-75, 1 M acetic acid) of radioactivity released after 5 h of incubation indicated no conversion to lower molecular weight forms, although ~20% of the radioactivity appeared in the excluded column volume (results not shown). Thus, release of bound IGF-I does not appear to involve degradation of the polypeptide.

Binding of tracer concentrations of ^{125}I-labeled IGF-I to normal fibroblasts at 15°C was inhibited by unlabeled IGF-I in dose-dependent fashion. In 18 experiments, specific binding was 5.7±0.5% (SEM) of input radioactivity/10^6 cells. Half-maximal inhibition occurred with 12.3±1.5 (SEM) ng/ml of IGF-I.[3] Partially purified NSILA was 21% as potent as IGF-I (data not shown).

The specificity of the IGF-I receptor was established

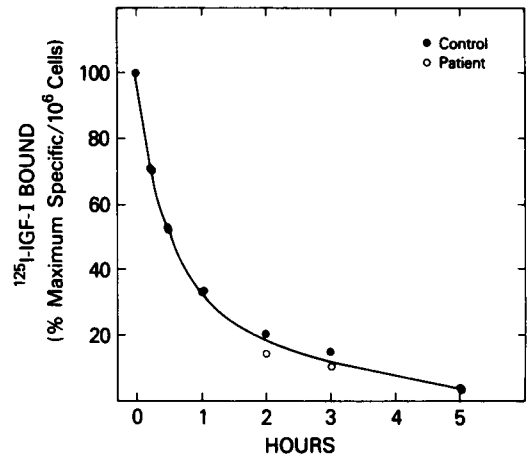

FIGURE 1 Dissociation of ^{125}I-labeled IGF-I from patient's and control fibroblasts at 37°C. ^{125}I-labeled IGF-I was incubated with 0.69 × 10^6 control fibroblasts and 0.64 × 10^6 patient's fibroblasts for 3.25 h at 15°C. Cells were pelleted and resuspended in an equal volume (0.5 ml) of Hepes binding buffer without IGF-I, and incubation continued at 37°C. At the indicated times, replicate incubation tubes were harvested and cell-associated radioactivity determined as previously described. Total binding per 10^6 control fibroblasts was 13.36%; nonspecific binding was 2.03%. Total binding per 10^6 patient fibroblasts was 6.14%; nonspecific binding was 0.98%. Percentage of maximum specific binding per 10^6 cells is plotted for different times of dissociation. Control (●); patient (○).

by competition experiments summarized in Table I. IGF-I, IGF-II, MSA II, and MSA III-2 inhibited ^{125}I-labeled IGF-I binding completely and with parallel dose-response curves. On the basis of the peptide concentration required to inhibit binding by 50%, MSA III-2 and MSA II were 23 and 5.0±0.6% (SEM) as potent as IGF-I, respectively (Table I). Insulin inhibited ^{125}I-labeled IGF binding with 6.5±1.5% (SEM) the potency of IGF-I; proinsulin was ~0.3% as potent as IGF-I. Chemically unrelated polypeptides—EGF, nerve growth factor, and human growth hormone—did not inhibit binding at higher concentrations.

Binding of IGF-I to patient's fibroblasts is decreased. Binding of ^{125}I-labeled IGF-I to control and patient's fibroblasts was studied under steady-state conditions at 15°C in suspension. The results of eight paired experiments are presented in Table II. The concentrations of ^{125}I-labeled IGF-I were chosen to measure maximum binding; addition of unlabeled IGF-I at four- to eightfold greater concentration did not decrease the bound/free (B/F) ratio (Fig. 2). The maximum

[3] This dose-response curve is somewhat less sensitive than was previously reported (11) (half-maximal binding at ~4 ng/ml) with a different preparation of IGF-I and a slightly different iodination scheme. A similar decrease in sensitivity also was observed in chick embryo fibroblasts with ^{125}I-labeled IGF-I (half-maximal inhibition of binding by 8.3±0.8 ng/ml [n = 5] of unlabeled IGF-I instead of 2 ng/ml) and with ^{125}I-MSA tracer, which suggests minor differences in the unlabeled IGF-I preparations.

TABLE I

Relative Potencies of Competitive Inhibitors of ^{125}I-labeled IGF-I Binding to Normal Human Fibroblasts

Peptide	Potency
IGF-I	100
IGF-II (n = 1)	43
MSA III-2 (n = 1)	23
MSA peak II (n = 6)	5.0±0.6
Insulin (n = 8)	6.5±1.3
Proinsulin (n = 2)	0.3±0.2
EGF	0*‡
Human growth hormone	0*‡
Nerve growth factor	0*

Relative potencies were determined by the ratio of concentrations of indicated peptide and IGF-I that inhibited ^{125}I-labeled IGF-I binding by 50%.

* No inhibition at 1 μg/ml.

‡ No inhibition at 10 μg/ml.

total binding to 10^6 patient's cells was 29±3% of that to 10^6 control cells. Specific binding per 10^6 cells was decreased by 75%. The decreased binding to patient's fibroblasts was observed throughout the dose-response curve. (Fig. 2).

The decreased binding to the patient's fibroblasts appears to be an intrinsic property of the cells, rather than a result of the binding assay conditions. (*a*) ^{125}I-labeled IGF-I recovered from the supernatant media after incubation with patient's cells and examined by gel filtration on Sephadex G-75 in 1 M acetic acid remained physically intact (results not shown), which suggests that the low binding to patient's fibroblasts did not result from degradation of the tracer. (*b*) ^{125}I-labeled IGF-I (0.25 ng/ml) bound rapidly to both patient's and control fibroblasts at 15°C. Maximal binding was observed by 2 h, with a steady state of binding persisting for up to 7.5 h (results not shown). Thus, the decreased binding to patient's fibroblasts did not simply result from a failure to achieve maximum binding. (*c*) Decreased binding to patient's fibroblasts also was observed throughout a 5-h time-course at 37°C (results not shown). (*d*) Decreased binding to patient's fibroblasts was observed not only at pH 8.0, but also at pH 7.4, 7.6, and 7.8 (results not shown). (*e*) Patient's fibroblasts were not unusually sensitive to the trypsin treatment used to suspend them for binding assays (Table III). Although binding experiments were performed routinely on fibroblasts incubated with trypsin-EDTA for 3–5 min, no consistent decrease in binding of ^{125}I-labeled IGF-I was observed until significantly longer times of incubation: > 20 min for control fibroblasts and >10 min for patient's fibroblasts. (*f*) Binding to control fibroblasts in the eight paired experiments did not differ significantly from the results in a total of 18 experiments using this control (n = 11) and two 21–22-yr-old female volunteers (n = 7). The percentage of ^{125}I-labeled IGF-I bound/10^6 cells was 6.7±0.5 (SEM) in the 18 experiments, and 5.7±0.5 (SEM) for the eight paired experiments. Half-maximal inhibition was observed with 12.3±1.5 ng/ml unlabeled IGF-I in the 18 experiments, and with 15.1±2.4 ng/ml in the eight paired experiments.

Binding of EGF to patient's and control fibroblasts. To evaluate the specificity of the decrease in IGF-I binding, we examined binding of a chemically unrelated growth factor, EGF, to patient's and control

TABLE II

^{125}I-labeled IGF-I Binding to Control and Patient's Fibroblasts

	Control			Patient			Patient/control	
Experiment	Bound per tube	Cells/ml	Bound per 10^6 cells	Bound per tube	Cells/ml	Bound per 10^6 cells	Maximum total binding/ 10^6 cells	Maximum specific* binding/ 10^6 cells
	%	×10^{-6}	%	%	×10^{-6}	%	%	
201	7.6	1.9	8.2	2.3	1.7	2.7	33	28
212	6.4	2.3	5.7	1.7	2.7	1.2	21	15
321	6.0	2.2	5.4	1.9	3.1	1.2	22	17
331	18.0	4.9	7.3	6.8	5.1	2.7	37	35
424	6.8	1.4	9.4	1.3	1.2	2.2	23	13
807	7.8	1.4	10.8	1.8	1.7	2.1	19	17
813	6.4	1.4	8.2	2.6	1.6	3.3	36	35
821	5.3	1.7	6.4	1.5	1.2	2.5	39	36
						Mean±SEM	29±3	25±4

* Calculated after subtraction of nonspecific binding (i.e., radioactivity bound in the presence of 2 or 4 μg/ml NSILA).

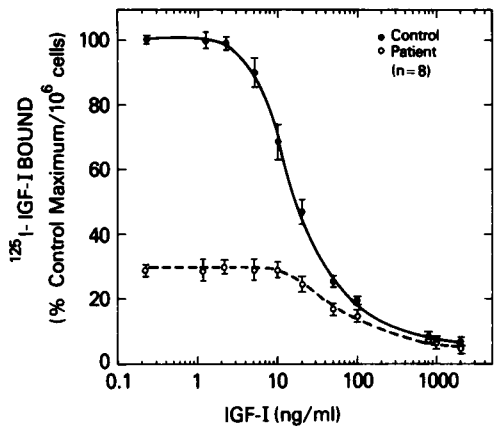

FIGURE 2 Dose-response of inhibition of ^{125}I-labeled IGF-I binding to patient's (○) and control (●) fibroblasts by unlabeled IGF-I in eight paired experiments. Conditions of binding assays were as described in Methods. The eight paired experiments are those presented in Table II. Results are expressed as percentage of maximum binding to 10^6 control cells in the same experiment. Mean±SEM are plotted for each IGF-I concentration (ng/ml). The three points representing the highest concentrations of IGF-I (plotted as 800, 1000, and 2,000 ng/ml) were obtained with 4,000, 5,000, and 10,000 ng/ml of partially purified NSILA, respectively.

fibroblasts. Although EGF binds initially to cell surface receptors at 37°C, the EGF-receptor complex forms clusters, is internalized by endocytosis, and EGF (and perhaps the receptor) is degraded in lysosomes (23). To inhibit EGF degradation and achieve a steady state at 37°C, we performed the binding incubation in the presence of 10 mM NH_4Cl. Under these conditions, ^{125}I-labeled EGF bound to normal fibroblasts reached a stable maximum in 3–6 h of incubation (not shown). ^{125}I-labeled EGF recovered from the incubation media after 3 h of incubation with cells at 37°C coeluted with intact ^{125}I-labeled EGF on Sephadex G-75 gel filtration in 1 M acetic acid (not shown).

^{125}I-labeled EGF binding to patient's and control fibroblasts was examined in the presence of 10 mM NH_4Cl in six paired experiments (Table IV). The iodinated peptide was added at concentrations of 50 or 100 pg/ml, concentrations that did not decrease the bound EGF/free EGF ratio (data not shown). Maximum binding to patient's fibroblasts compared to control fibroblasts was 87±9% (expressed per culture well) and 95±14% (expressed per cell). The dose-response curves for EGF binding to patient's and control fibroblasts did not differ significantly (Fig. 3). We conclude that the defect in the patient's fibroblast does not affect EGF binding.

Quantitative analysis of IGF-I competitive binding data. The eight paired IGF-I dose-response curves obtained in competitive binding experiments and summarized in Table I were each evaluated separately by computer curve-fitting using the LIGAND program system (21). This program fits the raw binding data, including nonspecific binding, in an untransformed coordinate system. The Scatchard plot derived from the results of a representative experiment (experiment 424, Table II) is linear (Fig. 4, left panel), consistent with a single class of binding sites. The equilibrium constant (K_a) and receptor numbers per cell obtained in these eight experiments are tabulated in Table V. No significant difference in receptor number between patient's and control cells was observed: $4.10\pm0.79 \times 10^4$ molecules/cell to control fibroblasts and $5.56\pm1.65 \times 10^4$ molecules/cell to patient's fibroblasts. By contrast, the K_a observed in patient's fibroblasts was only 25±8% of that observed in control fibroblasts: $8.68\pm1.60 \times 10^8$ M^{-1} for control and $1.74 \pm0.33 \times 10^8$ M^{-1} for patient.[4]

The dose-response curves from the eight experiments were further analyzed by simultaneous curve-fitting using the LIGAND program. The eight paired experiments were fit on the assumption that the receptor

TABLE III
Effect of Trypsinization of Fibroblast Monolayers for Different Duration on the Binding of ^{125}I-labeled IGF-I

Time	Maximum specific binding of ^{125}I-labeled IGF-I/10^6 cells	
	Control	Patient
min	%	
2	100	100
4	104	102
6	90	88
8	94	80
10	78	95
20	109	52
30	27	42

Fibroblast monolayer cultures were suspended by incubation with 0.01% trypsin-0.5 mM EDTA for the indicated intervals as described in Methods. Soybean trypsin inhibitor was added to terminate the reaction, and the cells collected by centrifugation, resuspended, washed, centrifuged, and resuspended in Hepes binding buffer. Cell number was determined on an aliquot of the suspension. The remaining cells were incubated under standard conditions with ^{125}I-labeled IGF-I in the presence and absence of 4 μg/ml of partially purified NSILA. Specific binding/10^6 cells was calculated. Binding to cells trypsinized for 2 min was taken as 100%.

[4] The K_a determined for IGF-I binding to age-matched male control fibroblasts was similar to the K_a determined from a similar analysis of results of IGF-I binding to fibroblasts from a 21-yr-old normal female volunteer: $K_a = 15.2\pm4.5 \times 10^8$ M^{-1} ($n = 4$).

TABLE IV

Binding of ^{125}I-labeled EGF to Control and Patient's Fibroblasts

Experiment	Control			Patient			Patient/control	
	Bound per well	Cells/well	Bound per 10^6 cells	Bound per well	Cells/well	Bound per 10^6 cells	Maximum binding per well	Maximum binding per cell
	%	$\times 10^{-6}$	%	%	$\times 10^{-6}$	%	%	
07	8.0	0.20	37.5	10.0	0.30	31.5	125	84
08	12.5	0.37	30.9	9.7	0.42	22.5	78	73
09	14.7	0.41	35.5	9.7	0.24	40.3	66	114
11	16.9	0.24	71.0	16.0	0.36	45.4	95	64
12	15.1	0.27	55.9	11.0	0.26	42.3	73	76
13	7.1	0.64	11.1	6.1	0.35	17.4	87	157
						Mean±SEM	87±9	95±14

For each experiment, 0.4–0.5 × 10^6 fibroblasts were plated in 35-mm multiwell tissue culture plates in growth medium and incubated at 37°C. Binding assays were performed 2 d later as described in Methods. Incubations were at 37°C in 10 mM NH_4Cl with a tracer mass of 100 pg/ml for experiments 07, 08, and 09; and 50 pg/ml for experiments 11, 12, and 13. Total ^{125}I-EGF bound (%) is compared per cell and per 35-mm culture well in patient's and control fibroblasts.

The exceptionally high binding to control fibroblasts (expressed per 10^6 cells) in experiment 11 resulted, in part, from incomplete trypsinization resulting in an underestimate of cell number. Although in most experiments the cell number/well recovered at the end of the experiment was somewhat lower than the number of cells plated, the efficiency of plating for patient's and control fibroblasts appears to be similar.

concentration was the same for patient and control in a given experiment, allowing for variation from experiment to experiment. The results are plotted in Fig. 4, right panel. The K_a determined for control fibroblasts was 7.22±0.86 × 10^8 M^{-1} K_a for patient's fibroblasts was 1.66±1.77 × 10^8 M^{-1}. By contrast, it was not possible to fit these data acceptably with a model in which K_a was constant for patient and control, and receptor concentration was allowed to vary (not shown). Thus, these results suggest that the decreased IGF-I binding to the patient's fibroblasts results from a decreased affinity for IGF-I.

The apparent decrease in K_a for IGF-I might have resulted from a decrease in the association rate con-

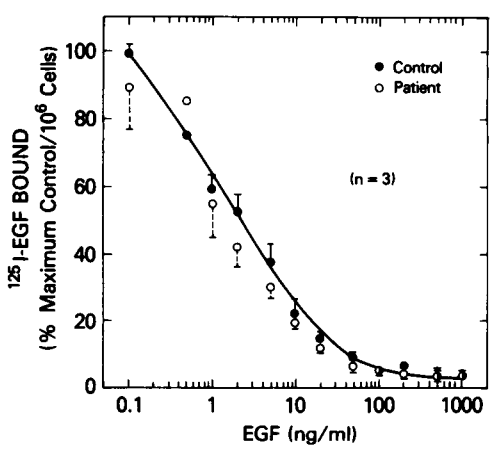

FIGURE 3 Dose response of inhibition of ^{125}I-labeled EGF binding to patient's (○) and control (●) fibroblasts in three paired experiments. Conditions of binding assays were described in Methods. The three paired experiments are 07, 08, and 09 from Table IV. Results are expressed as percentage of maximum binding to 10^6 control cells in the same experiment (mean±SEM) for each total EGF concentration. The 0.5-ng EGF point was determined only in experiment 09.

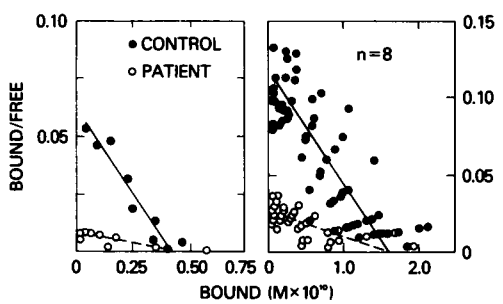

FIGURE 4 Scatchard plot based on computer curve fit of IGF-I binding to patient's (○) and control (●) fibroblasts. Left panel: results of binding to patient's and control fibroblasts were separately fit using the LIGAND program. Data are from experiment 424 (Table II). For comparison, binding to control fibroblasts was adjusted to the same cell concentration as patient's fibroblasts (1.16 × 10^6/ml). Right panel: Results from the eight paired experiments (Table II) were simultaneously fit for the patient's and control fibroblasts using the LIGAND program. A single class of binding sites was assumed. Receptor number was assumed to be constant for patient and control fibroblasts in a given experiment, but allowed to vary from experiment to experiment. The composite fit was kindly performed by Dr. Peter Munson, NICHHD.

TABLE V
Computer Analysis of IGF-I Binding to Patient's and Control Fibroblasts

Experiment	Equilibrium constant			Receptor number		
	Control	Patient	Patient/control	Control	Patient	Patient/control
	$M^{-1} \times 10^{-8}$			molecules/cell $\times 10^{-4}$		
201	5.38	0.71	0.13	5.58	14.65	2.63
212	8.75	1.80	0.21	2.40	1.64	0.68
321	16.70	2.92	0.17	1.21	1.02	0.84
331	3.85	2.96	0.77	8.13	3.11	0.38
424	14.40	1.89	0.13	2.21	2.28	1.03
807	7.64	0.77	0.10	5.04	8.55	1.60
813	6.58	2.09	0.32	4.58	4.81	1.05
821	6.11	0.77	0.13	3.64	8.42	2.31
Mean±SEM	8.68±1.60	1.74±0.33	0.25±0.08	4.10±0.79	5.56±1.65	1.32±0.28

Results of the eight paired competitive binding experiments presented in Table II were analyzed. Each dose-response curve was fit separately to a single site model using the LIGAND program (21).

stant, k_a, or an increase in the dissociation rate constant, k_d. When ^{125}I-labeled IGF-I was bound to patient's and control fibroblasts at 15°C, the dissociation at 15°C following resuspension in medium without tracer was slow (<30% in 6 h, results not shown). Dissociation at 37°C was more rapid and complete, and was indistinguishable for control and patient's fibroblasts (Fig. 1).

Specificity of ^{125}I-labeled IGF-I binding to control and patient's fibroblasts. The relative abilities of unlabeled IGF-I, MSA, and insulin to inhibit ^{125}I-labeled IGF-I binding to control and patient's fibroblasts were compared (Fig. 5). As expected, higher concentrations of unlabeled IGF-I were required to produce the same inhibition in patient's fibroblasts as in control cells. Unlabeled MSA was ~6% as potent as IGF-I as an inhibitor of binding in control fibroblasts. As with IGF-I, higher concentrations of MSA were required to produce equivalent inhibition in the patient's fibroblasts. Unlabeled insulin was ~7% as potent as IGF-I in inhibiting ^{125}I-labeled IGF-I binding to control fibroblasts. Surprisingly, the same concentrations of unlabeled insulin gave equivalent inhibition of binding to patient's and control fibroblasts. In patient's

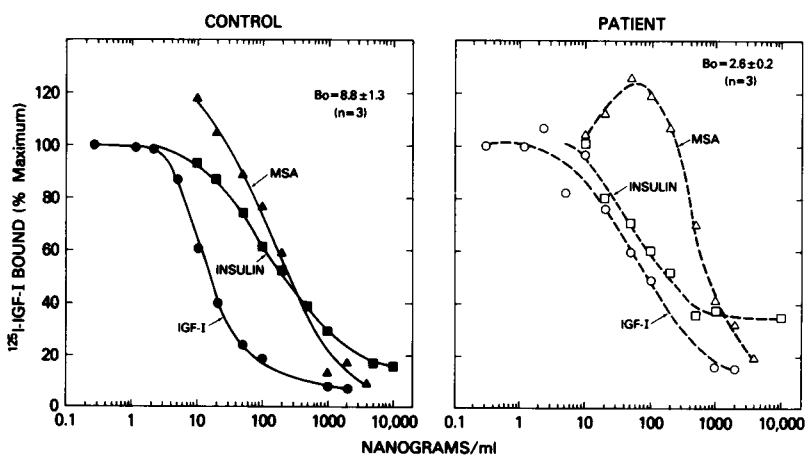

FIGURE 5 Inhibition of ^{125}I-labeled IGF-I binding to control fibroblasts (solid symbols) and patients fibroblasts (open symbols) by insulin and MSA. Results of three paired experiments (807, 813, and 821) are combined. Insulin (■, □); MSA II (▲, △); IGF-I (●, ○). Percentage of maximum ^{125}I-labeled IGF-I bound is plotted against nanograms per milliliter of peptide. Maximum binding/10^6 control fibroblasts was 8.8±1.3%; maximum binding/10^6 patient's fibroblasts was 2.6±0.2%. A small increase in binding of ^{125}I-labeled IGF-I was noted at low MSA concentrations, especially in patient's fibroblasts. This observation has not been examined further and its significance is unclear.

fibroblasts, insulin was ~50% as potent as IGF-I, significantly greater than its relative potency in control cells. Thus, the alteration in the patient's IGF-I receptor appears to decrease its affinity for IGF-I and MSA, but has little effect on its ability to recognize insulin.

DISCUSSION

In this report, we have characterized the binding of ^{125}I-labeled IGF-I to normal human fibroblasts in order to evaluate IGF-I binding in fibroblasts from a patient with leprechaunism. Under steady-state conditions at 15°C, binding of ^{125}I-labeled IGF-I to normal fibroblasts was specifically inhibited by unlabeled IGF-I, IGF-II, MSA III-2, MSA II, insulin, and proinsulin, in order of potency. The same peptides inhibit ^{125}I-labeled MSA binding to human fibroblasts (24). But, in contrast to ^{125} I-labeled IGF-I, which binds to a single receptor site, ^{125}I-labeled MSA (and ^{125}I-labeled IGF-II) appears to bind to two receptor sites in human fibroblasts (11). We used ^{125}I-labeled IGF-I as the radioligand for these comparative studies with the leprechaun patient's fibroblasts to avoid the complexity of binding to two receptor sites.

Binding of ^{125}I-labeled IGF-I to fibroblast cultures established from the leprechaun patient was decreased by 75% compared with fibroblasts from an age- and sex-matched control subject studied in the same experiments. The culture histories of patient's and control cells were virtually identical, and all manipulations (subcultivation, feeding) were performed in parallel. Results obtained with fibroblasts from this 7-d-old male control are quantitatively similar to results obtained with skin fibroblasts from a 21-yr-old female normal volunteer studied on several occasions during the same time period in which the experiments reported were being performed.

Decreased binding of IGF-I appears to be an intrinsic property of the leprechaun patient's cells and has been observed under different incubation conditions of temperature (15 and 37°C) and pH (7.4–8.0). The decreased binding in patient's cells does not arise from excessive degradation of ^{125}I-labeled IGF-I tracer, from failure to achieve steady state, or from excessive sensitivity of the patient's receptor to the trypsin-EDTA treatment used to suspend fibroblasts from the cell monolayer for binding experiments (Table III).

Decreased IGF-I binding to the patient's cells appears to be selective, and not simply a reflection of decreased cell surface area. (*a*) EGF binding to EGF receptors was indistinguishable for patients' and control fibroblasts examined at steady state at 37°C in the presence of 10 mM NH_4Cl to inhibit lysosomal degradation of EGF (22, 23) and with low concentrations of ^{125}I-labeled EGF to ensure that the observed B/F ratio was maximal. (*b*) Quantitative analysis of IGF-I binding data by computer curve-fitting assuming a single class of binding sites strongly suggests that the decreased binding to patient's fibroblasts can be accounted for by a decreased binding affinity (K_a) and not by a decreased receptor concentration per cell (R_0). (*c*) The ability of the IGF-I receptors on the patient's fibroblasts to recognize IGF-I and MSA is affected more severely than their ability to recognize insulin (Fig. 5). This qualitative difference in binding specificity is consistent with an alteration in the properties of the binding site, rather than a simple decrease in the number of sites.

MSA (3), IGF-I[5], and IGF-II[5] stimulate glucose incorporation in control fibroblasts with a high potency (relative to insulin), which suggests that they act via one or more IGF receptors (e.g., the IGF-I receptor or the second IGF receptor to which ^{125}I-labeled MSA and IGF-II also bind) and not via insulin receptors. The decreased affinity of the IGF-I receptor for IGF-I and MSA, however, is unlikely to completely account for the impaired stimulation of glucose incorporation by MSA in the leprechaun patient's fibroblasts, since high concentrations of MSA did not elicit a response of normal magnitude (3). Either MSA acts through the second IGF receptor and the concentration of this receptor is decreased in the leprechaun patient's fibroblasts, or the impaired biological response to MSA arises at least in part from a postbinding site alteration. At present it is not possible to evaluate independently whether the second IGF receptor is affected in the leprechaun patient's fibroblasts.

In summary, we have demonstrated four defects in fibroblasts from this patient with leprechaunism: (*a*) impaired insulin binding to insulin receptors, (*b*)reduced affinity of the IGF-I receptor for IGF and MSA, (*c*) decreased stimulation of glucose incorporation by insulin via insulin receptors, and (*d*) decreased stimulation of glucose incorporation by MSA via IGF receptors. Although these defects may have separate molecular and genetic bases, the possibility that these phenomena may be related also should be considered.

Several independent observations suggest possible relationships between insulin and IGF receptors, and their coupling to biological responses. (*a*) Human fibroblast insulin receptors (19, 20) and IGF-I receptors (Table I) interact with the same peptides (insulin, proinsulin, and the insulinlike growth factors), which suggests similarities in the two binding sites. (*b*) ^{125}I-labeled IGF-I incubated with human fibroblasts (25, 26) and covalently crosslinked with disuccinimidyl suberate forms an IGF-I: receptor complex with the specificity of an IGF-I receptor, but with subunit size

[5] Knight, A. B., and M. M. Rechler. Unpublished results.

(mol wt 130,000 reduced) and aggregation properties (mol wt > 300,000, without reduction) indistinguishable from those of an insulin receptor. The subunit of mol wt 130,000 may contain both insulin and IGF-I binding sites, or, alternatively, the binding sites for the two hormones may be on separate but similar molecules. (*c*) In the leprechaun patient's fibroblasts, insulin receptor-mediated and IGF receptor-mediated glucose incorporation are impaired, whereas insulin receptor-mediated and IGF receptor-mediated amino acid transport are spared (3). This led us to propose that the two receptors use common mechanisms of coupling to different effector pathways and that the lesion in the patient's cells might affect a common coupling component closely linked to both insulin and IGF binding sites. (*d*) In rat adipocytes, physiological concentrations of insulin acting through insulin receptors increases the affinity of the adipocyte MSA receptor for MSA (27). If similar interactions were to occur in other cell types, they would provide a mechanism by which a primary defect in the insulin receptor might affect the binding affinity of an IGF receptor.

It remains to be determined whether IGF-I receptors are affected in other patients with leprechaunism. These patients exhibit considerable heterogeneity in the apparent mechanisms for insulin resistance. In our patient (1–3) and one other (28), a primary cellular defect of insulin binding has been described. In other patients, defective insulin binding in circulating cells (which suggests a secondary defect) or normal insulin binding but defective responsiveness to insulin (which suggests a postreceptor defect) have been reported (4, 5, 29). Identification of an IGF-I receptor defect in our patient's fibroblasts reopens the question whether some of the clinical manifestations of leprechaunism (especially intrauterine and neonatal growth retardation, poorly developed muscles, and absent fat) might result from a deficiency of IGF biological effect mediated through the IGF receptor.

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