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## Immunoreactive growth hormone receptor/binding protein is present on fibroblasts and in serum of Laron-type dwarfs <sup>\*</sup>

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### Summary

Laron-type dwarfism is an autosomal recessive disorder characterised by extreme growth retardation and growth hormone (GH) resistance and has been shown in some cases to be associated with mutations in the GH receptor gene. Limited data suggest that in this condition specific liver GH binding is absent. In the majority of reported cases specific GH binding is also absent in serum. However it is not known whether the GH receptor and/or the serum GH binding protein are expressed in this condition.

Using the techniques of immunohistochemistry and Northern blotting we have demonstrated that in cultured skin fibroblasts derived from four patients with Laron-type dwarfism the GH receptor gene is transcribed and the GH receptor protein is expressed on the cell surface. Further study of one of these patients, who has not previously been reported, has also revealed low but detectable levels of GH binding protein in serum using a two-site immunoradiometric assay which does not depend on GH binding.

These results indicate that the growth hormone receptor/binding protein is expressed in Laron-type dwarfism.

### Introduction

Laron-type dwarfism is an autosomal recessive condition characterised by extreme growth retardation and apparent GH resistance (Laron et al., 1966). Affected children have an appearance typical of isolated GH deficiency but GH levels in plasma are found to be elevated and GH secretory responses to insulin-induced hypoglycemia and arginine infusion are normal or exaggerated (Laron et al., 1968, 1972). The GH present in plasma reacts normally in a GH radiorecep-

tor assay suggesting that it is biologically active (Jacobs et al., 1976) and plasma IGF-I levels are very low and do not respond to GH administration (Daughaday et al., 1969). An underlying receptor defect in Laron-type dwarfism is suggested by the absence of specific hepatic microsomal GH binding activity in two patients (Eshet et al., 1984) and the absence of specific serum GH binding activity in most cases described (Daughaday and Trivedi, 1987; Baumann et al., 1987). This binding activity in serum has been shown to be due to a protein which has extensive homology to the extracellular binding domain of the GH receptor (Leung et al., 1987) and is thought to be a cleavage product of the GH receptor. Mutations affecting the GH receptor gene have been described in a number of patients with Laron type dwarfism (Godowski et al., 1989; Amselem et al., 1989, 1991; Berg et al., 1992). In vitro studies have confirmed GH resistance in erythroid and lymphoid cell lines derived from two patients (Geffner et al., 1987).

We have had the opportunity to examine in detail a new case of Laron-type dwarfism and to examine cul-

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tured fibroblasts from three previously described cases. In particular, we were interested to determine whether the GH receptor protein and the serum GH binding protein were expressed despite their inability to bind GH. We now report the presence of GH receptor protein and mRNA in cultured fibroblasts from four patients and the presence of immunoreactive GH binding protein in the one patient whose serum was available for study.

### Human subjects

Clinical details on Laron subjects 1, 2 and 3 and the basis for their diagnosis have previously been reported (Daughaday and Trivedi, 1987). GH receptor genomic studies have shown a normal hybridisation pattern in Laron subjects 2 and 3 and a 200 bp insertion in the 3' untranslated region in Laron subject 1 (Godowski et al., 1989), suggesting that major deletions are unlikely to be present. The genetic defect in Laron subject 4, a male now 10.3 years old, has not been characterised. He was born at 32 weeks gestation in India to parents who were first cousins and had Turkish and Pakistani ancestry. His birth weight was 2.7 kg and his birth length was not recorded. Apart from an episode of gastroenteritis requiring hospitalisation at the age of 3 months his general health was good and his developmental history was normal. Poor weight gain was evident by the age of 5 months. Hypopituitarism was diagnosed at the age of 2 years; however treatment was not available. At the age of 7.2 years he presented to the Royal Children's Hospital, Melbourne for assessment. Examination revealed a height of 78.6 cm (SDS -8.6) and weight of 10 kg which was 32 cm and 9 kg respectively below the 3rd centile for chronological age. Body proportions were normal and there were no dysmorphic features. His appearance was typical of isolated GH deficiency. Two male siblings were of normal height and there was no family history of short stature. Investigation with sequential arginine-insulin stimulation revealed a basal GH level of 18  $\mu\text{g/l}$  (reference range  $< 5 \mu\text{g/l}$ ) and an arginine-stimulated peak GH level of greater than 65  $\mu\text{g/l}$ . Following insulin GH decreased from 29  $\mu\text{g/l}$  to 15  $\mu\text{g/l}$  (nadir plasma glucose 2.0 mmol/l; maximum plasma cortisol 1010 nmol/l). The lack of additional response following insulin may be due to the large preceding arginine response. Published data in Laron subjects for isolated insulin stimulation have reported exaggerated, normal or subnormal GH responses (Laron et al., 1972). Following exercise GH increased from 7 to 22  $\mu\text{g/l}$ . Serum IGF-I levels (INCSTAR, Somatomedin C) in five separate samples over two days were  $< 10 \text{ nmol/l}$  (age/sex matched normals 6.5–24.8 nmol/l, mean 13 nmol/l,  $n = 8$ ). Thyroid and other serum investigations were normal. Bone age was assessed at 3.5 years and

skull X-ray was normal. At the age of 8.4 years therapy was commenced with methionyl human GH (Somatorm, Kabi Vitrum, Stockholm, Sweden) at a total weekly dose of 1 IU/kg given as 5 injections per week. This resulted in an increase in growth velocity from 2.1 cm/year in the year prior to GH therapy to 5.8 cm/year in the first year and 4.7 cm/year in the second year of therapy. After 11 months of GH therapy, the serum IGF-I level (Royal Prince Alfred Hospital, Sydney) was 4.9 nmol/l (age related reference range 9–30 nmol/l). Forearm skin biopsy specimens from Laron subjects 1 to 4 were used to establish fibroblast cell lines.

### Methods

#### *Clinical methods*

Bone age was determined using published standards (Greulich and Pyle, 1962).

#### *Hormone assays*

GH was measured using the Pharmacia HGH-RIA 100 assay (Pharmacia Diagnostics AB, Uppsala, Sweden). Initial IGF-I measurements used the INCSTAR Somatomedin-C assay (INCSTAR Corporation, Stillwater, MN, USA). Subsequently IGF-I was assayed at the Royal Prince Alfred Hospital, Sydney, Australia, using a previously published radioimmunoassay method following removal of IGF binding proteins by acid-ethanol extraction (Baxter et al., 1982).

The GH radioreceptor assay was performed as previously described using pregnant rabbit liver microsomal membranes (Herington et al., 1974). hGH (NIAMDD-hGH-I-1) used for iodination was a gift of the National Hormone and Pituitary Program (NIADDK, National Institutes of Health, Bethesda, MD). hGH used for assay standards was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia.

#### *Serum GH binding studies*

Total specific GH binding activity in serum was determined using previously described methods (Herington et al., 1986). Total specific binding of GH to serum detects both high and low affinity GH binding sites including the GH binding protein. Iodinated GH was incubated with serum in the absence (total binding) or presence (non-specific binding) of an excess (0.1  $\mu\text{M}$ ) of unlabelled GH and bound and free hormone were separated by gel filtration using mini-columns.

#### *Gel filtration profiles*

Serum gel filtration studies were performed using a modification of a previously described method (Herington et al., 1986). For the control subject, 3.0 ml serum was applied to an Ultrogel AcA 34 column ( $2.6 \times 90$

cm) and specific GH binding activity was determined on 200  $\mu$ l aliquots of successive 7.5 ml eluted fractions. For Laron subject 4, the same method was used except that 1.0 ml serum was applied and successive pairs of 7.5 ml fractions were combined, dialysed, lyophilised and reconstituted in 2 ml buffer. 100  $\mu$ l aliquots were used for measurement of specific binding. Ultrogel AcA 54 mini-columns were used to separate bound and free fractions as previously described.

#### *GH binding protein two site immunoradiometric assay*

A more detailed description and validation of the GH binding protein assay will be published elsewhere (M.J. Waters, manuscript in preparation). Mouse anti-GH receptor monoclonal antibody MAb 263 was used as the solid phase antibody and MAb 43 or MAb 5 was used as the labelled antibody. The production and characterisation of these antibodies (Barnard et al., 1984, 1985) and their reaction with the human serum GH binding protein (Barnard et al., 1989) has been described elsewhere. Glutaraldehyde-activated 96-well microtitre plates were incubated with MAb 263 (100  $\mu$ l of a 100 mg/l solution in 0.1 M NaHCO<sub>3</sub>), blocked with BSA and rinsed in PBS/0.5% Tween 20. For assay, 10  $\mu$ l of normal male rat serum diluted 1:5 in 2.5% BSA/PBS containing 300  $\mu$ g/l of hGH was added to the wells, followed by 100  $\mu$ l of sample (diluted in 2.5% BSA/PBS, or undiluted) or standard (recombinant human GHBP made in *E. coli*, a gift of Genentech, South San Francisco, CA) also diluted in 2.5% BSA/PBS. Plates were incubated for 24 h at 4°C, then rinsed six times in PBS/0.05% Tween 20. <sup>125</sup>I-labelled MAb 5 or 43 (100 000 cpm, 15–70  $\mu$ Ci/ $\mu$ g by lactoperoxidase method) diluted in fresh PBS (pH 7.4)/0.05% Tween 20/4% polyethylene glycol 6000 was then added to the wells in 100  $\mu$ l volume, and the plates incubated for a further 48 h at 4°C. Plates were finally rinsed six times in PBS/0.05% Tween 20, tap dried and wells counted on a gamma spectrometer (LKB 1274).

#### *Tissue culture*

Fibroblasts were obtained by primary culture of forearm skin biopsies in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and containing penicillin 100 IU/ml, streptomycin 100 mg/l, gentamicin 8 mg/l and amphotericin B 2.5 mg/l. Cells were grown in 75 cm<sup>2</sup> flasks (Linbro, Flow Laboratories, Australia) in a 5% CO<sub>2</sub>/room air atmosphere at 37°C and maintained by passage at a 1:4 split ratio. Laron fibroblasts were matched for age and sex with control fibroblasts derived from genital skin specimens of normal healthy children obtained at circumcision.

#### *Immunocytochemistry*

Laron and control fibroblasts of passage 6–10 were grown to subconfluent density in slide chambers (Labtek, Nunc, Naperville, IL) and washed in phosphate buffered saline (PBS) prior to fixation at room temperature for 30 min in 4% formaldehyde/PBS. The primary antibody used for immunohistochemistry was a mouse monoclonal anti GH receptor antibody, MAb 263 (Barnard et al., 1985), used at a concentration of 18 mg/l. This monoclonal antibody was raised against rat liver membrane GH receptor but it also recognises the human GH receptor, as evidenced by immunoprecipitation of solubilised, <sup>125</sup>I-labelled IM-9 lymphocyte GH receptor (Asakawa et al., 1986), and the human serum GH binding protein (Barnard et al., 1989). An unrelated anti-Brucella monoclonal antibody was used as a control antibody at the same protein concentration. The Vectastain (Vector Laboratories, Burlingame) avidin-biotin-peroxidase kit and protocol was used for immunostaining. Slides were then haematoxylin counterstained and mounted.

#### *Northern blotting*

Poly A+ RNA was prepared from four Laron and five age and sex matched control fibroblast cell lines and Northern blotting, probe labelling, hybridisation and washing performed as previously described (Oakes et al., 1992). Rabbit liver total RNA was used as a positive control. The filter was initially probed with the <sup>32</sup>P-labelled 847 bp human GH receptor cDNA clone pghr 501.1 (Leung et al., 1987), a gift from Dr. W.I. Wood (Genentech, South San Francisco, CA). The filter was then stripped and reprobed with a <sup>32</sup>P-labelled 193 bp control rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Piechaczyk et al., 1984) to check for equivalent loading, used with kind permission of M. Piechaczyk (Laboratoire de Biologie Moléculaire, USTL, Montpellier, France). The GAPDH probe was labelled to a lower specific activity of  $2 \times 10^5$  cpm/ $\mu$ g DNA by inclusion of cold CTP in the reaction mixture. The filter was washed at room temperature in  $2 \times$  SSC/0.1% SDS, then at 42°C in  $0.1 \times$  SSC/0.1% SDS for 30 min and exposed to photographic film.

## **Results**

#### *GH radioreceptor assay*

Serum from Laron subject 4 was examined by a previously published method (Herington et al., 1974) and was found to dilute out in a manner parallel to human GH (data not shown). This indicates that the endogenous GH in this patient's serum competed equally with human pituitary GH for rabbit liver membrane binding sites. This data excludes inability of

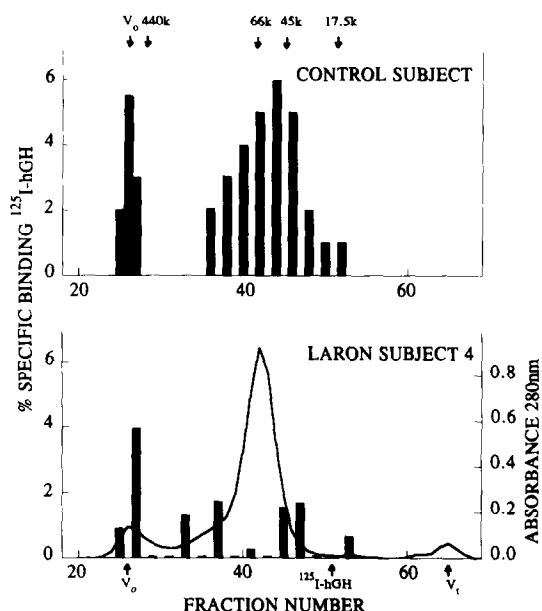


Fig. 1. Gel filtration profile of specific GH binding activity in serum from Laron subject 4 (lower panel) and a control subject (upper panel). Aliquots of successive fractions from a 2.5 × 90 cm Ultrogel AcA 34 column were incubated with <sup>125</sup>I-GH in the absence (total binding) and presence (nonspecific binding) of an excess (0.1 μM) of unlabelled GH. Bound and free fractions were separated using AcA 44 minicolumns (Herington et al., 1986). Absorbance at 280 nm (solid line) indicates relative protein content of column fractions.

endogenous GH to bind to its receptor as the cause of the apparent GH resistance in this Laron subject and is in agreement with previously published data from other Laron subjects (Jacobs et al., 1976).

#### Total serum GH binding activity and gel filtration profile

Total specific GH binding activity in serum from Laron subject 4 and an age and sex matched control was 0.36% and 4.79% respectively. After adjustment of the control serum to the GH concentration present in Laron subject 4 (45 μg/l), control total specific GH binding was 3.46%. The gel filtration profile of specific GH binding activity seen in Laron subject 4 and in an age matched control subject is shown in Fig. 1. In the control serum a broad peak of specific binding is seen in the molecular weight range 60 to 70 kDa due to the GH binding protein. In the Laron subject this peak is absent with low and inconsistent specific binding in these fractions. In contrast, the high molecular size GH binding activity was found in both the control and Laron serum. This peak is consistent with the presence of the very low affinity 'peak 1', non-GH receptor related binding protein (Herington et al., 1986; Baumann and Shaw, 1990). These findings are consistent with previous studies of serum GH binding in Laron dwarfs (Daughaday and Trivedi, 1987; Baumann et al., 1987; Rosenbloom et al., 1990).

#### GH binding protein immunoassay

With MAb 263 as the solid phase antibody and MAb 5 as the labelled antibody, GHBP concentration in Laron subject 4 was at the limit of sensitivity of the assay, i.e. < 1 μg/l. This value was < 7% of the level in adult sera (15.2 ± 1.3 μg/l, mean ± SE, n = 5), and < 19% of the level in age-matched controls (5.3 ± 0.6 μg/l, mean ± SE, n = 6). With this combination of MAbs, the intra assay CV was 5.1% and 5.8% for high

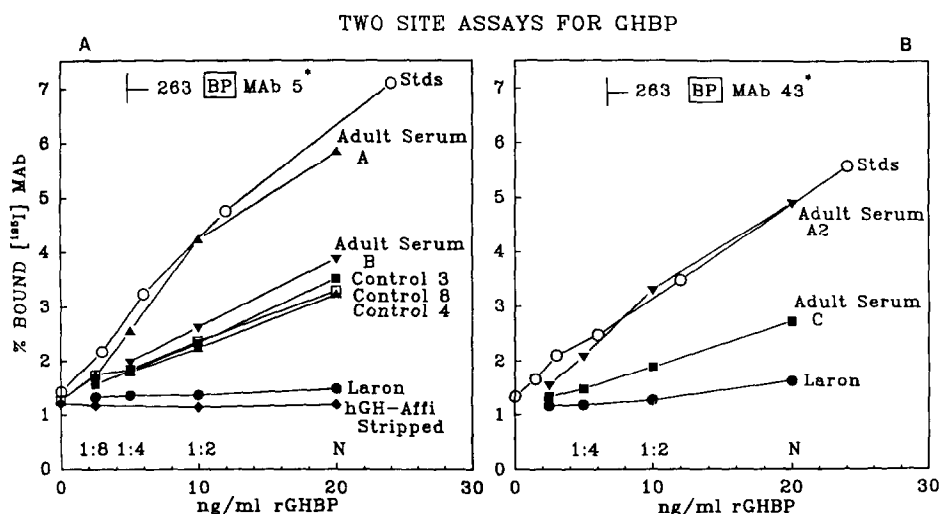


Fig. 2. Standard curves for human GH binding protein immunoradiometric assay. Solid phase capture antibody was MAb 263. Radiolabelled antibodies were MAb 5 (Fig. 2A) and MAb 43 (Fig. 2B). Recombinant human GH binding protein standard is plotted in ng/ml and human sera are plotted as serial dilutions from neat (N) to 1:8. Shown are normal adult sera (A, B, A2, C), normal control sera from children-age matched to subject 4 (controls 3, 4, 8) and serum from Laron subject 4. Also shown is normal adult serum A following removal of GH binding protein by hGH affinity-stripping. Binding protein is detectable in controls with both assay configurations. In Laron subject 4 binding protein is only detectable with MAb 43 as labelled antibody (Fig. 2B).

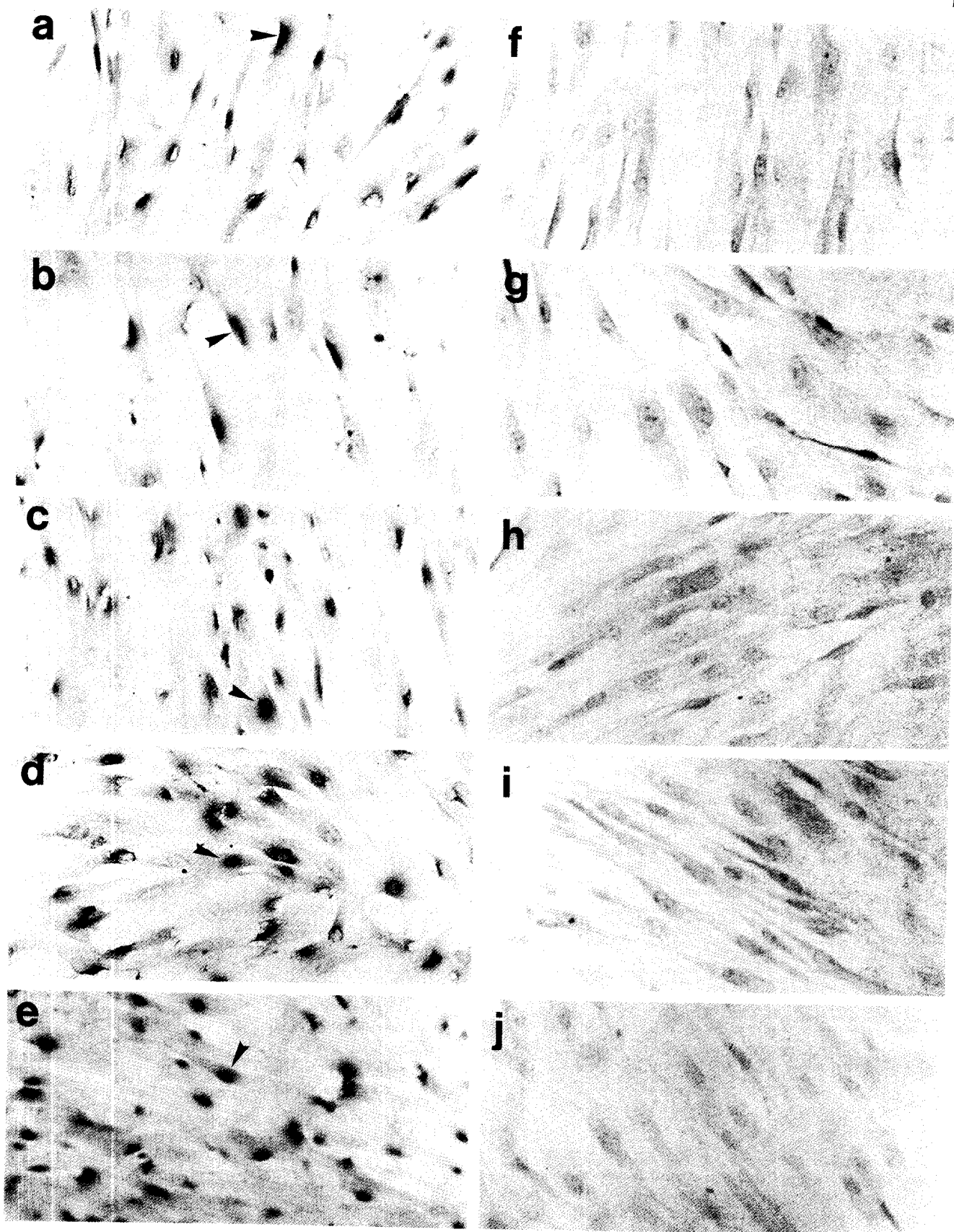


Fig. 3. Immunohistochemical demonstration of GH receptor antigen on cultured human skin fibroblasts. Staining of fibroblasts (indicated by arrows) from Laron subjects 1, 2, 3 and 4 (a, b, c and d) is similar to that seen in normal fibroblasts (e). Monolayer cultures were formalin fixed, washed and then immunostained using MAb 263 as primary antibody and the Vectastain avidin-biotin-peroxidase detection procedure. Control antibody staining was negative in Laron (f-i) and normal (j) fibroblasts (magnification  $\times 80$ ).

and low control sera, respectively. Assay standard curve is shown in Fig. 2A.

With the assay configuration using MAb 43 as labelled antibody, however, GHBP was detectable at a low level in Laron subject 4. This concentration of  $1.5 \pm 0.3 \mu\text{g/l}$  (mean  $\pm$  SE of 3 separate assays), based on the recombinant GHBP standard, was approximately 14% of adult GHBP levels ( $10.5 \pm 1.4 \mu\text{g/l}$ , mean  $\pm$  SE,  $n = 9$ ), and 24% of the level in age matched controls. With this combination of MAbs, the intra assay CV was 6.0%. Assay standard curve is shown in Fig. 2B.

#### Immunohistochemistry

Positive immunohistochemical staining for GH receptor was obtained in fibroblasts from Laron subjects

1, 2, 3 and 4 (Fig. 3a, b, c and d, respectively). The staining was highly localised to one region of the cell membrane and was similar to that seen in normal control fibroblasts (Fig. 3e). This suggests that in the four Laron subjects studied immunoreactive GH receptor is present on fibroblast cell membranes. The pattern of staining has been described and discussed previously (Oakes et al., 1992) and is consistent with 'capping' of surface membrane GH receptor accentuated by the double antibody detection procedure. Staining with a control antibody was negative in both Laron fibroblasts (Fig. 3f–3i) and normal fibroblasts (Fig. 3j).

#### Northern blotting

In support of the immunohistochemical evidence of the presence of GH receptor on Laron fibroblasts, Northern blots of mRNA prepared from the same cell lines showed a single strongly hybridising species of approximately 5.2 kb (Fig. 4). Control cell lines showed the same mRNA species. Allowing for the variation in sample loading (as indicated by the GAPDH hybridisation pattern) there was considerable variation in message abundance in both normal and Laron cell lines. However, the least abundant and most abundant message levels were in Laron cells. Laron cells also showed some heterogeneity in message size. Control rabbit liver total RNA showed the expected mRNA species of 4.6 and 3.2 kb (Tiong et al., 1989).

#### Discussion

We have examined cultured fibroblasts from four patients with Laron-type dwarfism for expression of the GH receptor gene. We have demonstrated that the GH receptor gene is transcribed into mRNA and that receptor protein is produced and expressed on the cell membrane. Previous studies of GH receptors in patients with Laron-type dwarfism have demonstrated absent specific GH binding in liver microsomes (Eshet et al., 1984) and reduced GH responsiveness of peripheral blood erythroid and lymphoid cell lines (Geffner et al., 1987). Our data is consistent with the hypothesis that GH resistance in this condition is due to the expression of a non-functional GH receptor protein.

Specific GH binding activity in serum has been previously reported to be absent in Laron subjects 1, 2 and 3 (Daughaday and Trivedi, 1987) and is very low in subject 4 (10% of an age matched control). Using a two site immunoradiometric assay, which is not dependent on the ligand binding activity of the GH binding protein, we have found reduced but detectable levels in the serum of Laron subject 4. This suggests that in at least some patients with the syndrome of Laron type dwarfism the GH binding protein, like the GH receptor, is expressed albeit at a low level. A priori, it might

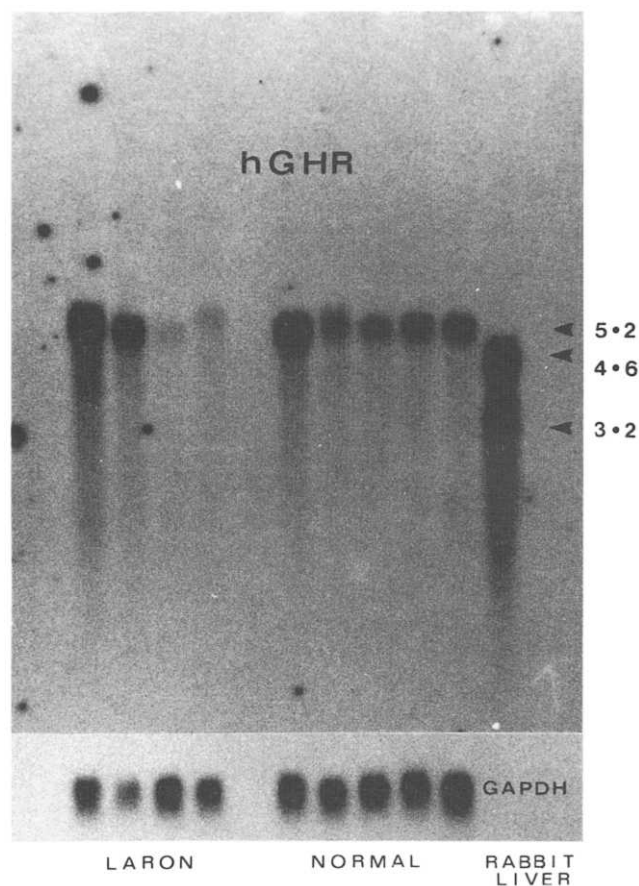


Fig. 4. Northern blot analysis of poly A<sup>+</sup> RNA from Laron and normal fibroblasts (15  $\mu\text{g/lane}$ ) and control female non-pregnant rabbit liver total RNA (10  $\mu\text{g/lane}$ ) probed with a <sup>32</sup>P-labelled 847 bp human GH receptor cDNA clone pghr 501.1. From left to right, Laron cell lines are from subjects 1, 2, 3 and 4. A single hybridising species of 5.2 kb is seen in all fibroblast cell lines. Rabbit liver shows two species of 4.6 and 3.2 kb. The same blot was stripped and reprobed with a <sup>32</sup>P-labelled 193 bp glyceraldehyde-3-phosphate dehydrogenase cDNA to indicate equivalence of RNA loading.

be anticipated that normal levels of a non-functional GH binding protein might be present in Laron patients. The finding of a low level may indicate that GH receptor binding is required for generation of the GH binding protein. Carlsson et al. (1991) have reported undetectable GH binding protein in two Laron subjects; however, this assay used is absolutely dependent on the ability of the binding protein to bind GH and thus is theoretically unsuitable for the detection and measurement of binding protein when the ability to bind GH has been lost. The low level of specific GH binding activity found in subject 4 is consistent with levels reported in other Laron subjects (Rosenbloom, 1990). When the low binding activity in subject 4 is considered in conjunction with the low level of immunoreactive GH binding protein detected in this patient it is apparent that no conclusion can be drawn regarding the functional activity of his GH binding protein. The absence of serum GH binding activity in Laron dwarfism has been considered to reflect defective GH receptor binding. The data obtained in Laron subject 4 suggest that caution should be exercised in drawing conclusions as to likely GH receptor binding activity based on the demonstration of low or absent serum GH binding activity in this condition, especially as the origin of the binding protein in humans remains unclear.

The mutations present in these patients are not yet known. However, it is evident that they do not prevent the processing and membrane insertion of the GH receptor, and at least in Laron subject 4, the export of the GH binding protein. Genomic studies have shown a normal hybridisation pattern in Laron subjects 2 and 3 and a 200 bp insertion in the 3' untranslated region in Laron subject 1 (Godowski et al., 1989), suggesting that major deletions are unlikely to be present. The finding of membrane expression of the GH receptor in Laron fibroblasts contrasts with a previous study (Duquesnoy et al., 1991) which suggests that a Phe<sup>96</sup> to Ser mutation detected in a patient with Laron dwarfism prevents normal processing and plasma membrane insertion of the GH receptor in COS-7 cells. Other workers however have not found any difference in membrane expression between the wild type and Phe<sup>96</sup> to Ser mutant GH receptor (Edery et al., 1993).

Laron subject 4 shows the typical clinical features of Laron-type dwarfism with typical laboratory evidence of GH resistance. He was treated with high doses of GH (1 U/kg/week) which resulted in an increase in growth rate from 2.1 to 5.8 cm/year in the first year. This response, which represents a growth velocity around the 10th centile for age, is considerably less than that seen in patients with isolated GH deficiency, who typically achieve growth velocities of 8–10 cm/year in similar doses of GH given thrice weekly (Milner et al., 1979, 1987; Kaplan et al., 1986). However, the

growth response does indicate that the resistance to GH in this patient is incomplete and may be partially overcome using a high GH dose. Previously reported growth responses to GH in Laron patients have ranged from no response to 2–3-fold increases in growth velocity (Laron et al., 1971). This may reflect variable severity of and/or heterogeneity in the nature of the underlying defect. The ability of GH in Laron subject 4 to elicit a growth response at high GH doses may indicate reduced receptor affinity or a partial signal transduction response.

It is of interest that the Laron fibroblasts showed considerable variation in GH receptor message abundance compared to normal fibroblasts. As all cell lines were grown in the absence of human GH this variation in mRNA abundance cannot be attributed to possible regulatory disturbances resulting from altered interaction between GH and its receptor. The variation may reflect altered mRNA stability or disturbance of an unknown intrinsic regulatory mechanism; however, undetected differences in growth conditions between cell lines cannot be excluded. It is of interest that despite well documented effects of GH to both increase (Baxter et al., 1984; Maiter et al., 1988b) and acutely decrease (Maiter et al., 1988a) the number of GH binding sites in liver, GH has little or no acute effect on hepatic GH receptor mRNA levels (Tiong and Herington, 1991; Mathews et al., 1989). Similarly, in cultured fibroblasts, GH acutely decreases GH binding sites (Murphy et al., 1983) without affecting GH receptor mRNA levels (S. Oakes, unpublished data). This suggests that regulation of GH receptor number by GH may not occur at the level of gene transcription.

No correlation was evident across the four Laron cell lines between the abundance of GH receptor mRNA and the intensity of immunochemical staining. The significance of this is not clear as the linearity of the enzymatically linked immunochemical detection system with respect to antigen concentration and the effect of possible antigenic heterogeneity on epitope recognition by MAb 263 has not been established. Similarly, the significance of both low fibroblast GH receptor mRNA abundance and low immunoreactive GH binding protein in Laron subject 4 is uncertain given the possibility of antigenic differences and altered GH binding affecting the reactivity of the binding protein in the immunoassay.

*In summary*, we have demonstrated that GH receptor protein is expressed in cultured Laron fibroblasts and that GH binding protein measured by a ligand-independent immunoassay is present, albeit at a low level, in a Laron patient with deficient serum GH binding activity. Examination of GH receptor and binding protein expression in cases of Laron dwarfism provides additional information about the GH receptor defect in this heterogeneous syndrome.

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