CLINICAL STUDY

IGFs and IGF-binding proteins in short children with steroid-dependent nephrotic syndrome on chronic glucocorticoids: changes with 1 year exogenous GH

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Abstract

Objective: Children with steroid-dependent nephrotic syndrome (SDNS), despite being in remission on glucocorticoids, continue to have growth retardation and short stature. The mechanism is uncertain as both chronic glucocorticosteroids and the nephrotic syndrome may independently affect growth. We investigated the changes in the IGFs and IGF-binding proteins (IGFBPs) in a group of short SDNS children, and studied the changes prospectively with 1 year’s treatment with GH.

Design and Methods: Total and ‘free’ IGF-I, IGFBP-3 and acid-labile subunit (ALS) were studied in eight SDNS boys (mean age 12.6 years; mean bone age 9.1 years) on long term oral prednisolone (mean dose 0.46 mg/kg per day) before, during, and after, 1 year’s treatment with GH (mean dose 0.32 mg/kg per week). Pretreatment comparisons were made with two control groups, one matched for bone age (CBA; mean bone age 9.2 years), and another for chronological age (CCA; mean chronological age 13 years). Subsequently, three monthly measurements of serum and urine IGFBPs were carried out in the GH-treated SDNS patients using Western ligand blot and Western immunoblot.

Results: Pre-treatment serum total IGF-I levels and the IGF-I/IGFBP-3 ratio were elevated significantly in SDNS compared with CBA, and were similar to CCA. Serum free IGF-I levels were elevated significantly compared with both control groups, but serum IGFBP-3 did not differ significantly. Urinary IGFBP-2, IGFBP-3 and ALS were detectable in the SDNS children only. With GH treatment, IGF-I and IGFBP-3, but not IGF-II, increased significantly compared with pre-treatment values, and returned to baseline after cessation of GH treatment. Urinary IGFBPs did not change significantly with GH treatment.

Conclusions: There is persistent urinary loss of IGFBP-2, IGFBP-3 and ALS in children with SDNS in remission with growth retardation. However, the significant elevation in serum IGF-I suggests that glucocorticoid-induced resistance to IGF is the main factor responsible for the persistent growth retardation in these children. Exogenous GH was able to overcome this resistance by further increasing serum IGF-I.

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Introduction

Children with steroid-dependent nephrotic syndrome (SDNS) on treatment with chronic glucocorticoids may have persistent growth retardation despite being in remission (1). The mechanism by which this occurs is still poorly understood, as nephrotic syndrome and chronic systemic glucocorticosteroids may individually and independently interact and interfere with the process of growth (2, 3).

In animal models and in children with active nephrotic syndrome, urinary loss of insulin-like growth factor (IGF)-I and the insulin-like growth factor-binding proteins (IGFBPs) has been demonstrated in several studies, and this mechanism has been suggested as the cause of the growth retardation observed (4–6). However, growth retardation persists in some children despite effective treatment of the nephrotic state, and this has usually been attributed to the chronic glucocorticoids. The mechanism by which these may cause growth retardation in these children with nephrotic syndrome in remission and on chronic glucocorticoids has not been well studied. Some studies have suggested that chronic glucocorticoids inhibit the secretion of growth hormone (GH) at the hypothalamic-pituitary level, which would therefore cause slow
growth (3, 7, 8). However, despite this, serum levels of IGF-I, which are dependent on GH secretion, have been reported to be low, normal, or elevated in children on long term glucocorticosteroids (3, 9). Similarly, IGFBP-3, which is GH dependent, has also been reported as low, normal or increased in glucocorticoid-treated children (3, 9–12). Thus another mechanism has been postulated, namely that glucocorticosteroids may directly inhibit osteoblastic activity in bone and cartilage, inducing a state of IGF resistance affecting bone growth (10, 13, 14).

We recently demonstrated in a prospective study of eight SDNS children in remission on long term prednisolone, that exogenous high dose GH given for 1 year could increase growth velocity significantly (15).

In the present study, we compared the serum and urine IGFBPs in these children with two groups of normal control children, and describe the changes in serum IGFs and IGFBPs when they received GH treatment for 1 year.

**Subjects and methods**

**Subjects**

The individual details of the study patients, their prednisolone doses, and response to GH treatment, have been described in detail previously (15). Briefly, eight boys (mean chronological age 12.6 years, bone age 9.1 years) with well defined SDNS and short stature, who were all in steroid-dependent remission for at least 4 years prior to the commencement of GH treatment, participated in the study. All the patients were steroid dependent and were on daily or alternate day prednisolone only (0.46 mg/kg per day), with a mean height SDS of −1.4. All the patients had a decreased GH response of not >7 mg/l in response to insulin-induced hypoglycemia. Recombinant human GH (Novo-Nordisk, Denmark) was administered daily (0.32 mg/kg per week) for 1 year. Fasting serum and urine samples were collected before, during and after GH treatment. In response to GH, the patients had significant increases in mean height velocity from 3.7 cm/year to 9.4 cm/year, and mean lumbar bone mineral density from 0.50 g/cm² to 0.64 g/cm², but creatinine clearance did not change significantly.

Two groups of controls were recruited (Table 1): controls matched for bone age (CBA, n = 18; mean bone age 9.2 years) and controls matched for chronological age (CCA, n = 13; mean age 13.0 years).

The study was approved by the institutional and national review committees, and informed consent was obtained from the children and their parents. However, limitations were placed on the quantity of blood taken from the children for the purposes of measurements not essential for the monitoring of safety and efficacy parameters in the study.

**Assays**

Serum total IGF-I, IGF-II, and urinary IGF-I, were measured by immunoassay after acid-ethanol extraction, and serum IGFBP-3 and ‘free’ IGF-I were measured directly by immunoassay (Diagnostic Systems Laboratories, Webster, TX, USA). Each assay was carried out with all the samples in a single assay, and the intra-assay coefficients of variation were all <10%.

**Western ligand blots**

A 2 μl aliquot of serum or 100 μl urine were electrophoresed on 10% SDS–PAGE under non-reducing conditions, electroblotted to nitrocellulose, incubated with an equal combination of [125I]IGF-I and [125I]IGFBP-3 (Amersham, Aylesbury, Bucks, UK), and exposed to X-ray film for at least 5 days as described previously (16).

**Western immunobLOTS**

ImmunobLOTS were performed using a commercial enhanced chemiluminescence system (ECL) for visualization (Amersham, UK). Briefly, samples were subjected to 10% SDS–PAGE and electroblotted as

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Chronological age, bone age and serum levels of IGFs and IGFBP-3 in SDNS children and two control groups. Values are mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variables</strong></td>
<td><strong>Children with SDNS in remission, on steroids, before GH (n = 8)</strong></td>
</tr>
<tr>
<td>Mean chronological age (years)</td>
<td>12.6</td>
</tr>
<tr>
<td>Mean bone age (years)</td>
<td>9.1</td>
</tr>
<tr>
<td>Serum total IGF-I (μg/l)</td>
<td>368.7 ± 31.8*a</td>
</tr>
<tr>
<td>Serum total IGF-II (μg/l)</td>
<td>625.5 ± 41.4*a,b</td>
</tr>
<tr>
<td>Serum IGFBP-3 (μg/l)</td>
<td>2719.8 ± 197.0</td>
</tr>
<tr>
<td>Serum IGF-I to IGFBP-3 ratio</td>
<td>0.14 ± 0.01*a</td>
</tr>
<tr>
<td>Serum ‘free’ IGF-I (μg/l)</td>
<td>5.37 ± 0.94*a,b</td>
</tr>
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*aP < 0.05 compared with control A subjects. bP < 0.05 compared with control B subjects.

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The serum IGF-I/IGFBP-3 ratio, an index of bioavailable IGF-I, was significantly higher in the SDNS children in remission on prednisolone compared with bone age matched controls (Fig. 2A). This shows a Western ligand blot of urine from the SDNS patients (17) with a broad band at 37–41 kDa of urinary IGFBP-3 (best seen in lanes 5 and 7), and two bands at 34 and 28 kDa of urinary IGFBP-2. Urine from normal control children (lane 2, Fig. 2A) did not show any detectable bands on blots exposed for the same duration. Figure 2B shows a Western blot of SDNS urine after immunoprecipitation with antibodies to IGFBP-2 (aBP-2) and IGFBP-3 (aBP-3) confirming that the bands in SDNS children in remission are that of IGFBP-3 and IGFBP-2. Lane 3 shows a sample of pooled urine without immunoprecipitation from SDNS children deliberately overexposed to demonstrate correspondence with lanes 1 and 2. An additional lower molecular weight band at 24 kDa is seen in SDNS urine corresponding to the known molecular weight of IGFBP-4.

Finally, we studied serum and urine acid-labile subunit (ALS), which circulates normally in excess unbound in serum or as part of the 140 kDa ternary complex with IGFBP-3/IGF (18), using Western immunoblotting. Figure 3 shows a Western immunoblot of serum (Fig. 3A) and urine (Fig. 3B) from SDNS patients.

Statistics

All values of IGFs and IGFBP-3 measured by immunoassay were compared by two-factor ANOVA for repeated measurements with the general linear models procedure using the Statistical Analysis Systems package (version 6.0, SAS Institute).

Results

**IGF-I, IGFBPs, and ALS before treatment with GH**

All the SDNS patients were in remission on maintenance daily or alternate day prednisolone for at least 4 years prior to GH treatment. Table 1 shows the mean (±S.E.) serum levels of IGF-I and IGFBP-3 in the 6 months before GH treatment compared with the two control groups; group A, matched for bone age (CBA) and group B, matched for chronological age (CCA). Children with SDNS on prednisolone had significantly higher levels of serum total IGF-I compared with bone age-matched controls (P < 0.05), but had similar levels to chronological age-matched controls. By contrast, although mean serum IGFBP-3 levels were higher in the SDNS children, the differences between the three groups were not statistically significant.

The serum IGF-I/IGFBP-3 ratio, an index of bioavailable IGF-I, was significantly higher in the SDNS children than in the bone age-matched controls (P < 0.05), but did not differ significantly from the values in chronological age-matched controls. Similarly, serum ‘free’ IGF-I levels measured with a commercial immunoassay were significantly higher in the SDNS children compared with both the bone age-matched and the chronological age-matched controls (P < 0.05).

Further analysis using Western ligand blotting of the IGFBPs in the serum of patients with SDNS in remission on prednisolone is shown in Fig. 1 with serum from bone age-matched control subjects for comparison. The serum from five SDNS patients (Fig. 1, lanes 1–5) and four representative CBA control subjects (lanes 6–9) had two major bands with molecular weights of 37–43 kDa, corresponding to the well known glycosylated forms of serum IGFBP-3 seen on Western ligand blots. Other minor bands in the serum from control and SDNS patients with molecular weights of 33 kDa, 29 kDa and 24 kDa corresponded to IGFBP-2, -1 and -4 respectively. There was no obvious difference between the controls and the SDNS patients.

We also investigated the urinary excretion of IGFBPs in SDNS children in remission on prednisolone compared with bone age matched controls (Fig. 2A). Figure 1 shows a Western ligand blot of serum from representative SDNS children before GH treatment and bone age-matched controls (group A). Western ligand blot after SDS–PAGE on a 10% gel of individual samples of SDNS serum (lanes 1–5) and bone age-matched controls (lanes 6–8). Two major bands with a molecular weight ranging from 37 to 43 kDa, correspond to the glycosylated form of IGFBP-3. Other bands with molecular weight of 33 kDa, 29 kDa and 24 kDa correspond to IGFBP-2, -1 and -4 respectively. Comparison of arbitrary densitometry units for each IGFBP of SDNS patients and control group A showed no significant difference.
and controls. The typical doublet with molecular weights of about 87 kDa was present in the serum from control children (lanes 1–4, Fig. 3A) and serum from SDNS children in remission on prednisolone (lanes 5–9, Fig. 3A). There was no obvious difference between serum from SDNS children and the controls. An identical band at 87 kDa was also identified by immunoblotting of the urine from SDNS patients (lanes 2–5, Fig. 3B), which indicated that in addition to the excretion of the IGFBPs, there was also excretion of ALS in the urine from SDNS children in remission. This doublet was not detectable by immunoblotting in control pooled urine (lane 1, Fig. 3B).

**Changes in IGF-I and IGFBPs with GH treatment**

The children with SDNS on prednisolone received daily GH for 1 year, which resulted in statistically significant gains in height in them all (15). Figure 4 summarizes the changes of serum levels of total IGF-I, total IGF-II, IGFBP-3 and 'free' IGF-I, as measured by immunoassay, before, during and after treatment with GH. The mean serum IGF-I level of the eight SDNS children was significantly higher than before treatment \( P < 0.05 \) ANOVA, Fig. 4A). Six months after stopping the GH treatment (on the 18th month), the mean serum IGF-I level dropped back to pre-treatment levels \( P < 0.05 \); ANOVA Fig. 4A). By contrast, the changes in IGF-II were not statistically significant when comparing mean levels before, during, and after cessation of GH treatment \( P > 0.05 \); ANOVA Fig. 4A). The mean serum IGFBP-3 levels of the eight SDNS children also increased significantly during the treatment with GH \( P < 0.05 \) ANOVA, Fig 4B). On stopping GH treatment, the mean values decreased significantly and returned to pre-GH treatment values \( P < 0.05 \), Fig. 4B). Serum 'free' IGF-I levels, which were already significantly higher than...
controls, also showed a further significant increase during GH ($P < 0.01$ ANOVA, Fig 4B), and decreased back to pre-treatment values on stopping the treatment.

For comparison, Fig. 4C summarizes the changes in mean serum total, high-density lipid and LDL-cholesterol before, during, and after GH treatment in these children with SDNS on prednisolone. Although there was a rebound in serum cholesterol after stopping GH, which led to further additional measurements after the completion of the study, at 24 months the changes were not statistically significant ($P > 0.05$, ANOVA).

Figure 5 shows a Western ligand blot of pooled urine from the SDNS children undergoing GH treatment. Urinary excretion of IGFBP's continued throughout GH treatment (lanes 2–5), and did not show any obvious changes during this time.

Figure 5 Western ligand blot of urine from SDNS patients treated with GH. Western ligand blot after SDS–PAGE on a 10% gel of pooled urine (100 ml/lane) of eight SDNS children at each time point. Lane 1, before GH treatment; lanes 2–5, the 3rd, 6th, 9th and 12th month with GH treatment; lane 6, pooled urine from control group A.

Discussion

The pathophysiological mechanisms for persistent growth retardation in SDNS children on long term glucocorticoid treatment are complex and have not been well studied. In the present study, we have measured the serum IGF-I, IGFBPs and ALS in a group of eight children with SDNS on chronic prednisolone, and compared these with a control group of normal bone age-matched children and a second control group matched for chronological age. We then measured the changes in the IGFs and IGFBPs when GH was administered for 1 year to this group of children.

Our study demonstrated that serum levels of IGF-I in SDNS patients on prednisolone were significantly higher compared with bone age-matched controls despite their short stature. Serum IGF-I was assessed as serum total IGF-I, serum ‘free’ IGF-I and as the total IGF-I/IGFBP-3 ratio, and was significantly elevated in SDNS patients compared with bone-age matched controls. The ‘free’ IGF-I was elevated greatly in the SDNS children compared with bone-age matched controls and also the chronological age-matched controls. By contrast, serum IGF-II was elevated only in the SDNS children, and there was no difference between the two groups of normal control children. To our knowledge, this is the first report of a significantly elevated serum IGF-I in these children compared with bone age-matched controls. Our study therefore differs
significantly from some previous studies (19, 20) that have reported serum IGF-I levels that were low or low normal in children on long term glucocorticoids. Differences in assay methods for serum IGF-I, especially if a bioassay is used (19), may be a possible source of discrepancy; however, a more likely and important reason is that the study patients in previous studies often had a mixture of various diagnoses including those whose disease processes (juvenile arthritis, post-renal transplant, etc) have a significant effect on their physical activity or on their catabolic state. The patients in our present study had no restrictions on their physical activity and were generally healthy and well apart from their SDNS, and is the only study we are aware of on such a homogeneous group of patients.

Our results on ‘free’ IGF-I using a commercially available assay, while pointing in the same direction as total IGF-I and IGF-I/IGFBP-3 ratio, show very elevated values, and a difference in being higher than both control groups. This data is limited partly by the absence of a gold standard for ‘free’ IGF-I, and by our inability to measure and compare with ‘free’ IGF-I values using other reported methods such as ultrafiltration (21) or neutral gel chromatography, which require greater volumes of serum. Further studies may be necessary to confirm these elevated values of ‘free’ IGF-I.

However, our findings are consistent with a recent report from Miell et al. (10), who found that short term 4-day treatment with dexamethasone increased serum IGF-I levels but had no effect on IGF-II levels in normal male volunteers. Our present study shows that the elevation in IGF-I persisted even with long term glucocorticoid treatment in this group of relatively healthy and mobile children, and that a state of IGF-I resistance existed in these patients who continued to have short stature despite the elevated IGF-I levels.

In addition, our study showed that with exogenous GH treatment that increased growth velocity significantly for 1 year, the serum IGF-I and IGFBP-3 all increased significantly in SDNS patients. These elevations persisted as long as the exogenous GH was continued for the 12 months. By contrast, serum total cholesterol changed only transiently, and did not persist in its elevated state (Fig. 4). This also suggests that the increase in IGF-I did not have a detrimental effect on the nephrotic syndrome.

Our study may also help to clarify recent reports on the interaction between glucocorticoids and growth. The recent report from the large National Cooperative Growth Study showed that, whereas there was a general trend in that the higher the glucocorticoid dose the more severe the growth retardation, children on an identical glucocorticoid dose could have growth velocities ranging from 0 to 15 cm/year (22). Such a wide and varying range of growth velocities may be the result of the differing underlying pathologies in the study patients rather than the glucocorticoid dosage.

Our study would therefore emphasize that such a grouping of various disease states may not be helpful in studies on the pathological mechanisms of glucocorticoid-induced growth retardation and that better selection of patients is necessary and essential for any reliable conclusion to be reached. We have attempted this in the present study by having a uniform group of patients, and having two separate control groups, one matched for bone age and another for chronological age. Our results demonstrate for the first time that children with SDNS in remission continue to have some measurable IGFBPs and ALS in their urine compared with completely normal children. However, this did not lead to lower serum levels of IGFBPs, and in fact, the serum levels of total IGF-I, and the IGF-I/IGFBP-3 ratio which is an index of bioavailable IGF-I, and ‘free’ IGF-I are all elevated compared with the bone age-matched controls. This therefore supports our conclusion that in physically active children with SDNS, glucocorticoid-induced IGF-I resistance is the most likely cause of the persistent short stature.

In conclusion, our study documents the serum and urine levels of IGF-I and the IGFBPs in short children with SDNS in remission, and the changes when effective supraphysiological doses of exogenous GH are given for one full year with significant increases in growth velocity (15). It confirms that the pathophysiological mechanisms of glucocorticoid-induced growth retardation are complex and that further study with uniform homogeneous disease groups are necessary. Our study supports the hypothesis that glucocorticoid-induced IGF-I resistance is the likely mechanism for growth retardation, and that this can be overcome by higher doses of GH.

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References
6 Hafner D, Tonshoff B, Blum WF, Vickers M, Siebler T, Cronin MJ et al. Insulin-like growth factors (IGFs) and IGF binding proteins,
14 Gourmelen M, Girard F & Binoux M. Serum somatomedin/insulin-like growth factor (IGF) and IGF carrier levels in patients with Cushing’s syndrome or receiving glucocorticoid therapy. *Journal of Clinical Endocrinology and Metabolism* 1982 54 885–892.

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