Circulating non-22 kDa growth hormone isoforms in healthy children of normal stature: relation to height, body mass and pubertal development

Cesar L Boguszewski, Chatarina Jansson1, Margaret C S Boguszewski1, Sten Rosberg1, Kerstin Albertsson Wikland1, Björn Carlsson and Lena M S Carlsson

Research Centre for Endocrinology and Metabolism, Department of Internal Medicine, Sahlgrenska Hospital and 1International Pediatric Growth Research Center, Department of Pediatrics, Ostra Hospital, University of Goeteborg, Sweden

(Correspondence should be addressed to C L Boguszewski, RCEM-Department of Internal Medicine, Sahlgrenska University Hospital, Bruna Strand 16-S-413 45, Goeteborg, Sweden)

Abstract

The proportion of non-22 kDa GH isoforms was evaluated in 93 healthy children (48 boys aged 6.8–18.4 years and 45 girls aged 3.9–18.4 years) of normal stature (height ± 2 s.d. score) at different stages of puberty. In addition, correlations among the proportion of non-22 kDa GH isoforms, auxology, spontaneous GH secretion and biochemical measurements were investigated. Serum non-22 kDa GH levels, expressed as percentage of total GH concentration in the samples, were determined by the 22 kDa GH exclusion assay, in which monomeric and dimeric 22 kDa GH are removed from serum and the non-22 kDa GH isoforms are quantitated using a polyclonal antibody GH assay. Samples were selected from spontaneous GH peaks in 24-h GH profiles. For boys, the median proportion of non-22 kDa GH isoforms was 8.5% (range 3.2–26.6%) and for girls it was 9.6% (1.8–17.4%), with no influence of age and no sex-related difference in prepubertal (boys, 7.2%; girls, 8.8%) or pubertal children (boys, 9.1%; girls, 9.9%). However, the median proportion of non-22 kDa GH isoforms was significantly higher in pubertal boys (9.1%) than in prepubertal boys (7.2%; P = 0.03). In pubertal boys, height S.D. scores (SDS) were inversely correlated to the proportion of non-22 kDa GH isoforms (r = −0.38; P = 0.02), especially at mid-puberty (r = −0.7; P = 0.01), indicating that the presence of increased amounts of circulating non-22 kDa GH isoforms was associated with less growth. In prepubertal children, positive correlations between non-22 kDa GH and weight SDS (r = 0.46; P = 0.03), weight-for-height SDS (r = 0.51; P = 0.01) and body mass index (r = 0.42; P = 0.04) were observed. No significant correlations were seen with spontaneous GH secretion or measurements of IGF-1, IGF-binding protein-3, insulin and leptin. These findings in normal children indicate that the proportion of circulating non-22 kDa GH isoforms may have physiologic significance for growth and metabolism in different stages of development, and emphasize the importance of evaluating the circulating ratio of 22 kDa and non-22 kDa GH in children with growth disorders.

Introduction

Pituitary secretion of growth hormone (GH) is pulsatile and regulated mainly by two hypothalamic peptides, GH-releasing hormone (GHRH) and somatostatin. After secretion by the pituitary gland, part of circulating GH binds to a high-affinity GH-binding protein (GHBp) in human blood (1, 2); this GHBp shares homology with the extracellular domain of the GH receptor (GHR) (3). In target cells, GH induces homodimerization of the GHR. This dimerization occurs sequentially, such that site 1 on GH binds to one GHR molecule and then site 2 on GH binds to a second GHR molecule (4–6), leading to a signal transduction cascade involving JAK2 kinase and STAT proteins (7, 8).

Human GH is a complex mixture of different molecular isoforms (9–11). The most abundant form of the pituitary-expressed GH gene is the 22 kDa GH, but other monomers with molecular weights of 27 kDa, 20 kDa, 17 kDa and 5 kDa have been shown in pituitary extracts and in the circulation (12), and oligomers have been demonstrated as native molecules (9, 10). The results of several mutational studies have shown that different GH analogs and fragments may interact as weak agonists or antagonists of the GHR, depending on the relative affinities of their binding sites 1 and 2 to the GHR (13). Accordingly, the presence of such peptides lacking site 1 or site 2 of the GH molecule in the circulation may have physiologic implications for growth and metabolism.
We have recently developed the 22 kDa GH exclusion assay (GHEA), in which monomeric and dimeric 22 kDa GH are removed from serum and the non-22 kDa GH levels are measured by a polyclonal antibody-based IRMA (GH-IRMA) (14). In the present study, the GHEA was used to evaluate the proportion of non-22 kDa GH isoforms in serum samples from a group of healthy children of normal stature and to investigate possible relationships among non-22 kDa GH isoforms, auxology, spontaneous GH secretion and serum measurements of insulin-like growth factor-I (IGF-I), IGF-binding protein-3 (IGFBP-3), insulin and leptin.

Participants and methods

Study participants
A total of 93 children, comprising 48 boys and 45 girls, were investigated at the Children’s Hospital, Göteborg, Sweden. The chronological ages ranged from 6.8 to 18.4 years in boys and from 3.9 to 18.4 years in girls; their heights, converted into S.D. scores (SDS), were within ± 2 SDS compared with the Swedish Growth Reference Values (15). All the children were healthy and well nourished, their bone ages were within the normal range, and they had normal thyroid, liver and kidney functions. Celiac disease was excluded. Puberty was assessed according to Tanner for pubic hair and breast development (16) and testicular volume was determined by Prader orchidometer (17). According to testicular volume, the boys were grouped as prepubertal (volume 1–3 ml; n = 13) and pubertal (n = 35); those in the latter group were sub-divided into early puberty (volume 4–9 ml; n = 16), mid-puberty (volume 10–15 ml; n = 12) and late puberty (volume ≥ 16 ml; n = 7) groups. Of the 45 girls, ten were prepubertal (breast stage 1) and 35 were pubertal (n = 35), including nine at early puberty (breast stage 2 or 3, all premenarcheal), ten at mid-puberty (breast stage 3, postmenarcheal; or breast stage 4, premenarcheal) and 16 at late puberty (breast stage 4 or 5, postmenarcheal). Table 1 shows the clinical characteristics of the children included in the study. Weight and weight-for-height were converted into SDS, using the Swedish Reference Values for healthy children (15), and body mass index (BMI) was calculated as weight/height² (kg/m²). The study was approved by the Ethics Committee of the Medical Faculty, University of Göteborg. Informed consent to inclusion in the study was obtained from the children and their parents.

Study protocol (spontaneous 24-h GH profiles)
The children stayed at the hospital for at least 24 h, during which time they received an ordinary diet and were allowed normal activity and sleep. A heparinized needle was inserted and blood sampling was initiated between 0800 and 0900 h, using a constant withdrawal pump (Swemed, Göteborg, Sweden) with a non-thrombogenic catheter (Carmeda AB, Stockholm, Sweden), according to a previously described method (18). The heparinized tubes were changed every 20 or 30 min over 24 h, giving a total of 48 or 72 samples per child. The 24-h GH profiles were analyzed with the Pulsar program (19) with the previously described setting for GH (20), giving the calculated baseline, number of peaks, peak amplitudes and area under the curve (AUC). The AUC was used to calculate the amount of secreted GH with the simplified formula derived from the deconvolution technique (20).

GH concentrations were measured using a polyclonal GH-IRMA (Pharmacia & Upjohn, Uppsala, Sweden). The samples were analyzed either with the WHO International Reference Preparation for human GH (hGH) (IRP 66/217) or with the First WHO IRP 80/505. A conversion factor of 1.55 was used to transform the values from the IRP 66/217 to the 80/505 system (21). Serum samples had been stored for 4.1 years (0.4–9.6 years) before analyses, and the proportion of non-22 kDa GH isoforms was not correlated to the storage time (data not shown).

22 kDa GH exclusion assay
The GHEA, an immunomagnetic-extraction-based assay for quantitation of non-22 kDa GH isoforms in serum, was recently described in detail (14). Briefly, a 100 µl aliquot of serum is incubated with an anti-22 kDa GH monoclonal antibody (MCB, Genentech Inc., South San Francisco, USA) (22). Paramagnetic beads coated with rat anti-mouse IgG, and a magnetic device (Dynal, Oslo, Norway), are used to remove monomeric and dimeric 22 kDa GH from serum. After extraction, the non-22 kDa GH levels are measured by a polyclonal antibody-based GH-IRMA (Pharmacia & Upjohn). In the GHEA, total GH concentrations are determined in another 100 µl aliquot of serum incubated only with assay buffer (without addition of MCB), and non-22 kDa GH levels are expressed as percentage of the total GH in the samples. The GHEA was systematically optimized by statistical experimental designs (23, 24), which were used to predict its performance at the 95% confidence interval in GH concentrations up to 50 µg/l (approximately 130 mU/l). The sensitivity of the assay is 0.02 µg/l (0.05 mU/l) and the intra-assay coefficient of variation (CV) for 22 kDa GH extraction is less than 1.5% (14). In the present study, the interassay C.V. for 22 kDa GH extraction was less than 3% as determined in two different serum pools with total GH concentrations ranging between 23.3 and 34.5 mU/l and between 6.4 and 7.7 mU/l respectively.

The GHEA was applied to a single sample collected at the time of a GH secretory peak observed during 24-h profiles from 30 children. In view of the volume of serum required for the assay (200 µl), pools consisting of two or three peak samples with similar GH concentrations were prepared from 49 and 14 children, respectively.
Biochemical assays

Serum levels of IGF-1 were determined by an IGFBP-blocked radioimmunoassay (RIA) without extraction and in the presence of an approximately 250-fold excess of IGF-II (Mediagnost GmbH, Tübingen, Germany) (25). Serum IGFBP-3 levels were measured by a previously reported RIA (Mediagnost GmbH) (25). Serum insulin concentrations were measured by RIA (Pharmacia & Upjohn). Serum levels of leptin were determined by RIA (Human Leptin RIA kit, Linco Research Inc., St Charles, MO, USA).

Statistical analysis

The descriptive statistical results are presented as the median and range, unless otherwise stated. The Kruskal–Wallis test was performed for comparisons among groups. Comparisons between two groups were performed using the non-parametric Mann–Whitney U test and correlations were sought by calculating the non-parametric Spearman rank correlation coefficient (Spearman’s rho, denoted ‘r’ in the correlations).

Results

Non-22 kDa GH isoforms in relation to age and sex

The proportion of non-22 kDa GH isoforms determined by the GHEA in all children is shown in Fig. 1, according to their age. No correlation was observed between the proportion of non-22 kDa GH isoforms and age. The median proportion of GH isoforms in all boys (8.5%; range 3.2–26.6%) did not differ from that in all girls (9.6%; range 1.8–17.4%).

Non-22 kDa GH isoforms in relation to puberty

The median proportion of non-22 kDa GH isoforms was similar in boys and girls both at prepubertal stage (7.2%
compared with 8.8% respectively) and at pubertal stage (9.1% compared with 9.9% respectively). The median proportion of non-22 kDa GH isoforms was increased in pubertal children (9.6%) compared with the prepubertal ones (8.1%; \( P = 0.02 \)). The median value was significantly higher in pubertal boys (9.1%) than in prepubertal boys (7.2%; \( P = 0.03 \)), whereas the median was similar in girls before (8.8%) and during puberty (9.9%; \( P = 0.1 \)). As shown in Fig. 2, the median proportion of non-22 kDa GH isoforms did not vary greatly during pubertal development in boys or in girls. In addition, the median values did not differ in boys and girls at different stages of puberty, regardless of the total GH levels in the samples (Table 2).

In six children (two boys and four girls), the proportion of non-22 kDa GH isoforms was determined longitudinally in samples collected at different pubertal stages (Fig. 3). In one boy, the proportion of non-22 kDa GH isoforms increased by 13.8% at early puberty, followed by a progressive decrease to his prepubertal value. In the other children, the values were more constant during puberty, with a maximal difference of 6% between the maximum and minimum values.

### Correlations between non-22 kDa GH isoforms and auxology

In prepubertal children, the proportion of non-22 kDa GH isoforms was positively correlated to weight SDS \(( r = 0.46; \ P = 0.03 \)\), weight-for-height SDS \(( r = 0.51; \ P = 0.01 \)\) and BMI \(( r = 0.42; \ P = 0.04 \)\) (Fig. 4). A stepwise linear regression analysis revealed that BMI was the main predictor of the proportion of circulating non-22 kDa GH isoforms in prepubertal children. In contrast, in pubertal children there was no correlation.

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**Table 2** Spontaneous GH secretion and the results of GHEA in the girls and boys studied, grouped according to their stage of puberty. Values are given as the median and range.

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous GH secretion (24-h profile)</th>
<th>Peak sample (GHEA)</th>
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<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>( \text{GH}_{t} ) (U/24 h)</td>
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<tr>
<td>Girls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prepuberty</td>
<td>10</td>
<td>0.4 (0.3/1.1)</td>
</tr>
<tr>
<td>Early puberty</td>
<td>9</td>
<td>1.2 (0.3/2.5)*</td>
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<tr>
<td>Mid-puberty</td>
<td>10</td>
<td>1.8 (0.6/2.6)*</td>
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<tr>
<td>Late puberty</td>
<td>16</td>
<td>1.3 (0.7/3.1)*</td>
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<tr>
<td>Boys</td>
<td></td>
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<tr>
<td>Prepuberty</td>
<td>13</td>
<td>0.6 (0.2/1.9)</td>
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<tr>
<td>Early puberty</td>
<td>16</td>
<td>0.9 (0.5/1.9)</td>
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<tr>
<td>Mid-puberty</td>
<td>12</td>
<td>1.1 (0.5/2.6)</td>
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<tr>
<td>Late puberty</td>
<td>7</td>
<td>0.9 (0.7/2.9)</td>
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**GH** \_t, GH secretion rate; \( \text{AUC}_{B} \), area under the curve over baseline; \( \text{GH}_{\max } \), maximum GH level in the profile.

\( P < 0.05 \): * compared with prepubertal values; † compared with boys.
between the amount of non-22 kDa GH isoforms and weight SDS, weight-for-height SDS or BMI. As shown in Fig. 5, an inverse correlation was observed between the proportion of non-22 kDa GH isoforms and height SDS in pubertal boys (r = 0.38; P = 0.02). This correlation was strongest at mid-puberty (n = 12; r = 0.7; P = 0.01), when peak height velocity occurs. In pubertal girls, no correlation with height SDS was observed.

**Spontaneous GH secretion and biochemical measurements**

Table 2 shows the changes in the spontaneous GH secretion during puberty in boys and girls. No correla-
tions were found between GH secretion rate and the proportion of non-22 kDa GH isoforms in any stage of puberty. Similarly, the proportion of non-22 kDa GH isoforms did not correlate with serum measurements of IGF-1, IGFBP-3, insulin and leptin in our group of healthy children of normal stature.

**Discussion**

The present study has characterized the proportion of non-22 kDa GH isoforms at spontaneous GH peaks in a group of healthy children of normal stature at different stages of pubertal development. We have used samples from GH secretory peaks because basal (trough) samples have very low or undetectable levels of GH. This is the first time that reference values for non-22 kDa GH isoforms have been obtained in relation to stages of puberty and auxology in a large group of children. Previous investigations of non-22 kDa GH isoforms have been limited by the use of laborious methods, with low sensitivity and limited sample capacity (for review, see ref. 10). The GHEA is suitable for clinical studies because it can be applied in large sets of samples, it has a broad detection range (0.05–130 mU/l; 0.02–50 µg/l) and its performance can be predicted by the use of statistical experimental designs (23, 24). It is to be hoped that the clinical application of the GHEA and novel GH assays recently described by other groups (26, 27) will help to determine the physiologic implications of the GH heterogeneity and increase our understanding of the structure–function relationships of GH (28).

In the GHEA, the non-22 kDa GH isoforms detected after extraction of 22 kDa GH from serum represent a mixture of the 20 kDa variant of GH, oligomeric isoforms and GH fragments. The 20 kDa GH is a variant produced from an alternatively spliced mRNA, which lacks amino acids 32–46 of the 22 kDa GH molecule. It comprises 5–10% of the pituitary GH and its physiologic role is not known (29, 30). It has been reported that 20 kDa GH does not bind to GHR in human liver or to the receptor-related GHBP in human plasma (31, 32). However, both 22 kDa and 20 kDa proteins stimulate linear growth in hypophysectomized rats and in transgenic mice, although they differ in terms of metabolic effects, such as acute insulin-like activity (33, 34). Immunoreactive GH fragments are generated by degradation of the GH molecule in peripheral tissues, mainly in the kidney (10). More important, however, is the presence of two naturally occurring fragments of GH in pituitary extracts, hGH 1–43 (5 kDa) and hGH 44–191 (17 kDa) (12). The latter was shown to circulate in significant amounts in human blood as measured by a specific RIA (35). Recently, Rowlinson et al. (36) found that both hGH 1–43 and hGH 44–191 have low or no affinity to GHR in vitro, and they suggested that specific receptors for these native fragments might exist.
In this study, we found that the proportion of non-22 kDa GH isoforms inversely correlated to height SDS in pubertal boys, especially at mid-puberty, indicating that the presence of increased amounts of circulating non-22 kDa GH isoforms was associated with less growth. A possible explanation for this finding is that non-22 kDa GH isoforms and fragments act as weak agonists or antagonists of the GHR. The former could, for example, be the case if receptor dimerization is achieved only at high concentrations of hormone, whereas the latter might result if the molecule binds normally to site 1 of the GHR but the affinity for site 2 is severely impaired (13). The lack of such correlation in girls suggests that the relation between weak agonist/antagonist molecules in the circulation may not be the same in boys and girls. It is known that the metabolic clearance of various GH molecules differs and that this factor is an important regulator of GH composition in human blood. For example, the elimination rate of 20 kDa GH (37) and oligomeric isoforms (38) is reduced in comparison with 22 kDa GH. The serum levels of estradiol have been shown to correlate inversely with the half-life of endogenous GH in the circulation (39), and this may be one explanation for possible sex-related differences in the molecular nature of circulating GH isoforms.

Figure 4 Proportion of non-22 kDa GH isoforms in prepubertal children, plotted against height s.d. scores (SDS), body mass index (BMI), weight SDS and weight-for-height SDS. Significant positive correlations were observed with BMI (r = 0.42; P = 0.04), weight SDS (r = 0.46; P = 0.03) and weight-for-height SDS (r = 0.51; P = 0.01), but not with height SDS (r = 0.29; P = 0.16). These correlations remained significant after multiple regression analysis (P < 0.01). The lines show linear regression of data points.

Figure 5 The proportion of non-22 kDa GH isoforms plotted against height s.d. scores (SDS) in pubertal children. In pubertal boys, a significant inverse correlation was observed (r = −0.38; P = 0.02), whereas in girls such correlation was not seen (r = −0.15; P = 0.4).
In the prepubertal children, the amount of non-22 kDa GH isoforms was directly correlated to body composition, as determined by BMI, weight-to-height SDS and weight SDS. This finding suggests that the proportion of 22 kDa to non-22 kDa GH molecules in circulation may be regulated by the nutritional state before puberty. The degree of adiposity seems to influence spontaneous GH secretion in prepubertal children (40, 41), in normal short children (42) and in normal boys just before starting puberty (43). However, it is not known if modifications in the pattern of secretion of GH are associated with changes in the nature of GH secreted by the pituitary gland. Another possibility is that the elimination rate of individual GH isoforms from the circulation changes according to body size, as determined by BMI, weight-to-height SDS and weight SDS in pubertal boys, suggesting the possibility that the different non-22 kDa GH isoforms may act as modulators of GH action in vivo.

Acknowledgements
We thank Birgitta Svensson, Lisbeth Larsson, Carina Ankarberg and the personnel of Ward 34T, the Children’s Hospital, Göteborg, for their support. We are grateful to Dr Werner F Blum for providing reagents for the IGF-1 and IGFBP-3 assays, and to Genentech Inc. for supplying the antibody MCB. This work was supported by grants from the Swedish Medical Research Society (No. 7509, 11285, 11331, 11502, 11576), Emil and Wera Cornell’s Foundation, Wilhelm and Martina Lundgren’s Foundation, Barnhusfonden, Stiftelsen Samariten, University of Göteborg and Pharmacia & Upjohn.

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Received 20 January 1997
Accepted 29 April 1997