CLINICAL STUDY

IGF-I, IGF-II, 'free' IGF-I and IGF-binding proteins-2 to -6 during high-dose oestrogen treatment in constitutionally tall girls

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Abstract

Objective: To investigate the effect of high-dose oestrogen treatment on IGF-I, IGF-II, free-dissociable IGF-I and the IGF-binding proteins (IGFBP)-2 to -6 in girls with constitutional tall stature.

Methods: In patient cohort 1, blood samples were drawn before and after 3 months of daily oral treatment with 0.1 mg ethinyloestradiol. In cohort 2, samples were collected at the end of the treatment period and 3 to 6 months afterwards. IGFs and IGFBPs were analysed by specific immunoassays and by Western ligand blot.

Results: Total IGF-I decreased significantly on oestrogen treatment and increased again after oestrogen withdrawal. Ligand blot analysis showed a clear reduction in a 34 kDa band, corresponding to IGFBP-2, and a strong induction of a 24 kDa band, corresponding to the non-glycosylated form of IGFBP-4. These changes were confirmed by specific immunological methods. The serum levels of IGFBP-3, IGFBP-5 and IGFBP-6 remained unchanged during the first 3 months of treatment. In cohort 2, IGFBP-3 and IGFBP-6 increased after oestrogen withdrawal. Free-dissociable IGF-I fell to $35 \pm 4\%$ during oestrogen therapy and rose again when the treatment was stopped.

Conclusions: Oestrogens modulate the serum concentrations of several components of the IGF system. The fall in total IGF-I is not explained by a decrease in IGFBPs but probably results from a decreased synthesis.

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Introduction

The effect of oestrogens on the serum concentration of insulin-like growth factor-I (IGF-I) depends on the dose of oestrogen and the age of the individual. In children and adolescents, ethinyloestradiol given to induce puberty up to a dose of 0.030 mg increases serum IGF-I (1) but doses higher than 0.100 mg, used to limit growth in constitutionally tall girls, decrease it (2–4). In adults, oestrogens invariably decrease IGF-I whether given in low doses as an oral contraceptive in young adult women (5, 6), as hormone replacement therapy in postmenopausal women (7–15), in high doses to human volunteers (16), in acromegalics (17) or in male to female trans-sexuals (18). A similar age-dependent effect of oestrogens on IGF-I was also reported in primates (19).

This decrease in IGF-I is not the consequence of a decrease in growth hormone (GH) secretion as shown during oestrogen treatment of acromegalics (17) and postmenopausal women (7, 11). Moreover, oestrogens

also reduce the growth response to exogenously administered GH in hypophysectomized rats (20) or Snell dwarf mice (21).

In the circulation, IGF-I is bound to at least six different high-affinity IGF-binding proteins (IGFBPs) (22–24). Since there is a 50% molar excess of binding proteins over IGF-I in serum, only a minute fraction is in the free, unbound form. Approximately 75% of IGF-I is bound to IGFBP-3. This IGFBP-3–IGF-I complex is, by itself, bound to an acid-labile subunit (ALS) to form a ternary complex that determines the pharmacokinetics and tissue distribution of IGF-I and therefore its biological activity (23). A decrease in serum total IGF-I caused by exogenous oestrogens may therefore be the consequence of a change in IGFBP levels.

The effect of oral oestrogens on IGFBPs has only been studied in women who take oral contraceptives (6) and during hormone replacement therapy in postmenopausal women (8, 13, 15, 25). Since the effect of oestrogens on IGFBPs may also be age- and dose-dependent we measured serum total IGF-I, IGF-II, IGFBP-2,

IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6 and free-dissociable IGF-I in tall girls who received a high dose (0.1 mg/day) of ethinyloestradiol to limit their final height.

Subjects and methods

Twenty-four constitutionally tall girls were included in this longitudinal study, approved by the ethics committee of the Antwerp University Hospital. Informed consent was obtained from patients and parents.

The diagnosis of constitutional tall stature was based on (a) familial occurrence of tall stature (height of one or both parents >2 s.d. above the mean), (b) height >2 s.d. above the mean for age, according to the standards of Freeman *et al.* (26), (c) predicted height of more than 180 cm and (d) exclusion of endocrine and other disorders that may explain their tall stature. Skeletal age was evaluated by X-ray of the left hand according to the method of Greulich & Pyle (27). Final height was predicted by the method of Bayley & Pinneau (28). The standard deviation score of the body mass index for age (BMI-SDS) was calculated using the reference data of Cole *et al.* (29).

Ethinylloestradiol (0.1 mg) was given every day at bedtime. This treatment was supplemented with 10 mg medroxyprogesterone acetate (Farlutal, Pharmacia) for the first 12 days of each month.

In 16 patients, blood samples were collected between 0900 and 1200 h at the start of therapy and after 2 to 4 months of treatment (cohort 1). In eight other patients, samples were collected at the end of 2 to 3 years of therapy and 3 to 6 months afterwards (cohort 2).

Total IGF-I was measured by radioimmunoassay (Biosource Europe, Nivelles, Belgium) after acid-ethanol extraction (30). Interassay variability was less than 10%. The concentration of IGF-II was determined after Sep-Pak C18 column extraction by a specific radioimmunoassay (31). IGFBP-2 was analysed by a radioimmunoassay developed by Diagnostic Systems Laboratories (DSL 7100; Webster, TX, USA) using a rabbit polyclonal IGFBP-2 antiserum. IGFBP-3 was measured in a solid phase immunoradiometric assay (BC 1040; Biocode, Liege, Belgium) using a mouse anti-IGFBP-3 monoclonal antibody. Serum IGFBP-4 levels were determined by a radioimmunoassay using recombinant human IGFBP-4 as tracer and standard, and antibodies against recombinant human IGFBP-4 raised in guinea pigs (32). Serum IGFBP-5 levels were determined by a radioimmunoassay using recombinant human IGFBP-5 as tracer and standard, and antibodies against recombinant human IGFBP-5 raised in guinea pigs (33). IGFBP-6 was measured by radioimmunoassay using a rabbit polyclonal antiserum raised against a synthetic fragment containing residues 90–118 that was also used as a tracer. Technical

details and data on reproducibility and stability after storage have been described elsewhere (34).

Easy dissociable or 'free' IGF-I was measured by a two-site immunoradiometric assay (DSL 9400; Diagnostic Systems Laboratories). The paired blood samples were always measured in the same assay and expressed as a fraction of their control. For cohort 1, the sample before treatment was set at 100%; in cohort 2, the sample at the end of treatment was designated as the control.

Ligand blot analysis was performed as described previously (35). Briefly, serum (1–3 μ l) was diluted in Laemmli sample buffer and separated by electrophoresis on a 12% SDS polyacrylamide gel in non-reducing conditions. The size-separated proteins were semi-dry blotted on nitrocellulose and incubated with biotinylated IGF-I for 1 h at room temperature. After washing, the membrane was incubated with streptavidin-horse-radish peroxidase and the bound IGF-I was visualized by enhanced chemiluminescence on ECL Hyperfilm (Amersham International plc, Amersham, Bucks, UK).

The results were analysed by Student's *t*-test for paired data.

Results

Table 1 summarizes the characteristics of the two patient cohorts. In cohort 1, treatment was started at a median calendar age of 13.7 years and a bone age of 13.25 years. Treatment was stopped in cohort 2 at a median bone age of 16.0 years when the median height was 178.5 cm.

The changes in total IGF-I, IGF-II and free-dissociable IGF-I are shown in Fig. 1. Total IGF-I decreased from 528 ± 31 ng/ml (mean $\pm$ s.e.m.) to 376 ± 27 ng/ml ($n = 16$ paired values, $P = 0.0001$) after 2 to 4 months of treatment. The total IGF-I value in eight other girls at the end of treatment was 243 ± 29 ng/ml and increased to 354 ± 27 ng/ml 3 to 6 months after ending treatment ($n = 8$ paired values, $P = 0.013$). Free-dissociable IGF-I was measured in thirteen out of sixteen patients in cohort 1 and in five out of eight in cohort 2. Free-dissociable IGF-I fell to $37 \pm 4\%$ after 2 to 4 months of oestrogen therapy ($n = 13$ pairs, $P = 0.002$) and rose again when the treatment was stopped ($n = 5$

Table 1 Characteristics of the patients who started (cohort 1) or stopped (cohort 2) oestrogen therapy.

	Cohort 1 at the start of treatment		Cohort 2 at the end of the treatment	
	Median	Range	Median	Range
Age (years)	13.7	11.8–15.6	16.7	13.1–17.8
Height (cm)	176.9	172–184.7	178.5	175.3–185.3
Bone age (years)	13.25	12.0–15.0	16.0	15.0–16.25
BMI-SDS	–0.2	–2.6–2.7	–0.4	–1.4–2.04

Figure 1 (A) Total IGF-I and (B) total IGF-II (mean and S.E.M.) in cohort 1 before treatment (grey bars) and after 2 to 4 months of oestrogen treatment (open bars) and in cohort 2 at the end of treatment (hatched bars) and 3 to 6 months after therapy (brick bars). NS, not significant; * $P \leq 0.05$ (Student's paired *t*-test). Change in free-dissociable IGF-I in (C) cohort 1 and (D) cohort 2. The paired samples were assayed simultaneously and the results are expressed as a fraction of the first measurement. E, oestrogen treatment.

pairs). IGF-II did not significantly change during oestrogen treatment (from 444 ± 32 to 541 ± 32 ng/ml, $n = 10$ pairs, $P = 0.12$) but increased in the second cohort from 430 ± 32 to 595 ± 32 ng/ml ($n = 6$ pairs, $P = 0.0004$).

The changes in IGFBPs during treatment were visualized by ligand blot analysis (Fig. 2). There were no significant changes in the IGFBP-3 doublet. Oestrogen reduced a 34 kDa band, corresponding to IGFBP-2 and strongly induced a 24 kDa band corresponding to IGFBP-4. In the 28–32 kDa area, four other binding species were detected and the two bands with the lowest molecular weight were increased by oestrogen therapy (Fig. 2B). One of these bands was probably an IGFBP-3 fragment that was identified by Western immunoblot (Fig. 2B, lane c).

Some of the changes found by ligand blot analysis were confirmed by specific immunological methods (Fig. 3). IGFBP-2 decreased at the start of treatment

Figure 2 (A) Ligand blot of 1 and 3 µl serum from a representative patient of cohort 1 at the start (lane A) and after 3 months of therapy (lane B) and from a patient of cohort 2 at the end (lane C) and 3 months after treatment (lane D). (B) Detailed view of the region between IGFBP-2 and IGFBP-4 of the ligand blot. Note the induction of IGFBP-2, IGFBP-4 and several bands in the 28–30 kDa region by oestrogens. Lane a and lane b are 1 and 3 µl serum at the start of treatment; lane c and lane d represent 1 and 3 µl serum after 3 months of oestrogen exposure.

from 556 ± 48 to 305 ± 22 ng/ml ($n = 16$, $P = 0.00002$) and increased again when the oestrogens were withdrawn (349 ± 47 versus 504 ± 52 ng/ml, $n = 8$, $P = 0.008$). The change in IGFBP-2 correlated with the change in body weight ($R^2 = 0.52$).

IGFBP-3 levels remained unchanged during the first 3 months of treatment: from 3919 ± 106 to 3963 ± 112 ng/ml ($n = 16$, $P = 0.68$). The mean IGFBP-3 of eight girls at the end of therapy was 3140 ± 207 ng/ml and increased to 3664 ± 139 ng/ml ($P = 0.011$) 3 months after the end of treatment. The ratio of IGFBP-3 over total IGF-I rose at the start of treatment from 7.8 ± 0.5 to 11.0 ± 0.5 ($P = 0.00015$).

Oestrogen treatment increased IGFBP-4 from 345 ± 9 to 544 ± 22 ng/ml ($n = 9$, $P = 0.0003$). IGFBP-4 decreased again after oestrogen withdrawal from 504 ± 70 to 339 ± 17 ng/ml ($n = 6$, $P = 0.11$).

IGFBP-5 remained unaffected in cohort 1 (from 466 ± 25 to 441 ± 22 ng/ml, $n = 9$, $P = 0.32$) and in cohort 2 (from 370 ± 27 to 450 ± 39 ng/ml, $n = 6$, $P = 0.18$). IGFBP-6 did not change during treatment but increased from 131 ± 9 to 158 ± 9 ng/ml ($n = 6$, $P = 0.044$) when oestrogens were stopped.

Discussion

High-dose oestrogen treatment for the reduction of final height in constitutionally tall girls reduced the levels of total IGF-I significantly as reported previously (3, 4). Several hypotheses have been proposed to explain this effect, such as (a) inhibition of GH secretion, (b) reduction in IGF-I synthesis or (c) a reduction in IGF serum carriers.

In a pilot experiment, we compared the blood samples from the same patient taken during the oestrogen-only period or during the oestrogen plus medroxyprogesterone period. We found no difference on the ligand blots, or for total IGF-I or IGFBP-3. Heald *et al.* (8) found a small antagonistic effect of medroxyprogesterone acetate on the changes induced by conjugated oestrogens in postmenopausal women. The higher dose of oestrogens in our study (0.100 mg ethinyl-oestradiol versus 0.625 mg conjugated oestrogens) may have overridden the effect of medroxyprogesterone. We therefore pooled the data for further analysis.

We visualized the serum IGF-binding species by ligand blot before and after 2 to 4 months of oestrogen treatment. No clear changes were seen in IGFBP-3, which binds 75% of IGF-I in serum (23). This was confirmed by a radioimmunoassay for IGFBP-3. The sharp decrease in IGF-I during oestrogen treatment can therefore not be explained by changes in IGFBP-3 levels. Since IGFBP-3 is also strongly GH-dependent, this result also rules out an acute effect of oestrogens on GH secretion or GH receptor expression (36). The lack of an important oestrogen effect on IGFBP-3 was also reported in postmenopausal women receiving oestrogen replacement therapy (8, 13, 15, 25). The IGFBP-3 levels in cohort 2 after 2 to 3 years of oestrogen exposure were somewhat lower than the IGFBP-3 concentrations after 2 to 4 months of oestrogen treatment in cohort 1 and rose again 3 to 6 months after treatment. Several explanations can be offered. First, it has been shown that oestrogens decrease the acid-labile subunit of the large ternary complex at least in postmenopausal women (15). The decrease in IGFBP-3 may therefore be the consequence of more rapid

Figure 3 Serum levels (means and s.e.m.) of IGFBP-2 to IGFBP-6 in cohort 1 at the start of treatment (grey bars) and after 2 to 4 months of oestrogen treatment (open bars) and in cohort 2 at the end of treatment (hatched bars) and 3 to 6 months after therapy (brick bars). NS, not significant; * $P \leq 0.05$ (Student's paired *t*-test).

clearance from the circulation due to prolonged lower ALS and IGF-I levels. Alternatively, it cannot be excluded that oestrogens have a slight effect on GH secretion, GH receptor expression (36) or IGFBP-3 synthesis after a more chronic use. A decreased GH activity may also explain our previously reported (4) and current observation that the total IGF-I concentration further decreases during oestrogen therapy.

IGF-I levels increased again after stopping therapy but not to pretreatment values. This may be due to the age-dependent decrease in IGF-I at the end of puberty.

The concentration of IGF-II was variable among individuals but had a tendency to increase during the first few months of therapy. The levels at the end of therapy in cohort 2 were again lower. One possible explanation is that IGF-II first rises because there are more binding sites available on IGFBP-3 due to the fall of IGF-I. As IGFBP-3 levels decrease gradually with more prolonged oestrogen exposure, IGF-II also declines. The sharp rise of IGF-II after therapy may be the consequence of a rise in IGFBP-3, IGFBP-2 and/or IGFBP-6.

Slight changes were observed in two faint bands in the 28–30 kDa region of the ligand blot. One of these bands represents a 30 kDa IGFBP-3 fragment that can be detected by our non-radioactive ligand approach (35). This may indicate that oestrogens stimulate the proteolysis of IGFBP-3 and the generation of a 30 kDa fragment which was shown to be a weak inhibitor of IGF-I action (37). We were unable to identify the other bands in the 28–32 kDa region but other investigators recently found IGFBP-1, IGFBP-6, glycosylated IGFBP-4 and IGFBP-5 in this region by two-dimensional ligand blot analysis (38). Because oestrogens were reported to dramatically increase IGFBP-1 levels in postmenopausal women (8, 13) and women on oral contraceptives (6) one of the bands is most likely IGFBP-1. We did not measure IGFBP-1 since it has a short serum half-life and is modulated by serum insulin levels, hence requiring fasting blood samples.

Oestrogen treatment clearly decreased a 34 kDa band on the ligand blot identified as IGFBP-2. The band became more prominent again after the end of therapy. These changes were confirmed by a radioimmunoassay for IGFBP-2. Oestrogens caused an average weight gain of 3 kg during the first 3 months of therapy. We were therefore not surprised to find a correlation (Pearson correlation = 0.52, $P = 0.056$) between the change in IGFBP-2 and the change in weight in cohort 1. A correlation between body weight and IGFBP-2 has been reported previously (39, 40). However, a direct effect of oestrogens on IGFBP-2 production is not excluded. Women have lower levels of IGFBP-2 than men between the ages of 10 and 40 years (41). On the other hand, IGFBP-2 levels remained unchanged by ligand blot analysis in postmenopausal women on oral replacement therapy (8) and in women taking oral contraceptives (6).

Oestrogen therapy induced a band at 24 kDa, most likely the non-glycosylated form of IGFBP-4. This effect was reversible as the band decreased again 3 months after the end of therapy. This finding was corroborated by the direct measurement of IGFBP-4. Oestrogens were reported to stimulate IGFBP-4 mRNA and protein in a human osteoblastic cell line (42) and in the human breast cancer cell line MCF-7 (43). Serum IGFBP-4 also increased in women who take oral contraceptives (6) and in postmenopausal women on hormone replacement therapy (8). Since IGFBP-4 is considered an inhibitor of IGF-I action (44, 45), it may contribute to the growth inhibition by oestrogens.

During puberty, low concentrations of oestrogen increase serum IGFBP-5 levels, probably mediated by an increase in GH secretion (46). In this study with supraphysiological doses of oestrogens, IGFBP-5 remained unchanged during the first months of treatment, supporting the hypothesis that GH secretion is not affected. Similar to IGFBP-3, the concentrations of IGFBP-5 were lower in cohort 2 than in cohort 1, which may again indicate that GH secretion decreases after a more chronic exposure.

IGFBP-6 remained unchanged under oestrogen therapy but increased afterwards. Since IGFBP-6 has a high affinity for IGF-II, the rise in IGFBP-6 may partly explain the increase in IGF-II in cohort 2 after treatment (34, 47). Our data do not support a role for oestrogens as a cause for the lower IGFBP-6 levels in females than in males (41).

Overall, oestrogens did not substantially decrease the IGF-binding capacity of serum. It is therefore highly unlikely that the drop in total IGF-I after 3 months of therapy is the consequence of a change in binding proteins. The dramatic fall in free-dissociable IGF-I that we found is also indirect evidence that the equilibrium between free and bound IGF-I is shifted. All the data therefore suggest that oestrogens directly decrease the production of IGF-I. At least three other observations support this hypothesis. First, IGF-I levels are less suppressed by transdermal oestrogen therapy as opposed to oral treatment, suggesting the importance of the local concentration of oestrogens in the liver, the predominant source of serum IGF-I (7, 25, 48). Secondly, oestrogens have been shown to inhibit hepatic mRNA synthesis for IGF-I in rats (49, 50) and in cultured hepatocytes (51). Finally, the type α -oestrogen receptor was recently shown to bind to the IGF-I gene promoter in a human liver cell line. In this model, IGF-I gene transcription was stimulated by oestrogen antagonists and inhibited by oestradiol (52).

In conclusion, this study and the data reported in the literature indicate that oestrogens modulate several components of the serum IGF system. The important decrease in total IGF-I and free-dissociable IGF-I are most likely the result of an inhibitory effect of oestrogens on IGF-I synthesis. Oestrogens decrease IGFBP-2 and increase the levels of IGFBP-1, IGFBP-4 and probably

IGFBP-3 fragments, some of which are also growth inhibitors. The contribution of these systemic alterations in the IGF system to the growth-inhibitory effects of high-dose oestrogen therapy remains unknown. Given the recent controversy on the importance of systemic versus locally produced IGF-I for growth, the circulating levels of the IGFBPs may not be relevant at all (53). The effects of oestrogen on the synthesis of components of the IGF system in the growth plate itself or actions of oestrogen that are independent of the IGF system (54, 55) may also be involved.

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