CLINICAL STUDY

The effects of GH and hormone replacement therapy on serum concentrations of mannan-binding lectin, surfactant protein D and vitamin D binding protein in Turner syndrome

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Abstract

Objective: Studies in animals and humans indicate that growth hormone (GH) and insulin-like growth factor-I (IGF-I) modulate immune function. Recently, it was reported that GH therapy increased the level of mannan-binding lectin (MBL) in normal patients, and that treatment of acromegals with pegvisomant decreased the levels of MBL. The effect on MBL was thought to be due to a specific action of GH, since IGF-I treatment did not affect MBL. Whether it is advantageous or not to have high or low levels of MBL is not known. Likewise, it is not clear how the modifications induced by GH affect immune function. In the present study we examined whether GH or hormone replacement therapy (HRT) in Turner syndrome (TS) influence the serum concentrations of MBL and two other proteins partaking in the innate immune defence, surfactant protein D (SP-D) and vitamin D binding protein (DBP).

Design: Study 1: a double-blind crossover study of 12 healthy TS adolescents examined during treatment with either placebo or GH for 2 months, and compared with a control group. Study 2: triple-blind crossover study of 9 healthy TS adolescents randomized to treatment with placebo, GH or GH + 17β-estradiol. Study 3: 60 adult TS patients (55 received HRT) compared with 59 age-matched controls. Study 4: 27 patients with TS were examined before and during sex hormone replacement with 17β-estradiol and norethisterone and compared with age-matched controls (n = 24).

Methods: Measurement of MBL, SP-D, DBP, and other inflammation markers.

Results: Study 1: the levels of MBL (P = 0.002) and SP-D (P = 0.012) increased during GH treatment, whereas no changes were observed in comparison with controls. DBP was unchanged by GH, but was significantly higher in TS compared with controls (P = 0.017). Study 2: treatment with GH increased MBL (P = 0.045) and SP-D (P = 0.05) concentrations in TS, while treatment with GH + 17β-estradiol did not increase levels further. DBP was unchanged by treatment. Study 3: levels of MBL, SP-D, and DBP were similar in adult TS and control subjects. Study 4: DBP levels decreased in response to HRT, while MBL and SP-D levels were unchanged. Levels of all three plasma proteins were similar to controls.

Conclusion: We show that treatment with GH significantly increases MBL and SP-D concentrations in TS, while HRT marginally decreases DBP. Whether the present findings, suggesting a link between the endocrine and the immune system, have clinical consequences needs to be studied further.

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Introduction

Middle ear infections are extremely frequent in Turner syndrome (TS), occurring in more than 50% of all patients (1, 2). This increased frequency of middle ear infections has been attributed to the congenital craniofacial malformation with possible distortion of the Eustachian tube and impaired ventilation of the middle ear. In keeping with this, auricular anomalies have been noted, primarily in females with the 45,X karyotype, but in fewer patients than were affected by middle ear infections (3). In addition to middle ear infections, pneumonia (relative risk (RR): 12 (95% confidence interval (CI): 4–26)) and diseases of the
respiratory system (RR: 8 (95% CI: 4–15)) are frequent causes of death in TS in comparison with the background population (4), suggesting a defective immune system leading to increased susceptibility to infections. Most patients with TS have no gonadal function and are thus, if untreated, deficient in female sex steroids from early childhood and onwards. Besides gonadal insufficiency, the cardinal stigmata of TS are growth retardation with reduced final height, and infertility. Furthermore, a number of congenital malformations and conditions are associated with TS (5). Growth hormone (GH) is widely used to increase final height, and, in those girls who do not experience spontaneous puberty, pubertal induction with estradiol and subsequent treatment with hormonal replacement therapy (HRT) is instituted when breakthrough bleeds occur.

Recently, we showed that mannan-binding lectin (MBL) is strongly affected by GH, but not by insulin-like growth factor-I (IGF-I) (6), in normal males given high to very high pharmacological (‘doping’) doses of GH. Furthermore, it was shown that untreated patients with growth hormone deficiency (GHD) had low levels of MBL that increased in response to GH, and that patients with acromegaly had elevated levels of MBL that decreased in response to octreotide as well as to pegvisomant – the new GH receptor blocking agent. MBL (also known as mannose-binding lectin) is an innate immune defence plasma protein synthesized in the liver. It binds to specific repetitive carbohydrate structures on microbial surfaces, and subsequently activates the complement cascade through MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3) (7, 8) – the so-called MBL pathway of complement activation. The concentration of MBL in human plasma is genetically determined. Because of the high frequency of three mutant MBL alleles, as well as mutations in the promoter region of the gene, very large inter-individual differences in MBL concentrations exist and the presence of MBL deficiency among 10% of the population makes it the most frequent immunodeficiency described (9). Studies indicate that MBL is of importance in first-line immune defence against a number of important pathogens (10–12), and low serum concentrations of MBL are associated with increased susceptibility to recurrent infections (13), although low levels of MBL in healthy individuals may well confer some selective advantage (9). This selective advantage could be due to MBL-mediated complement activation after hypoxia aggravating ischemic injury (14, 15), and inhibition of MBL activation of the lectin pathway protects the heart from ischemic lesions (16). Thus, it is not clear how changes in circulating MBL induced by GH will affect overall immune function, if at all. Recently, it has become clear that surfactant protein (SP)-D and SP-A, also collectins, may be the pulmonary counterparts of MBL; they participate in all aspects of the inflammatory response to pulmonary pathogens and are also located at extrapulmonary mucosal surfaces (17).

The aims of the present study were to examine the levels of MBL, and of SP-D and vitamin D binding protein (DBP) in TS; to examine the influence of GH treatment on MBL, SP-D, and DBP levels; and to examine the influence of another pituitary axis, the female sex hormone axis, on the synthesis and control of these proteins, and thus possibly establish another link between the immune and the endocrine system.

**Subjects and methods**

**Subjects and experimental designs**

We utilized serum from four previous studies from our own department. These studies fulfilled a design enabling us to answer the questions above.

**Study 1** We examined twelve girls with TS verified by chromosomal karyotyping in a randomized, placebo-controlled, cross-over design (Table 1), to study the effect of GH and to compare this with a control group. None of the participants had previously received estrogen. Four of the girls were spontaneously menstruating. Eight had the karyotype 45,X, two had 45.X/46.XX.

<table>
<thead>
<tr>
<th>Study</th>
<th>Age</th>
<th>Number</th>
<th>Tanner stage</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Turner syndrome</td>
<td>13 (10–15)</td>
<td>12</td>
<td>1: n = 7; 2: n = 2; 4: n = 3</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>12 (10–16)</td>
<td>16</td>
<td>4: n = 6, 5; n = 2</td>
</tr>
<tr>
<td>2</td>
<td>Turner syndrome</td>
<td>16 (13–18)</td>
<td>9</td>
<td>1: n = 1; 3: n = 4, 4: n = 4</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>37 (22–67)</td>
<td>60</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Turner syndrome</td>
<td>36 (22–66)</td>
<td>59</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>34 (21–49)</td>
<td>27</td>
<td>—</td>
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<tr>
<td></td>
<td></td>
<td>33 (21–46)</td>
<td>24</td>
<td>—</td>
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</tbody>
</table>
one had 46,X,i(Xq), and one had 45,X/46,X,i(Xq)/47, X.i(Xq)i(Xq). At least 5 months prior to inclusion in the study all girls with TS received GH (0.1 IU/kg/day). During the study subjects received GH (Norditropin, Novo Nordish, Denmark) 0.1 IU/kg/day s.c. at bedtime for 2 months, or placebo. The average daily dose was 3.5±0.9 IU GH during the study period. An age-matched control group (n = 16) was studied once. TS girls were studied twice during the end of each treatment period, while controls were studied once. Data from this study regarding the effects of GH on metabolism have previously been reported (18).

Study 2 We examined the effect of GH and estrogen in nine girls with TS verified by chromosomal karyotyping (Table 1), in a randomized, triple-blind, placebo-controlled, crossover study, with 2-month treatment periods. All of the participants were receiving GH and estrogen prior to entering the study. Four had the karyotype 45,X; one had 45,X/46,XX, three had structural abnormalities of one X chromosome (2 mosaics), and one had 45,X/46,X,r(Y). At least 5 months prior to inclusion in the study all girls with TS received GH (3.7±0.9 IU/day) and 17$\beta$-estradiol (0.33±0.16 mg/day). During the study subjects received GH (Norditropin) and 17$\beta$-estradiol, GH (Norditropin) and placebo, or placebo and placebo, at the same dose as at inclusion. The TS girls were studied thrice during the end of each treatment period.

Study 3 We examined adults with TS, most treated with HRT, and compared them with a control group (Table 1). Sixty patients with TS, diagnosed by chromosome analysis, and 59 age-matched controls were studied. Karyotypes among TS subjects were distributed as follows: 45,X (n = 29), 45,X/46,XX (n = 5), karyotypes with isochromosomes (Xq) or deletions (n = 16), karyotypes with Y chromosome material (n = 5), karyotypes with a marker or ring chromosome (n = 5). Treatment consisted of 17$\beta$-estradiol (2 mg) for the entire cycle and norethisterone (1 mg), medroxyprogesterone (10 mg) or levonorgestrel (0.25 mg) for 10 days every cycle. The average duration of HRT was 16±9 years, and the age at start of HRT was 21±10 (range: 9–60) years. Data from this study regarding bone metabolism have previously been reported (19).

Study 4 To further study the effect of HRT versus no treatment. 27 TS patients and an age-matched control group of 24 normal women (all premenopausal) with presumed normal karyotype were studied (Table 1). Karyotypes among TS patients were distributed as follows: 45,X (n = 17), 45,X/46,XX (n = 1), karyotypes with isochromosomes (Xq) or deletions (n = 7), karyotypes with Y chromosome material (n = 2). All TS patients were receiving female hormone replacement therapy, but prior to the initial examination (basal examination, TB) a 4-month washout period was introduced. None of the TS patients had experienced spontaneous puberty. Following the initial examination, patients were randomized to two regimens of hormone substitution (treatment period, TT): oral hormone replacement consisting of 2 mg 17$\beta$-estradiol/day from days 1 to 12, 2 mg 17$\beta$-estradiol/day and 1 mg norethisterone acetate/day from days 13 to 22 and 1 mg 17$\beta$-estradiol/day from days 23 to 28 (Trisek, Novo Nordisk, Bagsvaerd, Denmark), or transdermal estrogen replacement consisting of approximately 50 $\mu$g 17$\beta$-estradiol/55 kg/day for 28 days (Estadrerm, Ciba-Geigy, Copenhagen, Denmark) and 1 mg norethisterone (Noretisterone Dak, Nycomed DAK, Copenhagen, Denmark) administered orally from days 13 to 22. Fifteen subjects were randomly allocated to the group receiving transdermal estrogen and twelve subjects to the group receiving oral estrogens. Within the first month of treatment, three subjects from the group receiving transdermal estrogens had to be transferred to oral treatment due to irritative dermatitis. These three subjects were thus transferred to oral treatment and subsequently included as such in the statistical analysis. Preliminary analysis showed no difference in the level of the studied variables, and the two groups were merged for all calculations. All patients were then studied again after 6-months treatment, whereas all controls were examined once. Control subjects and TS patients during sex hormone treatment were studied in the early follicular stage (days 5 to 10) of the menstrual cycle. Data from this study regarding glucose metabolism and GH dynamics have previously been reported (20, 21).

All blood samples were collected following an overnight fast. The local ethics committees approved the protocols (#1991/2031, #1991/2030, #1993/2837, #1994/2929) and all subjects gave written consent to participate.

Analytical methods

Serum MBL concentrations were measured using an in-house time-resolved monoclonal immunoloumometric assay (TRIFMA) as previously described (16). SP-D was measured by a sandwich ELISA technique as previously described (22). DBP was measured by an immunonephelometric assay (23). Serum concentrations of C-reactive protein (CRP) were measured by an ultrasensitive latex-enhanced immunoturbidimetric method, haptoglobin and transferrin by an immunoturbidimetric method, all on a Cobas Integra 700 (Hoffmann-La Roche Ltd, Basel, Switzerland).

Statistics

Statistical calculations were carried out with SPSS for Windows version 11.0 (SPSS, Chicago, IL, USA). When comparing MBL, SP-D, and CRP levels, Wilcoxon’s
signed rank test or Friedman test for within group comparisons, or Mann–Whitney’s U test for between groups comparisons were employed, while parametric statistics (paired, unpaired $t$-test, and one-way ANOVA) were used for the other variables. Spearman correlation was used to examine the relationships among different variables at baseline and following treatment. All results are expressed as median and range (non-parametrically distributed data), or means $\pm S.D.$ (parametrically distributed data). $P$ values $<0.05$ were considered significant.

**Results**

**Effects of GH treatment on measures of the innate immune system in girls with TS (study 1)**

During GH treatment for 2 months levels of MBL increased by 96%, SP-D by 17%, while DBP was unchanged (Table 2). In comparison with controls, similar levels of MBL and SP-D were found, while DBP was increased in TS patients (Table 2). There was no significant correlation between the change in any of the studied parameters following treatment regimens.

The baseline level of CRP was higher, and the level of transferrin was lower in TS patients compared with controls, while haptoglobin was similar amongst the study groups (Table 2). There was no significant change in CRP or haptoglobin, while transferrin increased in response to GH treatment.

**Effects of GH and estrogen treatment on measures of the innate immune system in girls with TS (study 2)**

During GH treatment for 2 months levels of MBL increased by 57%, SP-D by 17%, while DBP was unchanged (Table 2). There was no further change in the levels of MBL, SP-D and DBP after combined treatment with GH and estrogen compared with the effect of GH alone.

The levels of CRP, haptoglobin, and transferrin were not affected by treatment with GH alone or GH and 17$\beta$-estradiol combined.

**Measures of the innate immune system in adults with TS (study 3)**

There was no difference in the levels of MBL, SP-D, and DBP among adult TS patients ($n = 60$) and controls ($n = 59$) (Table 3). The level of CRP was increased in TS patients compared with controls, while the levels of haptoglobin and transferrin were comparable to controls. In TS patients MBL and SP-D correlated significantly ($r = 0.370$, $P = 0.004$), but not in controls ($r = 0.082$, $P = 0.6$). There was no significant correlation between DBP and MBL or SP-D in TS patients, while SP-D and DBP correlated significantly and

**Table 2 Changes in MBL, SP-D, DBP, CRP, haptoglobin, and transferrin concentrations in healthy girls and adolescents, and in TS patients before (placebo) and during GH and estrogen (E) therapy. Results are expressed as median (range) or means $\pm S.D.$**

<table>
<thead>
<tr>
<th>Study 1</th>
<th>Study 2</th>
</tr>
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<tbody>
<tr>
<td>Variable</td>
<td>TS, placebo</td>
</tr>
<tr>
<td>MBL (mg/l)</td>
<td>1171 (337–3912)</td>
</tr>
<tr>
<td>SP-D (mg/l)</td>
<td>838 (344–1229)</td>
</tr>
<tr>
<td>DBP (mg/l)</td>
<td>211 $\pm$ 22</td>
</tr>
<tr>
<td>CRP (nmol/l)</td>
<td>26 (10–61)</td>
</tr>
<tr>
<td>Haptoglobin (mg/l)</td>
<td>9.3 $\pm$ 4.5</td>
</tr>
<tr>
<td>Transferrin (mg/l)</td>
<td>62.5 $\pm$ 7.3</td>
</tr>
</tbody>
</table>

*Paired $t$-test or Wilcoxon test; TS, placebo versus Controls; Unpaired $t$-test or Mann–Whitney test; Friedman test or one-way ANOVA (see Statistics section for further information).
Effects of HRT on measures of the innate immune system in adults with TS (study 4)

There was no difference in MBL, SP-D, and DBP levels among untreated TS adults and controls (Table 3), and HRT did not change the levels of MBL and SP-D in TS patients, while DBP decreased marginally in response to treatment. There was no difference in CRP, haptoglobin, and transferrin levels among TS patients and controls, although CRP was insignificantly increased in TS patients ($P = 0.08$). Treatment with HRT for 6 months increased the level of transferrin, while levels of CRP and haptoglobin were comparable in the two study situations. The observed changes in haptoglobin correlated significantly with changes in MBL ($r = 0.437$, $P = 0.024$), CRP ($r = 0.673$, $P < 0.0005$), DBP ($r = 0.731$, $P < 0.0005$), and transferrin ($r = 0.411$, $P = 0.033$). The changes in transferrin also correlated with CRP ($r = 0.434$, $P = 0.024$) and DBP ($r = 0.430$, $P = 0.025$).

Discussion

The main result of the present study is the pronounced effect of GH treatment in TS patients on the level of some of the studied proteins of the innate immune system. Previously, we showed that GH affects MBL in healthy subjects and GHD patients. Here, we have extended these data to girls and adolescents with TS, and in addition we have shown that two other components of the innate immune system, SD-P and DBP, are also affected by GH or estrogen. The results suggest that synthesis of MBL, SD-P and DBP, and thus probably control of the level of these proteins, are normal in TS patients. However, we confirm a pronounced effect of GH on MBL, and on SP-D levels, but no effect on the level of DBP. Thus, there seems to be a link between the endocrine and the innate immune systems. The serum half-life of injected MBL is 5–7 days (24), while the half-life of SP-D is unknown, and it is therefore likely that the increase in MBL, at least, is due to increased synthesis and not decreased degradation. The participants of the study were not tested for MBL gene mutations, but it is known that subjects with gene mutations in the MBL allele or in the promoter region of the gene have MBL levels in another magnitude than those with normal MBL genes (typically 5–50 versus 500–5000 μg/l) (25), and an indirect estimate of the frequency of gene mutations based on the MBL concentrations at baseline suggests that 10% in all groups had gene mutations.

A number of studies have shown minor deficiencies of humoral and cellular immunity (26–32), and a recent study showed increased apoptosis mediated by the tumor necrosis factor receptor and CD95 in cord blood T-cells (33). Furthermore, Turner syndrome is associated with increased susceptibility to bacterial infections, also a cause of death (1–4), but as the results from the present study show three proteins partaking in the innate immune defence, MBL, SP-D and DBP are essentially present in amounts comparable to the levels in age-matched controls. However, even though the levels of MBL and SP-D were normal, they increased in response to GH treatment. Whether this has clinical implications is difficult to say. SP-D (and MBL) is a member of the collectins, being produced by the alveolar type II cells and nonciliated bronchiolar cells and secreted into the alveolar lining layer, and was originally thought to have only surfactant function in the lungs (17). Interestingly, a recent study showed the presence of SP-D (and SP-A) in the epithelial cells of the porcine Eustachian tube (34), suggesting that they partake in the immune defence during middle ear infections. In view of the grossly increased risk of recurrent middle ear infections in TS patients, this finding could be of special importance. However, a recent trial of GH treatment in TS, with an 18-month placebo-controlled age-matched TS group, showed that...
otitis media was actually more frequent in the GH-treated group (35). GH is known to mediate water retention, and it is possible that this effect could worsen the unfavorable anatomical conditions present in the middle ear and lead to increased frequency of otitis media. Previously, GH given to acutely ill patients in intensive care approximately doubled the fatality rate (36), the reason for which has not been found. To date, it is not clear whether it is advantageous to have a high or a low level of MBL and SP-D. In another study of acutely ill patients in an intensive care unit treated either conventionally or with high dose insulin, high levels of MBL at baseline in conventionally treated patients was associated with better survival (37). The trial also showed lower levels of MBL in the insulin-treated group during the course of intensive care unit stay (37), which also had a highly significant reduction in morbidity and mortality (38). Thus, our finding of a GH-induced increase in MBL and SP-D and the above mentioned increased risk of middle ear infection during GH treatment could be linked, but further research will be necessary to establish such an association. The results from the present GH treatment studies must, of course, be viewed with caution because of the relatively few participants. DBP was not influenced by GH treatment, but decreased slightly in response to HRT (study 4), while baseline levels were comparable to controls. The vitamin D-binding protein is a multifunctional protein produced constitutively by the liver. It serves as a carrier protein for vitamin D, regulating its function thereof by delivering vitamin D metabolites, including 1,25-(OH)2-D3, to the cells, acts as an actin scavenger with subsequent activation of macrophages, and, in addition, it partakes in the inflammatory response (39, 40). It is a co-chemotaxin specific for the complement C5a and C5a-es-Arg and binds endotoxin (41). After removal of its galactose and sialic acid residues, DBP is converted to a very potent macrophage-activating factor (42). The mechanisms behind its immunomodulatory effects are elusive. Whether the slight decrease in DBP in response to HRT has any clinical significance is doubtful, but it is interesting that another endocrine mediator, 17β-estradiol (or possibly norethisterone), influences the immune system. Previously, the treatment of normal women with ethinylestradiol has been shown to increase DBP (43), and it remains to be seen whether the discordant results are due to different formulations of estrogen.

In order to study the effect of GH and HRT on other proteins involved in the acute phase response to inflammation, we found that the levels of CRP were increased in TS patients compared with controls, but they were unchanged in response to both GH and HRT. Previously, GH treatment to GHD patients has been shown to decrease the (previously increased) levels of CRP (6). The levels of haptoglobin, another acute phase protein, were similar in TS patients and in controls, and likewise the levels of the constitutive hepatic protein, transferrin, were similar to the levels in controls, except in study 1 where the levels were slightly decreased, and increased in response to GH, as seen previously (44).

Interestingly, in adults with TS, circulating levels of MBL and SP-D were correlated, while this was not the case in controls. On the contrary, SP-D and DBP were negatively correlated in controls, while not in TS patients. Furthermore, correlations were seen between DBP and SP-D and more traditional markers of inflammation (CRP, haptoglobin, and transferrin). In TS patients during HRT (study 4) changes in MBL and DBP in comparison with the washout phase were also correlated with the more traditional markers of inflammation. These results indicate that even the small changes in these markers observed here are all images of the current state of the immune system.

In summary, GH and HRT in Turner syndrome influence the immune system, as evidenced from increases in MBL and SP-D levels in response to GH treatment, and decreases in DBP levels during HRT. The endocrine and immune systems are interlinked, but whether this has positive or negative consequences remains to be resolved.

Acknowledgements

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