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Serum Osteocalcin and Bone Isoenzyme Alkaline Phosphatase in Growth Hormone-Deficient Patients: Dose-Response Studies with Biosynthetic Human GH

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Summary. Serum osteocalcin and bone alkaline phosphatase (BAP) were measured in samples drawn at 8 a.m. in 7 patients with GH deficiency treated with recombinant human growth hormone (rhGH) (2 IU/day subcutaneously at 8 p.m.), and 7 normal controls. Patients treated with 2 IU/day had lower BAP than controls ($P < 0.05$). Further, increasing doses of rhGH were given subcutaneously to each of the 7 patients for 3 consecutive 14-day periods (2, 4, and 6 IU/day at 8 p.m.) followed by 14 days off treatment. At the end of each period, the patient was hospitalized for frequent blood sampling from 8 p.m. to 11 a.m. the following day. A dose-dependent increase in area under the curve (AUC) was seen for osteocalcin ($P < 0.05$), whereas the increase in AUC for BAP just failed to reach significance ($P < 0.10$). The nocturnal patterns of serum osteocalcin in patients on 4 and 6 IU/day were statistically indistinguishable from those in controls. During treatment with 2 IU/day and the off-treatment period, the pattern was significantly different from controls ($P < 0.05$). In conclusion, rhGH has a dose-dependent effect on basal osteoblastic activity and the nocturnal pattern of osteocalcin. Serum osteocalcin increases within hours following rhGH administration. However, 2 IU/day is inadequate to maintain normal levels and nocturnal variation in markers of osteoblastic activity.

Key words: Growth hormone deficiency – Treatment – Bone GLA-protein – Bone isoenzyme – Alkaline phosphatase.

Previous recommendations on dosage and mode of administration of growth hormone (GH) in GH-deficient children have been a compromise between supply and effectiveness and only about 50% of the treated patients have grown to normal height [1]. The introduction of recombinant human GH (rhGH), however, has resulted in a potentially unlimited supply of the hormone [2] and therefore allows more thorough evaluations of its physiological effects and dose-response relationship [3]. Adjustment of dosage to the changing needs of the individual patient, for instance during puberty, may be one way to optimize substitution therapy in GH-deficient patients. A number of methods are currently used for measuring the effects of GH on bone. Measurements of the lower leg length by knemometry [4] or using a stadiometer may detect very small changes in growth rate. However, additional monitoring of more rapidly reacting biochemical indices of bone cell activity may be helpful in finding the best dose schedule for achieving normal final heights in these children. The growth-promoting effect of GH is mediated at least partially by insulin-like growth factor-I (IGF-I) [5], but most of the serum IGF-I derives from the liver [6] and serum levels of IGF-I may not be the best predictor of the growth response [7]. Serum osteocalcin (bone gamma-carboxy glutamic acid-containing protein) (BGP) [8, 9] reflects osteoblastic activity [10, 11] and has the advantage of a short half-life in serum [12]. Serum osteocalcin is low in patients with GH deficiency and increases in response to therapy [13]. Recently, GH has also been shown to affect the circadian rhythm of serum osteocalcin [14]. Serum levels of bone isoenzyme alkaline phosphatase (BAP), assessed as the lectin-precipitable fraction of total alkaline phosphatase (TAP) [15, 16], also correlates with bone formation [17], but BAP and

Table 1. Clinical data on the patients at the entry of the study

Group I—treated GH deficiency					Group II—normal controls				
Age (yrs)	Sex	Height (cm)	Weight (kg)	Tanner stage	Age (yrs)	Sex	Height (cm)	Weight (kg)	Tanner stage
11	M	131	28	1	9	M	139	30	1
12	F	135	37	1	9	M	139	31	1
15	F	143	43	2	12	F	141	37	2
17	F	162	50	2	12	F	159	39	3
16	M	168	59	2	14	M	180	57	3
17	M	156	46	2	14	M	175	65	3
19	M	168	51	3	14	M	170	67	3
Mean 15		152	45		Mean 12		157	47	

Normal controls were selected to match patients in Tanner stages. Patients in group I had received GH (2 IU/day sc) for 1–13 years

osteocalcin may reflect different aspects of bone metabolism [18]. In normal children, both serum osteocalcin and BAP parallel the growth velocity curve [19, 20] and serum osteocalcin correlates with height during puberty [19].

The aim of the present study was to evaluate the effects of increasing doses of rhGH (2,4,6, IU/day) on the variation in serum concentrations of osteocalcin and BAP in the same GH-deficient patients. We further compared the findings in these patients with a Tanner-stage matched control group.

Materials and Methods

Patients

The protocol was part of a clinical study on short-term metabolic response to rhGH [3]. We studied two groups of subjects. Seven GH-deficient patients (group I) who had been treated with 2 IU/day subcutaneously (sc) for 1–13 years, and 7 healthy controls (group II) (Table 1). All patients with GH deficiency had a maximum serum GH response of less than 5 μg/liter to two provocative tests, i.e., arginine infusion and elevation of body temperature [21]. One of the previously treated patients received additional hormonal replacement therapy with cortisone, 2 with cortisone and thyroxine, and 1 received cortisone, thyroxine, and testosterone. This treatment was continued unchanged during the study. Children in group II were selected to match patients in group I with respect to pubertal development (Tanner stages [22]) (Table 1).

Design

The subjects were admitted to the hospital during study periods of 15 hours. Conditions during these periods were kept as constant as possible. Blood samples were drawn through an indwelling catheter at 8 p.m., 9 p.m., 10 p.m., 11 p.m., 12 p.m., 2 a.m., 4 a.m., 8 a.m., 9 a.m., 10 a.m., and 11 a.m. Subjects rested in the supine position from 10 p.m. and throughout each study period. They fasted overnight and had breakfast at 8 a.m. Each patient received the same meal on all four occasions.

Each patient in group I was investigated during four study periods. At the time of the first study period, all patients in group I received 2 IU/day of natural sequence rhGH (Norditropin, Nordisk Gentofte, Gentofte, Denmark; biopotency 3 IU/mg) given as daily sc injections at 8 p.m. After the first study period the dose of GH was increased to 4 IU/day for the next 14 days and subjects were re-admitted to the hospital for another 15-hour study period. The dose was then further increased to 6 IU/day for the following 14 days, after which subjects were studied for the third time. Finally, all subjects had GH injections withdrawn for 2 weeks and were studied again. Group II went through one study period only; the study was performed from January to April.

Biochemistry

Serum osteocalcin (SOC) was measured by a radioimmunoassay modified from Price and Nishimoto [9] using rabbit antiserum against bovine osteocalcin. Intact, purified bovine osteocalcin [8] verified by amino acid analysis and antisera to bovine osteocalcin was generously provided by J. Poser (Procter and Gamble Company, Cincinnati, OH, USA). The antisera showed full cross-reactivity between human and bovine osteocalcin. The intra- and interassays coefficient of variations were 5 and 10%.

TAP in serum was measured spectrophotometrically using p-nitrophenylphosphate as substrate according to the method recommended by the Scandinavian Committee on Enzymes [23]. Interassay CV was 5% (mean = 218 U/liter, n = 24) and the intraassay CV was 2.5% (mean = 246 U/liter, n = 10).

Serum bone isoenzyme (lectin-precipitated) alkaline phosphatase activity (BAP) was determined by the method described by Rosalki and Foo [15]. Samples (300 μl) were pretreated with 30 μl Triton X-100 (20 g/liter) for 30 minutes at 37°C. An aqueous solution of wheat germ lectin (Sigma L-9640, 5 g/liter in distilled water) was then added, the samples were mixed, and were incubated for 30 minutes at 37°C. After centrifugation at 2,000 *g* in 10 minutes, the AP activity was determined as above and serum BAP was calculated as the difference between total and supernatant activity. The intraassay CV was 5% (mean = 309, n = 15) and the interassay CV 7% (mean = 322 U/liter, n = 12). TAP and BAP were only measured in samples drawn at 8 p.m., 8 a.m., and 11 a.m.

Statistical Analysis

Serum levels of the three parameters at 8 a.m. in group I and II

Table 2. Biochemical markers of osteoblastic activity in samples drawn at 8 a.m.

	Group I treated GH deficiency (n = 7)	Group II normal controls (n = 7)
S-osteocalcin (μg/liter)	57 ± 19	127 ± 38
SBAP (U/liter)	239 ± 48[a]	433 ± 69
STAP (U/liter)	349 ± 48	512 ± 74

[a] Significantly different from normal controls ($P < 0.05$)

were compared by unpaired *t*-test. In group I and II, area under the curves (AUC) for osteocalcin, TAP, and BAP profiles were calculated by trapezoidal integration and divided by the appropriate time intervals to give the integrated serum concentrations (S_iOC, S_iTAP, and S_iBAP, respectively). The differences between group I and II were tested by unpaired *t*-test and the dose-dependent effects in group I were analyzed by repeated measures analysis of variance (ANOVA).

In group I, the effects of time and rhGH dosage on the serum concentration profiles were analyzed by repeated measures ANOVA using Z-scores (difference from mean divided by SD). All statistical analyses were performed using SPSS (Statistical Package for Social Sciences). *P* values less than 0.05 were considered significant. All results are given as mean ± SE unless otherwise stated.

Results

The clinical data on the participating subjects are listed in Table 1. Table 2 shows the values of serum osteocalcin, BAP, and TAP obtained from measurements in samples drawn at 8 a.m. Patients on conventional replacement therapy (2 IU rhGH/day, sc) had lower levels of BAP than controls ($P < 0.05$).

In group I, a significant rise in the S_iOC was observed with increasing dosage of rhGH ($P = 0.02$) (Fig 1). The S_iBAP just failed to reach significance ($P = 0.08$), and the S_iTAP seemed unaffected by rhGH ($P = 0.50$) (Fig 1). Although the control group exhibited greater mean S_iOC, S_iTAP, and S_iBAP compared with the GH-deficient patients regardless of rhGH dosage, the observed differences did not reach significance due to a wide scatter of values in the control group.

In group II, serum osteocalcin increased gradually to maximum levels at 4 a.m.–8 a.m. followed by a subsequent decrease (ANOVA, $P = 0.14$) (Fig 2A). A similar pattern was seen during the first 13 hours in the GH-treated patients (ANOVA, $P < 0.01$) (Fig 2C–E). ANOVA revealed that in group I following 4 and 6 IU rhGH/day, the changes in serum osteocalcin with time were not different from those of group II (Fig 2A). However, the diurnal patterns observed in group I during treatment with

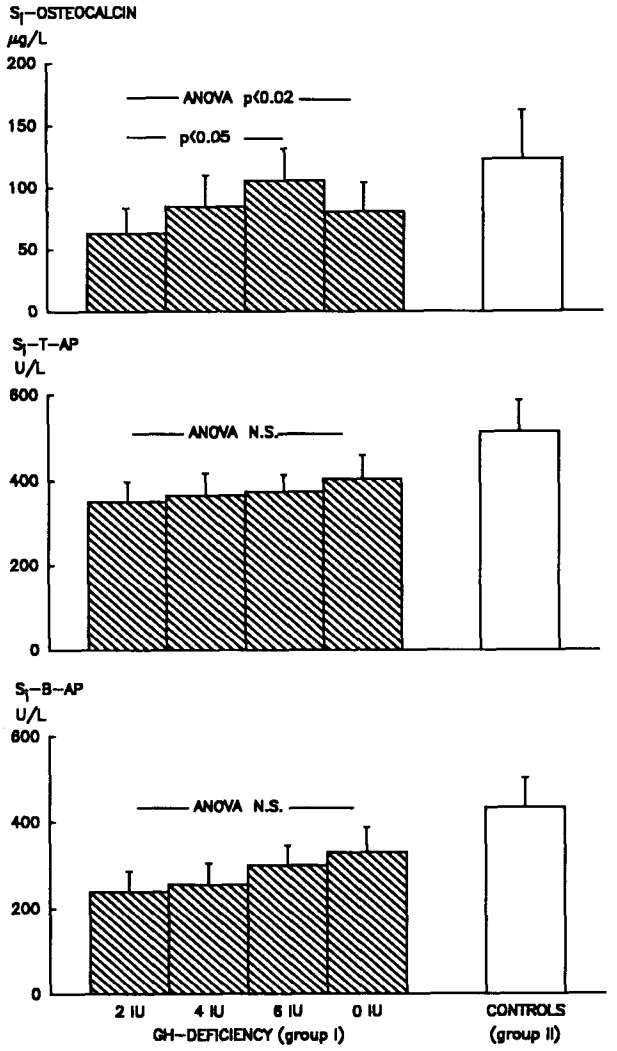

Fig. 1. Mean ± SE integrated serum levels of osteocalcin (S_iOS), total alkaline phosphatase (S_iTAP), and bone isoenzyme alkaline phosphatase (S_iBAP) (calculated from areas under the curves from 8 p.m. to 11 a.m.) in 7 GH-deficient patients treated with different doses of rhGH (group I) and 7 normal children (group II). Patients in group I were studied on four occasions: on conventional replacement therapy (2 IU/day), after 2 weeks administration of 4 IU/day, after additional 2 weeks with a further increase to 6 IU/day, and subsequently after 14 days off treatment. Injections were given sc at 8 p.m. In group I, a significant dose effect was seen in S_iOC (ANOVA, $P < 0.02$), the S_iBAP just failed to reach significance (ANOVA, $P = 0.08$), and the S_iTAP seemed unaffected by rhGH (ANOVA, $P = 0.50$). S_iOC was significantly lower during treatment with 2 IU/day compared with 6 IU/day ($P < 0.05$). The mean values of S_iOC, S_iTAP, and S_iBAP was higher in controls but these differences did not reach statistical significance.

2 IU rhGH/day (Fig 2C) and during the off-treatment period (Fig 2B) were significantly different from the pattern seen in controls ($P < 0.05$). Serum TAP and BAP did not display any significant variation with time in the two groups.

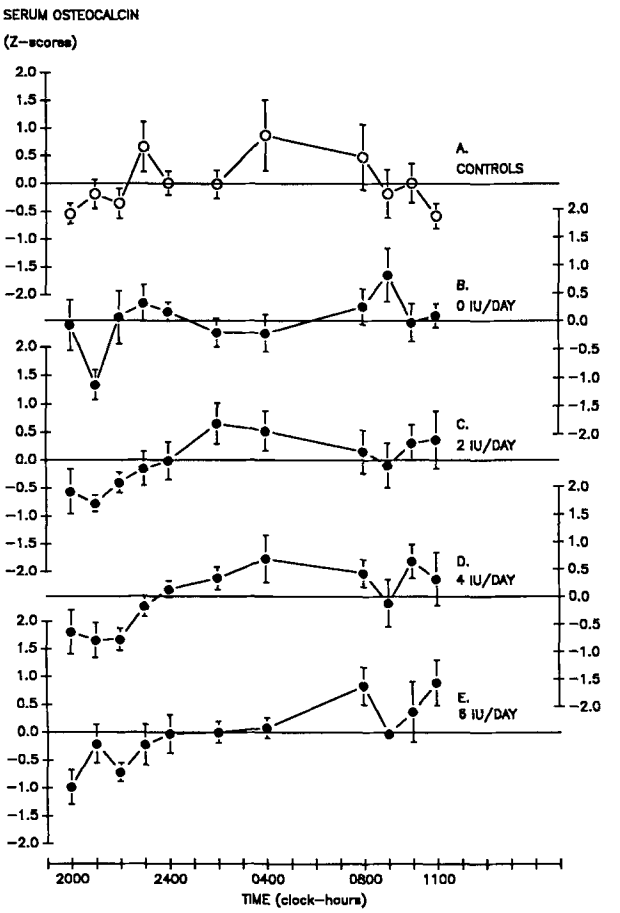

Fig. 2. Time-related variations in serum osteocalcin (Z scores) in 7 GH-deficient patients (closed circles) and in 7 normal controls (panel A, open circles). Patients were studied on four occasions: on conventional replacement therapy (2 IU/day) (panel C), after 2 weeks administration of 4 IU/day (panel D), after additional 2 weeks with a further increase to 6 IU/day (panel E), and subsequently after 14 days off treatment (panel B). Injections were given sc at 8 p.m.

Discussion

Patients with untreated GH deficiency have reduced serum levels of the indices of osteoblastic activity, when measured in single samples taken in the morning [13]. The present study suggests that patients on conventional replacement therapy (2 IU/day sc) have lower osteoblastic activity compared with controls, when evaluated on the basis of both BAP in morning samples and the nocturnal profile of serum osteocalcin. This is in contrast to the findings of Delmas et al. [13] who reported normal levels of osteocalcin during treatment with 4–8 IU given 2–3 times a week, corresponding to approximately 1–3.5 IU/day. The difference is further amplified by the observation that frequent injections by the subcutaneous route result in higher growth rates compared with less frequent intramuscular injections [24]. The discrepancy may be partly explained by different timing of blood sampling in relation to the GH administration, as our findings suggest an acute effect of rhGH on the postinjectional profile of serum osteocalcin.

The increasing levels of S_iOC during the study indicate a dose-dependent effect of rhGH on osteocalcin. However, as no wash-out periods were included, the increase in S_iOC observed following 2 weeks of 6 IU/day could have been partly induced by the first increase in dose from 2 to 4 IU/day.

In theory, serum levels of osteoblastic markers depend on the number of osteoblasts, the activity of single osteoblasts, and the metabolic clearance of the bone markers. As to the latter, serum levels of osteocalcin depend on renal function [25], which is not the case for BAP. The observed changes in osteocalcin during treatment cannot be explained by alteration in renal function, as both glomerular filtration rate and renal plasma flow are known to increase during treatment with hGH [26, 27]. This view is also supported by the almost parallel changes in S_iOC and S_iBAP. However, some differences between serum osteocalcin and BAP are to be expected because of their different serum half-lives, being 30 minutes for osteocalcin [12] and 1–2 days for BAP [28]. Further, some studies indicate that osteocalcin and BAP may be produced by osteoblasts differing with respect to phenotype or maturity [18, 29]. This may explain why serum osteocalcin decreased during the 2 weeks off treatment whereas serum TAP and BAP remained unchanged or even increased slightly.

The acute increase in serum osteocalcin seen after administration of rhGH during treatment with 4 and 6 IU/day most probably reflects the effect of rhGH on the activity of the present osteoblast population, as recruitment of new osteoblasts during the 15-hour study periods seems unlikely. On the other hand, both the effect of increased rhGH dosages on integrated serum levels and the observation that bone markers did not return to baseline values after 2 weeks off treatment support the theory of an expansion of the osteoblast population. However, it is not possible from our experiment to determine whether this was due to skeletal growth (modeling), activation of bone remodeling, or a combination of both. This would require invasive procedures (bone biopsy) and washout periods of several months which we judged unethical.

In normal adults, a diurnal rhythm in serum osteocalcin has been found by several investigators, showing a nocturnal acrophase around 2 a.m. and a nadir around noon [30–32]. Our results suggest that a nocturnal acrophase also exists in normal chil-

dren. Furthermore, the statistical analysis implies that the rhythm during rhGH treatment with 4 and 6 IU/day was not different from the physiological rhythm, whereas both 2 IU/day and discontinuation of therapy resulted in significantly different variations. Serial determinations of osteocalcin using hourly sampling has been shown to be a sensitive model system for evaluation of the effects of various hormones on osteoblastic activity [31, 32]. The present study suggests that patients lacking GH in serum do not have a normal physiological rhythm of osteocalcin. It also demonstrates that rhGH is capable of increasing nocturnal levels of serum osteocalcin and normalizing the nocturnal rhythm when given in doses of 4 and 6 IU/day but only partially in a dose of 2 IU/day. From inspection of the curves it seems that SOC may show a second increase during the hours from 10 a.m. to 11 a.m. in group I after 4 and 6 IU/day whereas SOC seems to fall in normals. There is no obvious explanation for this observation. Several hormones, capable of changing SOC, fluctuate during the day and mechanical load of the skeleton may play a role, but further studies are needed to clarify this point.

Patients and controls were matched according to pubertal development because serum levels of osteocalcin and BAP parallel the height velocity curve during the pubertal growth spurt in normal children [19, 20]. The study was completed within 3 months during wintertime and no significant seasonal variation in osteocalcin has been reported in these months of the year [33]. Moreover, the short individual observation period makes it unlikely that pubertal development or increase in height or weight during the study could account for the observed changes.

In conclusion, serial measurements of serum BAP and osteocalcin may be useful indicators of the response to treatment with rhGH. Our findings add to the evidence that substitution with 2 IU rhGH daily may be inadequate in GH deficiency [3], though the potential side effect of a higher GH dosage must be taken into careful consideration. The significance of the observed discrepancies between the bone markers, however, remains to be further elucidated.

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