Biochemical markers of bone metabolism in benign human osteopetrosis: a study of two types at baseline and during stimulation with triiodothyronine

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Abstract

Biochemical markers of bone remodelling were used to evaluate bone turnover in two types of autosomal dominant osteopetrosis (ADO) at baseline and during stimulation with triiodothyronine (T₃). Eight patients with Type I (aged 23–61 years, mean 40.4 years) and nine patients with Type II ADO (aged 20–49 years, mean 32.8 years) were compared with 10 normal controls (aged 22–54 years, mean 35.4 years). The participants were treated with 100 µg T₃ daily for 7 days and followed for a total of 16 weeks. Serum concentrations of T₃ increased and corresponding suppression of TSH was observed in all participants. Both formative and resorptive bone markers were normal at baseline. After stimulation with T₃, a significant increase was seen in all groups for the formative markers used. Secondary increments were observed at the end of the observation period for all groups, indicating activation of bone remodelling. A variety of resorptive markers was assessed, but no differences between patients and controls were seen. After stimulation, highly significant responses were found in all parameters in all groups, in accordance with stimulation of existing resorptive cells. However, no secondary increments were seen at the end of the observation period. A more pronounced response was found in crosslinks-related assays. The study demonstrates that it is possible to stimulate bone resorptive and formative cells with thyroid hormones in both types of ADO. Moreover, it indicates that the remodelling process is activated by a short course of T₃ treatment.

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Introduction

In order to evaluate bone metabolism at organ level and in a non-invasive manner, biochemical markers of bone remodelling and turnover are used increasingly (1). It is well recognized that the ideal marker has not yet been found. Several new assays, based in particular on Type I collagen degradation, have been developed, and new bone formative markers and second and third generations of specific formative markers have also been introduced (reviewed in (1) and (2)).

Osteopetrosis is a classical bone resorptive disorder, inherited in various mammalian species (3–7), that is characterized by normal bone formation and defective bone resorption. The different mutations involved exemplify defective bone resorption at different levels, all leading to a net accumulation of bone. In human osteopetrosis, two subtypes of the autosomal dominant inherited form are well described (8, 9). They have a typical different radiological phenotype on which the subtyping is based, and different clinical, biochemical, histological and ultrastructural pictures. The mutation in Type II has now been mapped, but not yet identified

(10). Bone metabolism is disturbed in the two subtypes, as judged by biochemistry (11), and, in a previous study (12), we showed that activation of bone metabolism with thyroid hormones was possible in benign osteopetrosis. However, this early report did not take the subtyping into account. In a previous report of the present study, we reported disturbed collagen metabolism in autosomal dominant osteopetrosis (ADO) (13).

In the present study, we evaluated different new biochemical markers of bone resorption and formation in two clinical disorders characterized by defective bone resorption, at baseline and after stimulation with thyroid hormones. Moreover, the aim was to study the possibility of activating bone remodelling with an exogenous stimulator, in the two subtypes of benign human osteopetrosis.

Material and methods

Participants and experimental design

Seventeen patients with autosomal ADO defined by standard radiographs and 10 normal controls

participated in the study. All were clinically and biochemically euthyroid before entering the study. There were eight patients with Type I ADO (five women and three men, aged 23–61 years, mean 40.4 years) and nine patients with Type II ADO (three women and six men, aged 20–49 years, mean 32.8 years). The normal controls (five women and five men, aged 22–54 years, mean 35.4 years) were recruited from the medical staff. All participants were treated with 100 µg triiodothyronine (T_3) daily for 7 days (13). The study was approved by the local ethics committee and conducted according to the Declaration of Helsinki II.

Biochemistry

Fasting blood samples were collected in the morning before the participant entered the study (week 0), after the treatment period (week 1), and 1 (week 2), 3 (week 4), 7 (week 8), 11 (week 12) and 15 (week 16) weeks after that. The samples were processed without delay, and serum stored at -40°C until required for assay.

Samples from individuals were measured in the same assay series. The serum concentrations of T_3 and thyroid-stimulating hormone (TSH) were analysed by RIA using standard laboratory methods.

Osteocalcin was measured by a first-generation assay (14) with intra- and interassay coefficients of variation (CVs) of 5% and 10% respectively (13). As a third-generation assay, intact osteocalcin concentrations were measured with a competitive-inhibition ELISA (provided by Osteometer Bio Tech A/S, Copenhagen, Denmark), which uses monoclonal antibodies (15). In addition to detecting intact osteocalcin(1–49) the N-terminal-Mid fragment(1–43) (N-MID) is also detected. The intra- and interassay CVs were 6.4% and 4.1% respectively.

Total serum alkaline phosphatase (ALP) activity was measured spectrophotometrically (intra- and interassay CVs of 2.5% and 5% respectively) and the serum concentration of bone isoenzyme alkaline phosphatase (S-LAP) was measured after lectin precipitation, as described in the previous report (13).

Urinary hydroxyproline was analysed using a kit from Organon Teknica (Netherlands). Intra- and interassay CVs were 10% and 12% respectively. The concentration of free deoxypyridinoline crosslinks in urine was measured using the Pylinks-D assay, D-Pyr (Metra Biosystems, Inc., CA, USA). The intra- and interassay CVs were 4–9% and 6–10% respectively.

Urinary concentrations of crosslinked N-telopeptides of type I collagen (NTx) were measured by ELISA using commercial kits from Ostex (Seattle, USA). The intra- and interassay CVs were 7.3% and 11.7% respectively.

Urinary concentrations of telopeptide-related fragment of type I collagen degradation products (β -CrossLaps and α -CrossLaps) were analysed using commercial kits from Osteometer Bio Tech A/S. The β -CrossLaps ELISA

(16) measures an isomerized form, whereas the α -CrossLaps RIA (17) reacts almost exclusively with the linear peptide form of the same amino acid sequence. According to the manufacturer, the intra- and interassay CVs were, respectively, 4.4% and 7.0% for the ELISA kit and 3.1% and 5.3% for the RIA kit. By calculating a ratio between the two CrossLaps assays (α/β), an index of degradation of isomerized collagen in relation to that of the non-isomerized form is obtained.

All urinary excretions were expressed as ratios relative to the urinary concentrations of creatinine.

Statistics

Differences between groups were compared by Mann–Whitney rank-sum test for unpaired data. For paired data, multiple analyses of variance (MANOVA) were performed *a priori*; if the outcome was significant, Wilcoxon's rank-sum test for paired data was performed *a posteriori*. Relations between variables were tested using Spearman's rank-correlation test and the level of statistical significance was chosen as $P < 0.05$.

Results

Thyroid hormones

After treatment with 100 µg T_3 daily for 1 week, the serum concentration of T_3 increased in all participants investigated and a corresponding suppression of TSH was observed (not shown), indicating increased effects of thyroid hormones on target tissues (13).

Bone formative markers

In order to evaluate the effects of stimulation by thyroid hormones on the bone remodelling process, serum osteocalcin and S-LAP were measured for the entire 16-week observation period. Serum osteocalcin concentrations were lower in both patient populations compared with those in normal controls, throughout the study period, but this was not statistically significant (Fig. 1A).

After stimulation with T_3 , a significant response was seen within all groups over time ($P < 0.001$, MANOVA), but not between the groups. During the stimulation week, serum osteocalcin increased in all groups, although not significantly in Type II patients (the delta-values between week 1 and week 0 are given in Table 1). Secondary increases were observed in the late observation period (Fig. 1A), although they were not significant for Type I patients.

The serum concentrations of bone-specific S-LAP over the entire study period are shown in Fig. 1B, and the delta values in Table 1. A significant response to T_3 treatment was observed ($P < 0.015$, MANOVA), but there were no differences between the groups. After stimulation, secondary increases were observed at

Figure 1 Serum concentrations of osteocalcin (BGP) (A), serum bone-specific leptin-precipitated alkaline phosphatase (SLAP) (B), and osteocalcin measured by the third-generation assay (N-MID) (C) in response to treatment with 100 µg T₃ daily for 1 week in patients with two types of ADO and in controls. Results are given as mean ± S.E.M. The *P* value in the upper right corner denotes the significance of the MANOVA test. The level of significance for each observation compared with baseline is given with a symbol for the group.

Table 1 Delta values (the difference between week 1 and week 0).

Marker	Delta value (%)			
	Control	Type I	Type II	Total
Formative				
BGP	47.7**	51.7*	34.8	44.7***
N-MID	58.6**	43.3*	34.9	45.6***
S-LAP	1.7	-1.7	-17.6*	-5.9
Resorptive				
OHP	44.2*	36.7*	34.2**	38.4***
NTx	78.4**	25.1	51.6*	51.7***
D-Pyr	20.7	38.7*	21.7	27.0***
α-CrossLaps	42.0*	49.2	28.4	39.9***
β-CrossLaps	75.8**	66.7*	46.5*	63.0***

BGP, osteocalcin. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 (Wilcoxon's rank sum test).

weeks 12 and 16; however, these were significant only for Type I patients at week 12 (*P* < 0.03).

Osteocalcin measured by the third-generation assay (N-MID) was only followed for the first 4 weeks of the observation period. N-MID showed a highly significant response to T₃ treatment (*P* < 0.001, MANOVA), but there were no differences between the groups (Fig. 1C). The delta values were 59% (*P* < 0.008), 43% (*P* < 0.02) and 35% (NS) for controls, Type I and Type II patients, respectively. The values returned to baseline at weeks 2 and 4.

The delta values for the formative markers are shown in Table 1. It may be seen that the third-generation assay for osteocalcin (N-MID) gave the highest value for each group (except for Type I patients) and also for the whole population. Correlations between the formative markers for the first 4 weeks are given in Table 2, both

Table 2 Bone formative markers: Spearman rank correlation relationship (*R*) at baseline (all groups) and after stimulation with 100 µg T₃ daily for 1 week and a 3-week observation period.

	<i>R</i>	
	BGP	N-MID
Baseline		
All groups		
N-MID	0.55**	
S-LAP	0.47*	0.65***
4 weeks		
Controls		
N-MID	0.94***	
S-LAP	0.54***	0.57***
Type I		
N-MID	0.14	
S-LAP	0.13	0.53**
Type II		
N-MID	0.84***	
S-LAP	0.39**	0.50**

BGP, osteocalcin. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

at baseline for the total population and for the 4-week observation period for each group. It can be seen that the results obtained using the two osteocalcin assays were highly positively correlated, both in the control group and in the Type II group. At baseline there was a positive correlation between all formative markers. After T_3 treatment, significant correlations were observed for all parameters in normal individuals and Type II patients. In Type I patients, only S-LAP and N-MID demonstrated significant correlations.

Bone resorptive markers

In order to evaluate the role of the resorptive markers in relation to the remodelling process, fasting renal excretion of hydroxyproline (OHP) and D-Pyr were followed for the total 16-week observation period. The urinary OHP/creatinine ratio did not differ between the groups, either at baseline or after stimulation, except for Type I patients, in whom the ratio at week 16 was significantly lower than that in the control group. After treatment with T_3 , a significant response was observed ($P < 0.001$, MANOVA; Fig. 2A). The delta values are given in Table 1. No secondary increases were observed at the end of the study period (weeks 12–16).

The fasting D-Pyr/creatinine ratio is shown in Fig. 2B. No differences were observed between groups at baseline. After stimulation, a significant response over time was observed ($P < 0.001$, MANOVA) within, but not between, the groups. The delta values were 21%, 39% and 22% respectively, significant only for Type I patients ($P < 0.03$). However, the values were still increased at the end of week 2, significant for Type II patients ($P < 0.03$). No secondary increases were observed.

The fasting NTx/creatinine ratio is shown in Fig. 2C. No significant differences were observed between the groups at baseline. After stimulation, a significant response over time was found ($P < 0.001$, MANOVA). The delta values were 78% ($P = 0.007$), 25% (NS) and 52% ($P < 0.04$) respectively. The value was still increased at the end of week 2 in the control group ($P = 0.05$).

The fasting β -CrossLaps/creatinine ratio is shown in Fig. 2D. There were no differences between the groups at baseline. A highly significant response over time was seen ($P < 0.001$, MANOVA) after stimulation with thyroid hormone. The delta values were 76% ($P < 0.007$), 67% ($P < 0.03$) and 47% ($P < 0.02$) respectively. The fasting α -CrossLaps/creatinine ratio is shown in Fig. 2E. No significant differences between the groups were observed at baseline. After stimulation, a significant response over time was seen ($P < 0.004$, MANOVA). The delta values were 42% ($P < 0.007$), 49% (NS) and 28% (NS) respectively.

Delta values for all resorptive markers are given in Table 1. NTx had the highest delta value, both in normal individuals and in Type II patients. In Type I

patients, however, and in the total population, β -CrossLaps demonstrated the highest delta values. Moreover, the delta values for Type II patients were lower than those for controls for all assays investigated, except D-Pyr. However, we could not demonstrate any statistically significant differences between these delta values.

At baseline, the resorptive markers were significantly correlated, except D-Pyr, which correlated with NTx and α -CrossLaps (Table 3). After stimulation with thyroid hormone, highly significant correlations between the markers were seen for controls. For Type I patients, the D-Pyr results correlated neither with the OHP nor with the CrossLaps results. For Type II patients, significant correlations were found for all assays.

The calculated ratio between the two CrossLaps assays (α/β ratio) during the 4-week observation period showed a significantly lower ratio for Type I patients ($P < 0.05$, area under the curve) compared with controls, whereas no significant difference was found concerning Type II patients.

Discussion

The present study shows that it is possible to activate bone metabolism in both types of benign human osteopetrosis, as judged by measurements of biochemical markers of bone resorption and formation. Exogenous T_3 has previously been shown to stimulate bone metabolism in normal individuals (18) and in patients with benign osteopetrosis (12). However, in the previous study the heterogeneity of benign human osteopetrosis was not taken into account. Moreover, the present study indicates that the remodelling process is activated, as the formative markers showed a secondary increase in the late phase of the 16-week observation period. This is best documented for the control group, in whom there were highly significant increases from week 8 to week 16 (Fig. 1A), whereas the secondary increases for the patient populations rely on a single P value for each of the patient groups (Fig. 1A, B).

A previous biochemical study of benign osteopetrosis indicated low concentrations of the formative marker, osteocalcin (11), and the same tendency was seen in the present study. However, the third-generation osteocalcin assay, which measured intact osteocalcin, did not show any difference between the groups and could thus not confirm this observation. It was not possible to demonstrate significant differences between the groups regarding delta values for any of the markers. Moreover, the groups did not differ qualitatively in response to thyroid hormone treatment. Thus, on the basis of the formative markers used, osteoblast function in both types of benign osteopetrosis seems to be normal, in accordance with previous radiogrammetric and histomorphometric studies (19–21).

The immediate and highly significant response to thyroid hormone for all groups, seen after the stimulation

Figure 2 Changes in urinary bone resorptive markers/creatinine ratios in response to treatment with $100 \mu\text{g}$ T_3 daily for 1 week in patients with two types of ADO and in controls. A, hydroxyproline (OHP)/creatinine ratio; B, free deoxypyridinum crosslinks (D-Pyr)/creatinine ratio; C, crosslinked N-telopeptides of type I collagen (NTx)/creatinine ratio; D, β -CrossLaps (β -CL)/creatinine ratio (by ELISA); E, α -CrossLaps (α -CL)/creatinine ratio (by RIA). Results are given as mean $\pm$ S.E.M. The P value in the upper right corner denotes the significance of the MANOVA test. The level of significance for each observation compared with baseline is given with a symbol for the group.

Table 3 Bone resorptive markers: Spearman rank correlation: relationship (*R*) at baseline (all groups) and after stimulation with 100 µg T₃ daily for 1 week and a 3-week observation period.

	<i>R</i>			
	NTx	OHP	D-Pyr	α-CL
Baseline				
All groups				
OHP	0.67***			
D-Pyr	0.49**	0.34		
α-CL	0.81***	0.68***	0.41*	
β-CL	0.71***	0.51**	0.35	0.77***
4 weeks				
Controls				
OHP	0.72***			
D-Pyr	0.49**	0.41**		
α-CL	0.91***	0.75***	0.44**	
β-CL	0.85***	0.56***	0.32*	0.77***
Type I				
OHP	0.66***			
D-Pyr	0.35*	0.33		
α-CL	0.73***	0.68***	0.28	
β-CL	0.73***	0.70***	0.28	0.92***
Type II				
OHP	0.53***			
D-Pyr	0.53**	0.62***		
α-CL	0.76***	0.56***	0.67***	
β-CL	0.78***	0.59***	0.73***	0.79***

α-CL, β-CL, α- and β-CrossLaps. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

week for all markers except for S-LAP, indicates a stimulation of existing formative and resorptive bone cells. It seems as if the immediate response on resorption lasts longer (best seen for the D-Pyr assay), in accordance with an activation of the remodelling process, as seen in normal individuals (18) and substantiated by the secondary increase in the formative markers. The time profile of the dynamics of the formative and resorptive markers in this study was in accordance with the duration of the remodelling process in trabecular and cortical bone (22, 23), demonstrated by histomorphometric reconstruction. The discrepancy between the response of the formative markers (osteocalcin and S-LAP) is most probably related to their different modes of expression during the formative process (2). This is in accordance with other dynamic studies of bone remodelling that used vitamin D (24) and growth hormone (25) as stimulators.

None of the assays was able to show decreased excretions of the resorptive markers in the patient populations, either at baseline or after stimulation, compared with that in normal controls. For most of the resorptive assays, the delta values were greater in the controls than in the patients, but none of these differences was significant. These observations are in contrast to findings in a previous biochemical study (11) and other documentation of defective bone resorption at tissue level (8, 9). The subjects in the present study were followed as outpatients and the urinary markers were measured in second-void urine. This might explain the lack of differences between the

groups at baseline, although the sensitivity of second-void samples for measuring resorptive markers, compared with 24-h urine collections, has been validated (26), at least for urine OHP. Another possible explanation might be that the control group was suboptimal when the markers were judged at baseline. In the present study, the same control group was used for both patient populations, as our earlier study (13) indicated that no significant differences might be expected to occur in either age or sex distribution. However, a more closely matched control group for each patient group might have given a better sensitivity. Obviously, the currently used resorptive assays, which have been developed for use in states of high turnover (osteoporosis) have a poor sensitivity in detecting decreased bone resorption.

For the formative markers, the third-generation osteocalcin assay (N-MID) seemed to give the greatest delta value for the subgroups, as it did for the whole population (Table 1). N-MID was very well correlated to the other formative markers in control and patient populations, except for osteocalcin in Type I patients. In the resorptive assays, the highest delta values were found for the crosslinks-related assays, NTx and β-CrossLaps. The good correlation between the resorptive assays corroborates the synchronization of the markers with the time of thyroid hormone stimulation. The significantly decreased α/β-CrossLaps ratio found for Type I patients suggests a more pronounced isomerization of the degraded bone collagen in Type I patients

(17), indicating a lower turnover of collagen in this disorder. A lower turnover is followed by an accumulation of old collagen, susceptible to isomerization. This is in accordance with the findings of a previous study, showing a decreased thermal stability of cortical bone collagen in Type I patients compared with controls (27), which also indicates accumulation of relatively old collagen. However, the alteration in cortical collagen turnover has not been confirmed histomorphometrically (19). This discrepancy is a subject for further investigation.

In conclusion, the present study demonstrates that it is possible to stimulate bone resorptive and formative cells with T_3 in both types of autosomal dominant osteopetrosis. Moreover, our results indicate that the remodelling process is activated. Of the resorptive assays evaluated, none was able to detect decreased resorptive function in the patients, either at baseline or after stimulation. However, the crosslinks-related assays seemed to give the most pronounced alterations, second to bone stimulation.

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