Abstract

Objective: Children and adolescents with type 1 (insulin-dependent) diabetes mellitus (T1DM) show several impairment of bone metabolism and structure, resulting in a higher risk of decreased bone mass and its related complications later in life. Alterations of the nuclear factor-κB ligand (RANK-L)/osteoprotegerin (OPG) system have been implicated in several metabolic bone diseases characterized by increased osteoclast differentiation and activation and enhanced bone resorption.

Design: We aimed to assess OPG levels and to investigate the possible relation between OPG levels, bone status and glycemic control in a group of prepubertal children with T1DM without microvascular complications.

Methods: Twenty-six prepubertal T1DM children (median age 9.9 years, range 4.1 – 13.1 years) were studied. In all patients, serum OPG, hemoglobin (Hb)A1c, parathyroid hormone (PTH) and 25-dihydroxyvitamin D (25-D) levels were evaluated. Bone quality was determined by measuring the attenuation of ultrasound waves by bone (broadband ultrasound attenuation (BUA)) at the calcaneal site. The data were compared with those of a group of 45 age-, sex- and body-size-matched healthy children.

Results: Children with T1DM showed a reduced Z-score BUA in comparison with the control group (Student's t-test, P < 0.0001). Plasma OPG levels were significantly higher in diabetic children than in controls (Student's t-test, P < 0.0001). In T1DM children, Z-score BUA values displayed a significant correlation with OPG (Student's t-test, r = -0.62; P = 0.001), and HbA1c (r = -0.59; P = 0.007). OPG levels were significantly correlated with HbA1c (r = 0.56; P = 0.008). In a multiple regression analysis including age, duration of diabetes, physical activity, calcium intake, mean HbA1c and Z-score BUA, only HbA1c significantly predicted serum OPG levels (beta 0.67; P = 0.003).

Conclusions: Prepubertal children with T1DM have a significant increase of OPG levels. OPG serum concentrations are correlated to calcaneal BUA and HbA1c values. OPG could be a new marker of reduced bone mass in children with T1DM.

European Journal of Endocrinology 153 879–885

Introduction

Bone metabolism and density in patients with type 1 (insulin-dependent) diabetes mellitus (T1DM) have been extensively investigated, and these patients seem to be at risk of decreased bone mass (1), impairing the attainment of peak bone mass and increasing the risk of osteoporosis with its related complications in later life (2–4). These patients also have accelerated atherosclerosis and a greater incidence of morbidity and mortality, secondary to premature cardiovascular disease, than in the general population (5, 6). Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor (TNFR) family (7). In mice, OPG mRNA expression has been demonstrated in numerous tissues, including calvaria, skin, liver, lung and heart (8). OPG is a circulating secretory glycoprotein without a transmembrane domain, and it works as a decoy receptor for the receptor-activator of the nuclear factor-κB ligand (RANKL) (9). RANKL and OPG are a key agonist/antagonist cytokine system, regulating important aspects of osteoclast biology, such as differentiation, fusion, survival, activation and apoptosis (8). RANKL increases the pool of active osteoclasts by activating its specific receptor RANK located on osteoclastic cells, thus increasing bone resorption, whereas OPG, which neutralizes RANKL, has the opposite effect. Alterations or abnormalities of the RANKL/OPG system have been implicated in different metabolic bone diseases characterized by increased osteoclast differentiation and activation,
and by enhanced bone resorption, including glucocorticoid-induced osteoporosis, hyperparathyroidism, Paget’s disease, rheumatoid arthritis and bone malignancies (10, 11).

OPG is expressed also in the heart and vascular wall in the rodent, and OPG-deficient mice exhibit severe osteoporosis and vascular calcification of the aorta and renal arteries (12). In vitro OPG prolongs endothelial cell survival by preventing apoptosis (12). In man, OPG has also been implicated in atherosclerosis (13). Serum OPG levels have been associated with fatal strokes and overall vascular mortality in elderly women (14) and coronary artery disease in men (15, 16). The quantitative high-frequency ultrasound technique (QUS) has been proposed to assess bone density and bone structure (17–19) in adults and children, as well as in T1DM patients (20). By this technique, two parameters can be simultaneously determined: speed of sound (SOS) and broadband ultrasound attenuation (BUA). In normal adults and children, BUA seems to be the parameter that shows the highest correlation index with bone mineral density (BMD) determined by dual energy X-ray absorptiometry (DEXA) (21, 22), currently considered the reference standard in the evaluation of bone mass (23, 24). However, this diagnostic procedure presents some limits, in that it exposes subjects to ionizing radiation and does not provide a measure of true bone density (25, 26). In this study, we evaluated OPG levels and bone status in a group of prepubertal T1DM children. We also investigated the possible relationship between OPG levels, bone status and glycemic control in these patients.

Subjects and methods

Subjects

The study consisted of 26 prepubertal children with T1DM, 18 boys and 7 girls (median age 9.9 years; range 4.1–13.1), recruited from September 2003 to May 2004 at Meyer Children’s Hospital in Florence, Italy. T1DM was defined by the National Diabetes Data Group (27). All children had T1DM for at least 1 year. The duration of their diabetes was 5.5 ± 3.2 years (range 1.4–10.2). They were taking no medications other than insulin at the moment of study. Children with hypertension, signs of diabetic retinopathy or nephropathy, and electrophysiologic abnormalities of autonomic or peripheral neuropathy were excluded. Forty-five age-, sex- and body-size-matched healthy children (median age 9.6 ± 3.3 years; range 6.3–12.8) were also recruited. Written informed consent was obtained from all participants after the appropriate institutional review boards had approved the study protocol.

Study protocol

Participants completed a questionnaire that was reviewed by an interviewer during the baseline examination. The questionnaire related to current medications, family and personal medical history, fracture history, age of diabetes onset, insulin regimen, calcium intake and physical activity. At baseline examination, we measured height, weight, body-mass index (BMI) and gross nutritional status. BMI was calculated as weight divided by height squared (kg/m²). Age-related reference values of height, bone age and BMI were those currently used in Italy, obtained from a wide sample of Italian children (28). Bone age was evaluated through radiographs of the left hand and wrist, and was calculated by Greulich and Pyle’s method (29). Height, bone age and BMI were normalized for chronological age by conversion to s.d. score (SDS). SDS was calculated by the following formula: patient value – mean of age-related reference value/s.d. of the age-related reference value. Gross nutritional status was evaluated by concentrations of serum albumin, urea, calcium, parathyroid hormone (PTH), 25-dihydroxyvitamin D (25-D), erythrocyte count and hemoglobin, and average corpuscular hemoglobin concentration and corpuscular volume. In these patients, clinical (diabetes duration, blood pressure and insulin dose) and laboratory data (glucose, hemoglobin (Hb)A1c and serum creatinine concentration) were also evaluated. Blood pressure was measured by a standard clinical sphygmomanometer on the right arm after a 10-min supine rest. Physical activity was assessed by a modified activity score composed of the scores for sports/leisure activities (0, < 2 or > 2 h per week) (30). This information was obtained with an activity questionnaire. In all T1DM patients, the presence of microvascular complications was evaluated. Retinopathy was excluded by stereoscopic fundal photography of seven fields. Microalbuminuria was assessed by the mean albumin excretion rate (AER), calculated from three consecutive, timed, overnight urine collections. Microalbuminuria was defined as AER of ≥ 20 µg/min in two of three samples or an albumin/creatinine ratio of ≥ 2.5 mg/mmol.

Laboratory methods

Serum OPG levels were measured by enzyme-linked immunosorbent assay (ELISA) with a mouse monoclonal antibody as capture antibody and a rabbit polyclonal antibody for detection (Immundiagnostik, Bensheim, Germany). The assay detects both monomeric and dimeric forms of OPG, including OPG dimer and intact (1–84) PTH concentrations were determined.
by a two-site chemiluminescent immunometric assay (Nichols Diagnostics, San Juan Capistrano, CA, USA). The normal range is 10–65 pg/mL. The interassay coefficient of variation was 10%. Serum 25-dihydroxy-vitamin D (25-D) was determined by competitive binding protein assay (Nichols Diagnostics). The interassay coefficient of variation was 8%. The normal range is 28–65 nmol/l.

### Calcaneal determination

Bone quality was determined by measurement of the ultrasound wave attenuation by bone (BUA) in the frequency range 200–600 kHz. BUA (dB/MHz) was measured at the calcaneal site by two 12.5 mm transducers mounted in hand-held calipers linked to the pediatric contact ultrasound bone analyzer (CUBA) (McCue Ultrasonics Limited, Compton, Winchester, UK) (31–33). The pediatric CUBA is a specific system containing normative data for children aged 5–15 years (Z-score = 0; S.D. = 1). We have established reference values for children younger than 5 years. These reference values were fully comparable to CUBA normative data. For the present study, we also evaluated an age-, sex- and body-size-matched control group of 45 children. Z-scores (difference between the patient value and the age-specific mean value divided by the normal group’s S.D.) were calculated in each patient (31, 34). The evaluations were made and analyzed by the same operator, and each value was the average of three consecutive calculations. Quality assurance was performed daily. The in vitro coefficient of variation for BUA using phantoms was 1.8%, and the in vivo coefficient of variation for BUA was 3.7%.

### Statistical analysis

The data was processed by the SPSSX (SPSS Inc., Chicago, IL, USA) statistical package. All results are expressed as mean ± S.D. Comparison among groups was made by Student’s t-test (unpaired). For noncontinuous variables, the chi-square test with Yates correction was used. Spearman’s (rank) correlation test was used to determine the correlation coefficients, and multiple stepwise regression was used to determine the variables that may correlate independently with Z-score BUA values. P values of <0.05 were considered statistically significant.

### Results

Clinical and laboratory characteristics of the subjects and controls are shown in Table 1. No statistically significant differences were found among our group of patients with T1DM and controls in height, bone age and BMI, all expressed as SDS (Table 1). As expected, HbA1c values were higher in children with T1DM than in controls (8.4 ± 1.1% vs 4.2 ± 0.2%; P < 0.0001). Z-score BUA (Table 1) appeared to be reduced in patients with T1DM ( −0.46 ± 0.84 vs 0.21 ± 0.65; P < 0.0001). Plasma OPG levels (Table 1) were significantly higher in children with T1DM than in controls (71.6 ± 14.6 pg/mL vs 40.2 ± 12.0 pg/mL; P < 0.0001). The quantitative assessment of physical activity in T1DM and controls showed no significant differences. The percentage of current physical activity levels of the three categories was similar in the two groups (0 h per week group, 21% and 25% respectively; <2 h per week group, 56% and 49% respectively; >2 h per week group, 23% and 25% respectively). No statistically significant differences were found between our group of children with T1DM and controls in nutritional status markers, particularly calcium intake, PTH and 25-OH-D vitamin levels (Table 1). Nor were statistically significant differences found between the two groups in history of fractures and mean creatinine and blood pressure values. Spearman’s rank correlation test showed that, in children with T1DM, Z-score BUA values displayed a significant correlation with OPG (r = −0.62; P = 0.001) (Fig. 1A), and HbA1c (r = −0.59; P = 0.007) (Fig. 1B). Furthermore, OPG levels were significantly correlated with HbA1c (r = 0.56; P = 0.008) (Fig. 1C). Duration of diabetes was not correlated with OPG levels and BUA Z-score measurements. In a multiple regression analysis including age, duration of diabetes, insulin regimen, physical activity, calcium intake, mean HbA1c and Z-score BUA, only HbA1c was identified as a significant predictor of serum OPG (beta 0.67; P = 0.003).

### Discussion

The major finding of this study is the presence of increased serum concentration of OPG in children with T1DM. OPG is an osteoclastogenesis inhibitory factor, a critical molecule for the morphogenesis and remodeling of bone, and a number of studies have been performed to assess its importance with respect to the human skeletal
system (35). Increased OPG levels have been demonstrated in several chronic diseases, such as arthritis, and in T2DM adults (35). OPG has been hypothesized as representing a compensatory response to bone and vascular damage. Children and adolescents with T1DM are at risk of decreased bone mass and its related complications later in life (2–4), and our data confirm that these patients show reduced bone mass (3–5, 23, 24) and significant impairment of bone quality (7). Patients with T1DM also seemed to show reduced bone mass at the time of clinical diagnosis (36), and cohort studies indicate that diabetic patients may have a higher risk of

Figure 1 Relationship between (A) Z-score BUA values and osteoprotegerin (OPG) serum levels ($r = -0.62; P = 0.001$); (B) Z-score BUA values and HbA1c ($r = -0.59; P = 0.007$); and (C) OPG serum levels and HbA1c ($r = 0.56; P = 0.008$).
Skeletal involvement in patients with T1DM has a complex pathogenesis (36), and, despite numerous papers on this problem, many questions remain unanswered. In these patients, several mechanisms may contribute to skeletal damage, such as increased urinary excretion coupled with lower intestinal absorption of calcium, inappropriate homeostatic response to PTH, impaired vitamin D metabolism regulation, reduced or increased insulin-like growth factor (IGF)-1 concentration, the effects of the accumulation of glycated end products on bone tissue, and extraskeletal factors due to neuropathic and microangiopathic complications (37). Furthermore, the results regarding the metabolic disturbances leading to skeletal involvement in patients with diabetes have given nonuniform, contradictory results, and these apparent discrepancies may be ascribed to the different selection criteria of several studies, in terms of possible confounders, such as patients’ age, pathogenesis of diabetes, degree of metabolic control, and presence or absence of complications (37). In these patients, urinary calcium excretion has been found to be normal (38), increased (37), and possibly associated with lower duodenal calcium absorption. Physiologically, the reduced intestinal absorption, together with the increased urinary calcium excretion, should induce a compensatory increase of PTH secretion. Even though increased circulating PTH has been reported in one study on a small group of poorly controlled T2DM patients (39), most studies demonstrated normal or even low PTH concentrations (38, 40, 41). These patients showed PTH secretion lower than expected for homeostatic needs (42), configuring a state of ‘functional hypoparathyroidism’, as confirmed by dynamic challenge studies, such as during citrate-induced hypocalcemia (43) or hyperinsulinemic hypoglycemia (44), or after an oral glucose tolerance test (45). This functional hypoparathyroidism has been considered responsible for the low bone turnover (46). Several studies have demonstrated, in T1DM, an altered balance among vitamin D metabolites, showing a marked reduction of 24,25-D levels (47), or a decreased synthesis of vitamin D-binding protein by the liver, decreased renal 1α-hydroxylase activity, and reduced vitamin D receptor concentrations (37). Patients with T1DM also showed impaired bone structure, and decreased collagen strength, probably derived from abnormal glycosylation and cross-linking of skeletal collagen, was observed in chronically uncompensated diabetes mellitus (37). These qualitative changes may induce bone fragility that exceeds even the effects of reduced bone mass. To the best of our knowledge, our study is the first to assess bone mass and quality by the ultrasound technique at the calcaneal site in children with T1DM. QUS is a useful method for measuring the physiologic bone development in childhood and adolescence (48); the diagnostic accuracy of ultrasound measurements in identification of fracture risk associated with osteoporosis has been demonstrated in adults in both retrospective (49, 50) and prospective (51, 52) studies. Previous studies utilizing single-photon absorptiometry of the distal forearm showed that, in T1DM, a moderate decrease in cortical appendicular BMD was already present in the first years after manifestation of diabetes (36, 53 – 55). In our patients, we did not find a significant history of fractures, even though we reported a degree of osteopenia, averaging 0.8 – 1.0 S.D. below that of controls. Since childhood and adolescence are crucial periods of life for the attainment of optimal bone mass (56, 57), we could speculate that T1DM children seem to be at risk of decreased bone mass (2, 3, 58 – 61), which may restrict the attainment of peak bone mass (62) and, eventually, lead to increased risk of osteoporosis and its related complications in later life (63). Our data also confirm that, in these patients, bone mass is inversely correlated with HbA1c (4). In adults with T2DM, increased OPG values appear to be related to cardiovascular risk (14) and microvascular complications (13). OPG is abundantly expressed in the media of large arteries (7), in atherosclerotic plaques (64), and in vascular smooth muscle cells (65). Furthermore, OPG has been demonstrated to act as a survival factor for endothelial cells (66), and mice lacking the ability to produce OPG develop vascular calcification (67). Diabetes mellitus is a well-established risk factor for the early development of accelerated atherosclerosis and microangiopathy (68, 69). These vascular complications of diabetes are not clinically evident in children (68). However, subclinical vascular involvement, in the form of impaired endothelial function and increased carotid intimal-medial thickness, has been demonstrated in young subjects with T1DM (68, 70). Further prospective studies are needed to establish whether increased OPG levels in diabetic children can predict later development of endothelial dysfunction and vascular complications. In conclusion, OPG levels are significantly higher in children with T1DM; a significant correlation between OPG, bone mass, bone quality and HbA1c values has also been demonstrated. OPG could be a marker of reduced bone mass and vascular damage in children with diabetes.

References


8 Horowitz MC, Xi Y, Wilson K & Kacena MA. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine and Growth Factor Reviews* 2001 **12** 9–18.


27 ADA. Diagnosis classification of diabetes mellitus. *Diabetes Care* 2005 **28** S37–S42.


