INCREASED SERUM OSTEOPROTEGERIN IN PATIENTS WITH PRIMARY ADRENAL INSUFFICIENCY RECEIVING CONVENTIONAL HYDROCORTISONE SUBSTITUTION

Patients treated for primary adrenal insufficiency (PAI) are at risk of steroid over-replacement, which may affect their skeleton. The study was aimed to investigate the effect of steroid substitution on serum osteoprotegerin and receptor activator of nuclear factor kappa-beta ligand (RANKL) levels in relation to bone mineral density (BMD) in PAI. Eighty patients (mean age 47.2±14.5 years, mean hydrocortisone dose 0.49±0.14 mg/kg/day) and 63 healthy subjects were included. Serum osteoprotegerin, RANKL, 25-hydroxyvitamin D₃, calcium, phosphate, alkaline phosphatase, intact parathormone, and dehydroepiandrosterone-sulfate levels were evaluated in patients and controls. BMD was assessed in affected subjects using dual-energy X-ray absorptiometry. Mean osteoprotegerin concentration in PAI patients appeared significantly higher than controls (p=0.002), while RANKL levels were similar (p=0.430). Serum osteoprotegerin increased with age (p<0.001), but showed no correlation with daily hydrocortisone dose. Osteoprotegerin was negatively correlated with serum dehydroepiandrosterone-sulfate (p=0.008) and with BMD at the lumbar spine (p<0.001) and femoral neck (p=0.003). RANKL correlated negatively with PAI duration (p=0.029) and positively with daily hydrocortisone dose (p=0.018). Lumbar spine osteoporosis and osteopenia were found in 12 and 31 patients, respectively, whereas in femoral neck: in 5 and 33 individuals. Patients with osteoporosis displayed higher osteoprotegerin levels, but after the age-adjustment the correlation was lost. In conclusion, increased osteoprotegerin in PAI might reflect a compensatory response to enhanced bone resorption due to exogenous steroid excess and/or result from a deficit in adrenal androgens. RANKL levels remain within normal range on standard steroid replacement.

Key words: glucocorticoids, osteoprotegerin, nuclear factor kappa-beta ligand, primary adrenal insufficiency, steroids, bone density

INTRODUCTION

Patients suffering from primary adrenal insufficiency (PAI) require lifelong glucocorticoid substitution, but individual dosage of exogenous steroids may be difficult to adjust (1). Hydrocortisone (HC), commonly prescribed in replacement therapy, is identical with the natural cortisol molecule. Unfortunately, with the currently used HC preparations, serum cortisol often rises sharply to supraphysiological levels after oral administration, and then rapidly declines within a few hours. Standard replacement scheme fails to mimic perfectly the natural circadian rhythm of cortisol secretion, leading to serum cortisol peaks and troughs, and to exacerbated adrenocorticotropic hormone (ACTH) secretion early in the morning, before the ingestion of the HC tablet. Hence, measurement of ACTH level is an unreliable indicator of adequate hormonal substitution in PAI (1). Moreover, attempts to bring the elevated ACTH back to the reference range may result in steroid over-replacement. One of the most prominent adverse effects of glucocorticoid excess involves the skeleton, especially its parts with high trabecular bone content. Steroids reduce bone formation and promote bone resorption via decreased intestinal calcium absorption, suppression of gonadal hormones, blunting of the growth hormone axis, and through direct inhibitory influence on osteoblasts (2). Furthermore, as evidenced in mice models, glucocorticoids contribute to prolonged osteoclast survival (3). Although high, anti-inflammatory doses of steroids are a widely recognized risk factor for osteoporosis, studies of bone mineral density (BMD) in hypoadrenal patients on steroid substitution have revealed inconsistent results (4-9). Likewise, evaluation of bone turnover markers did not provide unequivocal insights into specific features of the bone metabolism in these patients (7-10).

Recently, the central role of the receptor activator of nuclear factor kB (RANK) - RANK ligand (RANKL) - osteoprotegerin (OPG) signalling system was highlighted in bone remodelling (11, 12). Upon their differentiation from haematopoietic stem cells, osteoclast precursors begin to express RANK on their surface (13). RANK is activated by its ligand, RANKL, a cytokine that belongs to the tumor necrosis factor (TNF) family and is produced by osteoblasts, endothelium and activated T cells (11). RANK-RANKL interaction enhances osteoclast maturation and survival (12, 13). The biological effects of RANKL are counterbalanced by OPG, a soluble decoy receptor derived from osteoblasts, which competitively blocks the access of RANKL to its cognate receptor (12, 14). While RANKL is bound by OPG, osteoclasts cannot fuse, function and survive.
Therefore, OPG is a natural endogenous regulator of RANK-RANKL signalling. Transgenic animal models suggest that OPG overexpression might prevent bone loss and increase bone mineral density (14). Denosumab, a monoclonal human antibody, which specifically binds RANKL and mimics the bone-protecting action of OPG, has recently been approved for treatment of postmenopausal osteoporosis (15).

Many hormones, various metabolic states, inflammation, and bone malignancy may affect the RANKL/OPG equilibrium, thereby altering the bone turnover (16). According to in vitro studies, an imbalance between RANKL and OPG synthesis is considered one of the key mechanisms of glucocorticoid-induced bone loss (17). The aim of this study was to investigate the effect of the standard HC replacement therapy on serum OPG and RANKL levels in PAI patients with regard to their BMD. Potentially, these data might provide clinically useful information and guidance while adjusting the HC dosage in patients with PAI.

MATERIAL AND METHODS

Subjects

The study comprised 80 patients with PAI (56 females and 24 males) and 63 sex- and age-matched control subjects (44 females and 19 males) from the Polish Caucasian population. The mean age of the affected individuals was 47.2±14.5 years and their mean disease duration was 12.6±10.6 years. The diagnosis of primary adrenal failure was based on typical signs and symptoms, with low serum cortisol levels accompanied by elevated plasma ACTH, and confirmed by a lack of response of adrenal cortex to ACTH(1-24) stimulation (no rise in serum cortisol levels assessed after intravenous injection of 250 µg of tetracosactrin, a synthetic polypeptide composed of the first 24 amino acids contained in the naturally occurring ACTH) (18). The autoimmune origin of the disease was demonstrated with positive results of serum antibodies to 21-hydroxylase, the main adrenal autoantigen. Patients with autoimmune polyendocrine syndrome type 1, congenital adrenal hyperplasia, and individuals who underwent adrenalectomy were excluded from the study. At admission, mean daily HC replacement dose was 0.49±0.14 mg/kg, i.e. 18.7±4.8 mg/m² body surface. Additionally, 62 patients were on mineralocorticoid replacement with fludrocortisone (0.05–0.1 mg daily) and 16 used a dehydroepiandrosterone (DHEA) supplement (10–25 mg/day). All patients were either euthyroid or on adequate levothyroxine replacement, with the TSH and free T4 levels within normal reference ranges. Five PAI subjects were also suffering from type 1 diabetes. Thirty four (61%) of the 56 female patients were postmenopausal, and 4 of them received oestrogen replacement therapy. Individuals on bisphosphonate treatment were excluded. Patients were recruited from the inpatient and outpatient endocrine clinics at Poznan University of Medical Sciences. The control group was recruited among healthy blood donors from the Regional Blood Transfusion Centre in Poznan. Their mean age was 46.8±13.7 years. None of them was receiving any medication that could affect bone metabolism, although no data on osteoporotic fractures were available. All study participants presented with normal renal and liver function tests. The study protocol was approved by the Ethics Committee at Poznan University of Medical Sciences. Informed written consent was obtained from all participants.

Serum laboratory tests

Serum samples were collected after overnight fast, before the morning hydrocortisone dose, and stored in multiple aliquots at −70°C until analysed. Serum OPG and total RANKL levels were determined using commercially available ELISA kits: Osteoprotegerin (Biomedica, Wien) with detection limit 0.14 pmol/l (inter- and intra-assay CV: 5.5% and 4.6%, respectively), and Human sRANKL total (BioVendor GmbH, Heidelberg) with detection limit 0.4 pmol/l (inter- and intra-assay CV: 11.2% and 7.3%, respectively). 25-hydroxyvitamin D3 [25(OH)D3] levels were assessed by a radioimmunoassay, 25OH vitamin D3-RIA (DiaSource, Louvain-la-Neuve) with detection limit 3.0 pmol/l (inter- and intra-assay CV: 7.2% and 7.3%, respectively). The analyses were performed in duplicate. Serum calcium (total and ionized), phosphate, total alkaline phosphatase activity (ALP), intact parathormone (iPTH) and dehydroepiandrosterone-sulfate (DHEAS) concentrations were evaluated by means of the standard ECLIA assays (Cobas 6000, Roche Diagnostics).

Bone mineral density

BMD at the lumbar spine and femoral neck was analysed in the PAI patients by dual-energy X-ray absorptiometry (DXA) (Lunar DPX, Lunar Corp. Madison, WI). Based upon the WHO criteria, osteoporosis was diagnosed according to the BMD measurements with a T-score at or below 2.5 standard deviations (S.D.), whereas osteopenia was diagnosed with a T-score between 1.0 – 2.5 S.D. Z-score, which expresses sex- and age-adjusted BMD, was also considered, as it is often regarded as an indicator of secondary osteoporosis (19).

Statistical analysis

Statistical calculations were performed by means of SPSS 18.0 software (SPSS Inc., Chicago, IL). Data are presented as means ±S.D. Their distribution was evaluated using the Kolmogorov-Smirnov test. Normally distributed data were compared using the unpaired Student t-test, and those with non-normal distribution were analysed by the nonparametric Mann-Whitney test. Comparisons between three groups displaying different BMD were performed using one-way ANOVA or Kruskal-Wallis test for nonparametric data. Statistical correlations were assessed by calculation of Pearson’s or Spearman’s coefficient, depending on data distribution. Multiple regression analysis was used to determine which variables correlate independently with OPG and RANKL levels. Two-tailed p-values <0.05 were considered statistically significant.

RESULTS

Serum laboratory tests

The biochemical parameters, total and ionized serum calcium, phosphate, ALP, and 25(OH)D3, were within their reference ranges in both studied cohorts (Table 1). Total serum calcium and phosphate levels did not reveal significant differences between patients and the control group. Despite lower ionized serum calcium (p=0.031), iPTH levels were not increased among PAI patients. Serum DHEAS concentrations were significantly decreased in affected individuals, even with their mean value slightly elevated by DHEA supplementation in 16 patients. Mean OPG serum concentration in PAI patients (3.03±0.68 pmol/l) appeared significantly higher than in controls (2.47±1.05 pmol/l, p=0.002). In contrast, mean RANKL levels remained similar in both groups (502.3±489.5 vs. 477.8±358.3 pmol/l, p=0.430).

Bone mineral density

Mean BMD at the lumbar spine in PAI patients was 0.92±0.16g/cm², and 1.09±0.17g/cm² at the femoral neck.
Frequencies of osteopenia and osteoporosis among affected subjects are presented in Table 2. Patients stratified according to their bone mineral status displayed significant differences in age (p<0.001 for both the lumbar spine and femoral neck), with the highest values observed among subjects with osteoporosis. Correlations of osteoprotegerin and RANKL levels

Serum OPG was found to increase with age (p<0.001) and with disease duration (p=0.001) (Table 3), although the latter correlation was lost in multiple regression analysis with age-adjustment. In contrast, OPG presented no correlation with daily HC dose adjusted for body surface. OPG levels were negatively correlated with the serum DHEAS concentration (p=0.008) and with BMD at the lumbar spine (p=0.001) and the femoral neck (p=0.003). In a multiple regression model, patient’s age, together with serum DHEAS level appeared best predictors of the serum OPG concentration (r²=0.31, p<0.001), whereas the significance of BMD was lost. Accordingly, when the BMD was assessed using Z-scores in order to adjust the results for patients age, the correlation with OPG was no more detectable (p=0.097 for the lumbar spine, and p=0.662 for the femoral neck).

Soluble RANKL concentrations correlated negatively with PAI duration (p=0.029) and positively with daily HC dose/m² body surface (p=0.018). No correlation between RANKL and patients age, DHEAS levels or BMD at any skeletal location was found (for detailed results see Table 3).

DISCUSSION

The current study revealed a significant increase in circulating OPG levels among the PAI patients receiving conventional steroid replacement. This finding might derive...
from multiple reasons, although a mild glucocorticoid excess seemed likely to be involved. Bone formation is highly sensitive to glucocorticoids and even as little as 2.5 mg prednisone per day may increase the risk of fractures (20, 21). As estimated by stable-isotope dilution and mass spectrometry, physiological daily cortisol production rates in healthy individuals range between 5–10 mg/m2 of body surface (22). Allowing for bioavailability and first-pass hepatic metabolism, this would be equivalent to a replacement dose of 10–12 mg/m2 (about 15–25 mg HC daily), considerably lower than traditional steroid substitution regimens. During the study, the HC dosage in our cohort ranged from 6.6 to 31.3 mg/m2 (mean dose of 18.6±4.7 mg/m2 per day). This dosage is relatively high considering the current recommendations, and raises particular concern about long-term adverse effects on bone (1).

The biochemical investigations in our PAI cohort did not reveal major differences compared to the matched healthy subjects, apart from decreased ionized serum calcium levels (Table 1). However, total calcium was similar in both groups and the ionized calcium levels always remained within the reference range in PAI patients. Moreover, slightly lower ionized calcium in affected subjects was not associated with any remarkable rise in iPTH levels, which remains in line with previously published observations (10, 23). Serum 25(OH)D, vitamin levels ranged from an insufficient level of 28.4 nmol/L in PAI patients and 28.9 nmol/L in healthy controls, up to 132.5 nmol/L and 142.8 nmol/L in each group respectively, with no significant difference between both cohorts (p=0.082). These data indicate that conventional treatment of PAI is not associated with major disorders of calcium homeostasis, which might otherwise favour bone loss.

Given that in vitro studies in human osteoblastic cells reveal a suppressive effect of glucocorticoids on OPG synthesis (17), elevated serum OPG levels in our PAI patients may seem surprising. Additionally, according to some clinical data, short-term systemic glucocorticoid treatment leads to a rapid decrease in serum OPG within the first weeks of the therapy, without major changes in serum calcium, iPTH and ALP (24). However, an increase in serum OPG has been consistently described among subjects with Cushings’s syndrome, who suffer form long-lasting cortisol overproduction (25-27). Therefore, some authors suggest a bimodal OPG behaviour in response to glucocorticoid excess: a rapid initial OPG decline, which reflects a suppressive effect on osteoblasts, followed by a compensatory long-lasting increase in OPG synthesis to protect the bone from the deleterious steroid influence (27). This would be consistent with previously reported early severe reduction in BMD within the first months of systemic glucocorticoid therapy, and much slower decrease in bone mass observed thereafter (28).

Higher circulating OPG levels are often found in patients with osteoporosis and are usually regarded as a reflection of the increased bone turnover and a compensatory response to excessive osteoclast activity (29-32). Hence, it is possible that deteriored BMD might contribute to elevated OPG in the current cohort. A considerable proportion of our PAI group displayed decreased BMD: osteopenia or osteoporosis was found in 53.7% patients in the lumbar spine, and 47.5% in the femoral neck (Table 2). High frequency of osteopenia/osteoporosis might be attributed to steroid excess, but patients’ age may also play a role. Subjects with osteopenia or osteoporosis were significantly older and most of them were postmenopausal, thus at higher risk of bone loss. This would correspond with an age-related rise in OPG levels, commonly reported in the past (29, 32). Indeed, the correlation between OPG and BMD disappeared in the multiple regression model and when adjusted for age.

Of note, OPG in our study was negatively correlated with serum adrenal androgens, namely DHEAS levels. A lowering effect of androgen on OPG was demonstrated in human osteoblastic cells in vitro and confirmed in vivo in males with iatrogenic hypogonadism (33, 34). A similar relationship was also found in females with polycystic ovaries syndrome and androgen excess (35). Increased OPG levels in subjects with PAI might thus be related to a lack of adrenal androgens. Adrenal androgens are supposed to play a bone-protective role in these patients. Their serum levels were shown to correlate with BMD, and low DHEAs additionally contributes to increased risk for osteoporosis (36). In our study, the inverse correlation between OPG and DHEAS might therefore reflect changes in bone mineral status. However, it remained significant even after correction for BMD in the multiple regression model. A weak negative OPG-DHEAS correlation was also found in an elderly male population from Lebanon (37).

On the other hand, elevated serum OPG observed in the PAI patients may not directly result from the hormonal influence. Alterations in OPG/RANKL levels have been implicated in cardiovascular disease (26, 27), and increased mortality due to cardiovascular reasons was reported among Swedish PAI subjects (38). Another recent study revealed cardiovascular risk factors in an Italian PAI cohort (39). OPG is also produced by vascular endothelial cells, which, considering an immense surface of endothelium throughout the body, may significantly contribute to circulating levels of this cytokine. Hence, especially having found arterial calcifications in the OPG knockout mice, it was postulated that serum OPG might be involved in ongoing vascular disease (16, 40, 41). Elevated OPG levels were demonstrated in atherosclerosis, coronary artery disease, diabetes and fatal stroke (30, 41, 42). Moreover, OPG was found to be a marker of early atherosclerosis and a predictor of cardiovascular events (41, 42).

In contrast to OPG, serum RANKL levels did not display significant differences between affected PAI patients and healthy subjects. In vitro analyses reveal that glucocorticoids up-regulate RANKL expression in osteoblasts (17) and accordingly, RANKL concentrations in our study correlated with daily HC dose. This cytokine is a major osteoclast maturation factor therefore high serum levels were expected to correlate with enhanced bone resorption. However, despite some former reports of increased RANKL levels in osteoporosis (31, 43), no correlation with BMD was found in the current study. These inconsistencies may reflect the influence of extra-skeletal RANKL synthesis, which takes place in the endothelium and lymphoid tissues (11). Moreover, different techniques of RANKL measurement could also account for discrepant results in different studies (44). The majority of RANKL remains membrane-bound, hence circulating RANKL levels are a less reliable indicator of its production. Other methods, such as evaluation of the local RANKL mRNA expression, might provide more accurate results. However, their use in everyday clinical setting, especially with regard to bone metabolism, is impracticable (45).

This analysis is the first attempt to evaluate the effect of the standard HC replacement therapy on serum OPG and RANKL levels in patients with adrenal failure and to correlate them with bone mineral mass. Nevertheless, our study presents some limitations that need to be addressed. First of all, circulating OPG and RANKL may not perfectly reflect their local activity within the bone as considerable extra-skeletal synthesis might equally contribute to their serum concentrations. Additionally, classical bone turnover markers could also be assessed to elucidate fully the bone metabolic status in subjects receiving steroid replacement. However, the only consistent finding from the studies performed to date is an inverse correlation between glucocorticoid dosage and the osteocalcin level (8, 26). Decreased osteocalcin is a sensitive
indicator of suppressed osteoblastic function, but no relationship between OPG and osteocalcin has been reported (25). On the other hand, reduced osteocalcin levels were also found in hyperglycaemic subjects as assessed in animal models of diabetes and in humans (46, 47).

In conclusion, our study demonstrates increased circulating OPG levels in PAI patients on conventional glucocorticoid replacement therapy. This observation may reflect mild steroid excess, which enhances bone resorption, and/or may result from deficient adrenal androgens, although disturbed vascular metabolism might also contribute. Even though RANKL levels rise with the HC dose, they remain within the normal range on standard steroid substitution. Overall, serum OPG/RANKL measurements do not seem convenient for the routine clinical usage as markers of increased bone resorption in patients who require glucocorticoid replacement. Further, carefully designed studies, which will consider more confounding factors, are warranted to address this problem better.

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