ORIGINAL RESEARCH

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Elevated serum receptor activator of nuclear factor kappa B ligand and osteoprotegerin levels in late-onset male hypogonadism

Abstract

Purpose: Studies that analyze the levels of osteoprotegerin (OPG) or receptor activator of nuclear factor kappa B ligand (RANKL) in hypogonadal men, the majority of whom have prostate cancer or are undergoing androgen-deprivation therapy, are few and inconclusive.

Methods: 81 men aged 69.3 ± 0.8 years (39 men with late-onset hypogonadism and 42 age-matched controls) were recruited. Serum levels of OPG, total soluble RANKL (sRANKL), total and free testosterone (FT), estradiol (E₂), sex hormone-binding globulin (SHBG), follicle-stimulating hormone, luteinizing hormone (LH), prolactin, bone-specific alkaline phosphatase (BAP) and β -Cross Laps were assessed.

Results: Compared with controls, both OPG ($p = 0.023$) and sRANKL ($p = 0.010$) serum levels were increased in men with late-onset hypogonadism; however, when expressed as a ratio, sRANKL/OPG, the two groups were not significantly different. Simple and age-adjusted analyses showed that OPG was inversely related to FT and positively related to SHBG, E₂ and BAP. In the patient population, LH demonstrated statistically significant correlations with both OPG ($r = 0.274$, $p = 0.013$) and sRANKL ($r = 0.276$, $p = 0.018$). Multiple regression analysis retained age, SHBG, E₂ and BAP as independent predictors of OPG, explaining 27.71% of serum OPG variability.

Conclusions: Late-onset hypogonadism is associated with enhanced RANKL activity. Increased bone turnover-related OPG levels may act as a coupling factor between bone resorption and formation. The results suggest anti-RANKL-agents as therapeutic tools in osteoporotic men with late-onset hypogonadism.

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Receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) are members of the tumour necrosis factor receptor super family and play a pivotal role in bone remodelling. RANKL binds to RANK on the surface of osteoclastic precursors and thereby enhances the differentiation and activity of osteoclasts and inhibits their apoptosis [1]. The effects of RANKL are counteracted by OPG, which functions as a neutralizing decoy receptor.

Animal model and cell culture studies suggest a direct inhibitory effect of androgens on the OPG/RANKL cytokines system. In human osteoblastic cells, androgen receptor-induced specific inhibition of OPG mRNA expression by both testosterone and 5 α -dihydrotestosterone has been reported [2,3]. Androgens have also been shown to block RANKL-induced osteoclastic formation [4,5] while RANKL expression was found to be up-regulated in osteoblastic cells from androgen receptor-deficient mice [6]. In the rat, orchiectomy was associated with increased bone marrow-free soluble RANKL levels [7] and, notably, RANKL inhibition prevented orchiectomy-related bone loss [8], thus supporting the hypothesis that amplified RANKL activity promotes bone loss in androgen-deficiency states. In gonadotropin releasing hormone-induced male hypogonadism, testosterone replacement therapy significantly decreased serum OPG levels [9] while low serum OPG was observed in women with polycystic ovary syndrome [10], a disorder characterized by hyperandrogenemia.

Information on circulating OPG and RANKL changes is scarce in male hypogonadism and most studies originate from men under androgen-deprivation therapy for prostate malignancy. In an attempt to characterize the OPG/RANKL cytokines status in a naturally occurring model of androgen insufficiency, serum OPG/soluble RANKL levels were determined in elderly men with and without hypogonadism who tested negative at prostate cancer screening, and the relationships among these molecules and both sex hormones levels and bone remodelling parameters were analysed.

Methods

Study subjects

Eighty-one men aged ≥ 50 years entered this case-control study. According to ISA, ISSAM, EAU, EAA and ASA recommendations [11], late-onset hypogonadism (LOH) was diagnosed in 39 men aged 70.25 ± 1.26 years based upon loss of libido and/or erectile dysfunction and serum total testosterone (TT) < 350 ng/dl and/or low calculated serum free testosterone; 42 age-matched men (68.45 ± 1.23 years) with normal basal hypothalamus-pituitary-testicular function were used as con-

trols. Secondary causes of hypogonadism, hyperprolactinemia, advanced chronic renal failure and diabetes mellitus were exclusion criteria. Men with suspected prostate cancer, assessed by elevated prostate-specific antigen (PSA) levels, with or without abnormal histology in prostate biopsy samples, were not included. The study was done with approval of the local ethics committee and informed consent was given by all participants.

Serum assays

Fasting blood samples were collected from each patient. Serum total testosterone (TT), sex hormone-binding globulin (SHBG), estradiol (E₂), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), OPG and total soluble RANKL (sRANKL) were concomitantly assessed in all participants by ELISA (DRG Instruments GmbH, Marburg, Germany). C-terminal telopeptide of type I collagen (β -Cross Laps[®], CTx) and bone-specific alkaline phosphatase (Ostase[®], BAP) were measured by ELISA from Immunodiagnostic Systems Ltd, Boldon, UK). Free testosterone (FT) concentration was calculated according to Vermeulen's formula [12] and cut-off levels of 225 pmol/l were considered to define hypogonadism [11]. The intra-assay and inter-assay coefficients of variance (CV) were 4.4% and 5.7% for OPG and 4.6% and 9.1% for sRANKL, respectively. The sensitivity of the serum OPG assay was less than 0.14 pmol/l whereas the lowest detectable concentration of total sRANKL was 10 pmol/l.

Statistical analysis

Descriptive results are presented as mean $\pm$ S.E.M. One-way ANOVA or Kruskal-Wallis tests were used to test for differences between groups, as appropriate. Correlation analysis among continuous variables was performed, quantifying the strength of the associations by calculating either the simple or partial Pearson's correlation coefficient or the non-parametric Spearman's rank correlation coefficient. The stepwise multiple regression analysis was used to determine which variables predict serum OPG or RANKL levels. In all statistical tests performed, a p-value of < 0.05 was considered as statistically significant.

Results

Endocrine parameters of the pituitary-testicular axis and markers of bone turnover measured in both study groups are presented in Table 1. Compared with controls, LOH men had significantly lower TT, FT and E₂ levels but higher bone turnover rate. Serum PRL values for hypogonadal men were within

TABLE 1. Baseline endocrine parameters and markers of bone remodeling in elderly men with and without hypogonadism.

Variables	Hypogonadal Men (n=39)	Controls (n=42)	p-value
Age (years)	70.25 ± 1.26	68.45 ± 1.23	0.311
FSH (U/L)	21.280 ± 3.304	6.781 ± 0.896	< 0.0001
LH (U/L)	18.547 ± 2.403 (13.200)	7.150 ± 0.405	< 0.0001
TT (ng/ml)	3.203 ± 0.311	7.408 ± 0.370	< 0.0001
FT (pg/ml)	33.771 ± 3.597 (31.700)	162.076 ± 12.045	< 0.0001
SHBG (nmol/l)	94.832 ± 9.992	38.811 ± 2.908	< 0.0001
E ₂ (ng/ml)	24.263 ± 2.573 (20.19)	29.440 ± 2.121	0.023
PRL (ng/ml)	8.397 ± 0.656	6.254 ± 0.763	0.038
CTx (ng/ml)	0.314 ± 0.030	0.242 ± 0.019	0.046
BAP (μg/l)	11.522 ± 0.885	9.387 ± 0.577	0.045

Data is given as mean ± S.E.M. A p-value <0.05 was considered statistically significant. For non-parametrical parameters the median value is presented in parenthesis. TT = total testosterone, FT = calculated free testosterone, E₂ = estradiol, CTx = C-terminal telopeptide of type I collagen, BAP = bone-specific alkaline phosphatase.

normal limits. In the entire study population, serum CTx clearly correlated negatively to FT ($r = -0.227$, $p = 0.041$) and positively to serum SHBG ($r = 0.281$, $p = 0.011$). Moreover, testicular function accounted for the difference in bone resorption rate between groups, which was lost after adjusting for FT levels ($p = 0.621$). Spearman correlation analysis revealed a trend towards a negative relationship between serum BAP and testosterone ($r = -0.205$, $p = 0.066$ with TT and $r = -0.179$, $p = 0.109$ with FT).

Serum OPG levels were positively related to age, whereas sRANKL levels were not (Table 2). Cross-sectional analysis of serum OPG and sRANKL levels revealed significantly higher levels of both in men with LOH compared with controls (Fig.

1a, b). When sRANKL/OPG was considered, no significant difference was seen between hypogonadal men and controls ($p = 0.289$).

As depicted in Table 2, serum OPG was significantly and inversely associated with FT in simple and age-adjusted analysis ($r = -0.244$, $p = 0.029$) and positively with SHBG ($r = 0.338$, $p = 0.002$ in age-adjusted analysis) and E₂ ($r = 0.274$, $p = 0.014$ in age-adjusted analysis). Soluble RANKL showed only a tendency towards a negative correlation to FT while no relationship whatsoever was seen with serum E₂ (Table 2). Both cytokines were significantly and positively associated with serum LH levels (Table 2) and this association was primarily explained by age, SHBG and FT in multivariate analysis ($r =$

TABLE 2. Association of serum OPG and total sRANKL with gonadotropins, sex steroids, prolactin, SHBG and markers of bone turnover in elderly men with and without hypogonadism.

Variables	sRANKL		OPG	
	r	p	r	P
Age (years)	-0.161	0.174	0.302	0.006
FSH (U/L)	0.142	0.238	0.237	0.034
LH (U/L)	0.276	0.018	0.274	0.013
TT (ng/ml)	-0.189	0.112	-0.153	0.173
FT (pg/ml)	-0.194	0.101	-0.274	0.013
SHBG (nmol/l)	0.169	0.154	0.337	0.002
E ₂ (ng/ml)	0.033	0.778	0.225	0.042
PRL (ng/ml)	0.003	0.979	0.141	0.211
CTx (ng/ml)	-0.109	0.361	0.236	0.033
BAP (μg/l)	0.197	0.101	0.256	0.020

Results are expressed as mean ± S.E.M. Pearson's correlation analysis or Spearman's rank correlation was applied, as appropriate. A p-value <0.05 was considered statistically significant.

FIGURE 1. Serum osteoprotegerin (OPG) and total soluble receptor activator of nuclear factor kappa B ligand (RANKL) concentration in elderly hypogonadal men and age-matched controls. The box plots include the median (horizontal line), the mean (horizontal dotted line) and the interquartile range (box), and the whiskers indicate the 5-95 percentile. Outliers are presented as separate points and extend to a maximum of 1.5 times the interquartile range. $P = 0.023$ in OPG levels between men with and without hypogonadism by one-way ANOVA. $P = 0.010$ in total sRANKL levels between men with and without hypogonadism by one-way ANOVA.

0.074, $p = 0.520$ for OPG and $r = 0.209$, $p = 0.084$ for RANKL). Furthermore, the relationship between OPG/RANKL and bone turnover was analyzed (Table 2). It was observed that serum OPG was directly related to BAP levels in simple and age-adjusted analysis ($r = 0.270$, $p = 0.015$) and CTx concentration ($r = 0.192$, $p = 0.088$ after controlling for age). In contrast, no statistically significant relationship between sRANKL and either BAP or CTx was found.

Multiple regression analysis in a stepwise model applied to the study population of elderly men, with OPG as a dependent variable and age and markers of bone turnover as independent variables ($R^2 = 0.157$, F-ratio = 7.304, $p = 0.001$), revealed age (beta = 0.302, $p = 0.004$) and BAP (beta = 0.257, $p = 0.015$) as significant determinants of OPG levels, with the above mentioned predictors explaining 13.61% of OPG variability. Adding SHBG and sex steroids as independent variables improved the model ($R^2 = 0.313$, F-ratio = 8.664, $p < 0.001$), explaining 27.71% of the variation in OPG concentration. Age (beta = 0.302, $p = 0.001$), SHBG (beta = 0.337, $p = 0.002$), E_2 (beta = 0.226, $p = 0.012$) and BAP (beta = 0.257, $p = 0.015$) were further retained as independent predictors of OPG. None of endocrine or bone metabolism variables was found to independently predict serum sRANKL concentration in this study.

Discussion

In the present study, it is shown that men with LOH have elevated circulating OPG and sRANKL levels in comparison with

age-matched men exhibiting normal pituitary-testicular function. There is lack of convincing evidence that serum levels of these cytokines adequately reflect the protein activity in the skeleton [1,13]; nevertheless, these findings support the hypothesis that androgens act either directly or indirectly to suppress OPG/RANKL activity in human bone. In agreement with these results, increased skeletal expression of both RANKL and OPG genes has been found in the orchietomized rat [14] and high bone marrow RANKL levels have also been demonstrated⁷. Moreover, RANKL-mediated, osteoclast-induced bone resorption can be prevented in the animal rat model by testosterone undecanoate therapy⁷ or by osteoprotegerin infusion [8], suggesting that an increased functional RANKL/OPG ratio is associated with male hypogonadism by promoting bone loss.

A link between the OPG/RANKL system and circulating sex steroids in elderly men is evidenced by the close relationship among TT, FT and E_2 concentrations on one hand and serum OPG on the other hand. The present study confirms the findings of Gannagé-Yared [15] and Khosla *et al.* [16] that show an inverse relationship between OPG and the free androgen index or bio-available testosterone, respectively, in men over 50 but is contrast to the results of Oh *et al.* that show no relationship between OPG and total testosterone in middle-aged males [17] or to those of Szulc *et al.* that describe a positive correlation between OPG and both the free androgen index and the free estradiol index in men aged 19-85 years [18]. Nonetheless, total, but not free, testosterone was evaluated in

some studies in which no relationship was seen between OPG and androgens [17]. Moreover, this study demonstrates that both OPG and sRANKL are significantly correlated with serum LH levels - an independent indicator of low FT in older men [19]. As shown in a previous study [15], OPG and SHBG are positively related in aging men. Moreover, SHBG independently predicts serum OPG in stepwise multiple regression analysis, suggesting that an age-related increase in SHBG may account not only for the lower FT [19] levels but also for the significantly higher circulating OPG levels in hypogonadal older men.

The positive correlation between OPG and markers of bone remodeling in the ageing male population from our study is in line with the hypothesis that the increased OPG levels indicate a protective mechanism for counteracting the bone loss induced by accelerated RANKL-mediated bone resorption. Furthermore, in primary cell cultures of human osteoblasts, it has been shown that OPG directly increases alkaline phosphatase production and *in vitro* mineralization, and that all OPG-mediated effects can be prevented by RANKL neutralizing antibodies [13]. Presumably, compensatory increased OPG acts by stimulating osteoblasts, thus coupling bone resorption to bone formation.

In multiple regression analysis with variables fitted in a forward stepwise manner, serum OPG has been shown to be independently predicted by BAP, a solid formation marker. Thus, irrespective of the underlying pathological process, accelerated bone turnover may act as a determining condition of high OPG in elderly men. Power analysis for multiple regression showed that the sample size used in this model resulted in a high statistical power of >0.9 to detect significant interaction between predictors in multiple regression.

Studies of the association between serum RANKL and sex steroids are even less consistent than those of OPG, with most studies not revealing any relationship between these two parameters in men [17,20]. Taking into account the higher serum sRANKL level in the hypogonadal group, as well as the inverse correlation between sRANKL and LH, RANKL appears to intervene to promote bone loss associated with LOH. In clinical studies, few reported any interrelations between RANKL and markers of bone turnover in presumably healthy men [17] and most failed to obtain any consistent conclusion. Nevertheless, this might be due to circulating RANKL reflecting suboptimal skeletal expression of RANKL; in agreement with this theory, association studies have clearly shown that skeletal RANKL messenger RNA levels are significantly linked to histomorphometric bone resorption indices in both normal human cancellous bone²¹ and metabolic bone disease [22].

Moreover, agents with the potential to block RANKL effects in the skeleton have emerged as therapeutic tools in postmenopausal or glucocorticoid-induced osteoporosis [23] or malignancy-related bone disease [24]. Data showing favorable results with denosumab, a recently developed anti-RANKL monoclonal antibody that reduces the incidence of vertebral fractures in men with non-metastatic prostate cancer receiving androgen-deprivation therapy [25], also suggest potential use of denosumab as a therapeutic tool in endogenous hypogonadism-related male osteoporosis.

There are two limitations that need to be addressed regarding the present study. The first limitation concerns the relatively small sample size of participants. Second, the lack of information on bone mineral density (BMD) status for study subjects may hinder a deeper understanding of the relevance of the circulating OPG/RANKL changes to LOH-dependent low bone mass. Nonetheless, results from previous clinical studies, which examined the relationships between BMD, as assessed by dual X-ray absorptiometry (DXA), and serum OPG/RANKL levels in various male populations were conflicting and rather inconclusive [1,16-18,26]. Therefore, the more dynamic bone turnover parameters may better express the association between OPG/RANKL and bone status. In addition, in preclinical models, it has been shown that OPG treatment increases bone volume and also trabecular thickness and connectivity [1], thus suggesting a role for OPG in maintaining bone architecture and thus bone quality, a parameter poorly reflected by BMD measurement.

To conclude, late-onset hypogonadism is associated to an enhanced RANKL activity suggesting a role of RANKL-mediated bone resorption in androgen-deficiency induced bone loss. In turn, bone turnover-related OPG may act as a coupling factor, linking bone resorption to subsequent bone formation to maintain bone remodelling balance.

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