

Clinical Investigations

Relationships Between the Changes of Serum Levels of OPG and RANKL with Age, Menopause, Bone Biochemical Markers and Bone Mineral Density in Chinese Women Aged 20–75

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Abstract. The correlations between the serum levels of OPG, RANKL with age, menopause, bone markers, and bone mineral densities (BMDs) at the lumbar spine and proximal femur were studied in 504 pre- and postmenopausal Chinese women aged 20–75 years. We found that age was positively and negatively correlated with serum concentrations of OPG ($r = 0.442$, $P < 0.001$) and RANKL ($r = -0.263$, $P < 0.001$), respectively. Compared with premenopausal women, postmenopausal women showed higher serum OPG levels (107.6 ± 3.0 vs 72.0 ± 1.8 pg/ml, $P < 0.001$), lower serum RANKL concentrations (4.7 ± 0.4 vs 5.8 ± 0.3 pg/ml, $P < 0.001$) and RANKL/OPG ratios (0.045 ± 0.004 vs 0.099 ± 0.008 , $P < 0.001$). Neither serum levels of OPG nor RANKL or RANKL/OPG ratio correlated with BMDs after adjustment of age and menopause. They also showed no differences among normal, osteopenic and osteoporotic postmenopausal women. Serum levels of OPG were positively correlated with urinary excretion of NTx ($r = 0.1453$, $P = 0.006$). Serum levels of RANKL ($r = -0.1928$, $P < 0.001$) and RANKL/OPG ratio ($r = -0.1303$, $P = 0.013$) were inversely correlated with serum concentrations of OC. In multiple regression analysis, up to 20% variance ($R^2 = 0.106$ – 0.224) of the OPG-RANKL system in peripheral circulation can be explained by age, menopause and bone markers. These results suggest that although serum OPG and RANKL concentrations were unrelated with BMDs, the age- and menopause-dependent changes of serum OPG and RANKL might be a protective mechanism against the accelerated bone loss in postmenopausal women.

Key words: OPG — RANKL — BMD

The receptor activator of nuclear factor-kappa B ligand (RANKL) is a transmembrane ligand expressed on osteoblasts that bind to RANK. Interaction between RANK and RANKL induces differentiation, activation

and prevention of the apoptosis of osteoclastic cells, leading to enhanced bone resorption and bone loss [1, 2]. The effects of RANKL can be neutralized by its decoy receptor osteoprotegerin (OPG), which plays an important inhibitory role in the regulation of osteoclastic bone resorption [3, 4]. The OPG gene knockout mice demonstrated uncontrolled bone resorption and severe osteoporosis [5]. It was recently reported that homozygous deletion of the gene encoding OPG is the potential cause of juvenile Paget's disease in some patients [6]. On the other hand, RANKL knockout mice showed a decreased number of osteoclasts, increased bone mineral density (BMD), even osteopetrosis [7]. A single intravenous injection of recombinant OPG in young growing rats [8] and postmenopausal women with osteoporosis [9] caused significant gains in bone volume and density, together with a rapid and sustained suppression of osteoclastic bone resorption and a delayed reduction of bone formation.

However, the physiological and pathophysiological significances of the variations in serum concentration of OPG, especially the serum level of RANKL, are not fully understood [10]. In human studies, the correlation between serum levels of OPG and BMDs as well as bone biochemical markers are inconsistent in different reports [11–13]. Although the changes of serum RANKL were reported in Paget's disease [14], rheumatoid arthritis (RA) [15] and multiple myeloma (MM) [13], the studies of their associations with age and BMDs, their changes before and after menopause are rarely seen in relatively large cohorts of pre- and postmenopausal women.

The objectives of this study were to investigate the associations between serum concentrations of OPG, RANKL and age, menopause, BMD and bone biochemical markers, and to determine the factor(s) influencing serum levels of OPG and RANKL in 504 Chinese pre- and postmenopausal women aged 20–75 years.

Subjects and Methods

Subjects

We initiated our Genetic and Environmental Evaluation of Bone mass (GEEB) study in 2002. Several genetic markers, biochemical indices, and environmental factors were investigated in a cohort of healthy pre- and postmenopausal women from Shanghai, China [16].

Briefly, healthy female volunteers aged 20–75 were recruited through posters. The health status of the subjects was assessed by present and past history, as well as physical examinations and laboratory tests, including blood pressure, heart rate, fasting plasma glucose, lipid profile, and liver and kidney function. Self-reported fracture history was recorded. Those suffering diseases other than osteoporosis or taking drugs that might affect bone metabolism were excluded. A total of 504 healthy women, average age 46.6 ± 0.7 years, were eligible for the study. Menopause is recognized to have occurred after 12 consecutive months of amenorrhea, and postmenopause is defined as dating from the final menstrual period [17]. Thus, there were 282 premenopausal women with regular menstrual cycles, and 222 postmenopausal women in our cohort.

All the participants completed written informed consent before measurements. The ethical committee of Rui-jin Hospital, Shanghai Second Medical University, approved the study.

Measurement of BMDs

BMDs of lumbar spine and proximal femur, including femoral neck, Wards triangle, trochanter, total hip and L2–4 were assessed using dual-energy X-ray absorptiometry (DXA) (Lunar Expert 1313). BMD was automatically calculated from the bone area (cm^2) and bone mineral content (g) and expressed absolutely in g/cm^2 . The postmenopausal women were divided into normal, osteopenic and osteoporotic groups according to WHO criteria [18].

Sample Collection

Blood (in the follicular phase of the menstrual cycle in premenopausal women) and a second morning urine samples were collected after an overnight fast. Serum was separated and frozen immediately after blood drawing. Both serum and urine samples were stored at -80°C until analysis.

Measurement of Bone Biochemical Markers and Serum OPG, RANKL

Serum concentration of intact osteocalcin (OC) was measured by the immunoradiometric method (Diagnostic Systems Laboratories, USA). Urinary level of N-terminal crosslinking telopeptide of type I collagen (NTx) was measured by enzyme-linked immunosorbent assay (ELISA) (Ostex International, USA). Urinary determinations were expressed in relation to creatinine excretion.

Serum concentrations of OPG and RANKL were both measured by ELISA (Biomedica, Vienna, Austria). The RANKL/OPG ratio was obtained for all individuals. The detection limit of OPG and RANKL is 2.8 pg/ml and 1.6 pg/ml, respectively. All the samples had detectable levels for OPG, while 9 were below the test limit for RANKL.

All the samples were measured in duplicate according to the manufacture's instruction and then averaged. In cases of patients with undetectable values, the detection limit value was used for analysis. The intra- and interassay coefficients of variation (CV) for each marker, respectively, were as follow: for OC 1.4%–3.4% and 3.3%–5.3%, for NTx 5%–19% and 3%–5% for OPG <10% and <10%, for RANKL 3%–5% and 6%–9%.

Statistical Analysis

The results were expressed as mean \pm SEM. All the data not normally distributed were logarithmically transformed before analysis. Person's correlation and partial correlation analysis, ANOVA, covariance analysis and multiple regression analysis were used as appropriate. *P* values less than 0.05 were considered significant.

Results

In the present study, serum levels of OPG showed no association with serum concentration of RANKL ($r = -0.033$, $P = 0.514$). Age was positively associated with serum level of OPG ($r = 0.442$, $P < 0.001$), but negatively correlated with that of RANKL ($r = -0.263$, $P < 0.001$) and RANKL/OPG ratio ($r = 0.460$, $P < 0.001$).

Table 1 shows the significant differences of BMDs and bone biochemical markers between pre- and postmenopausal women. The serum concentration of OPG was higher, while serum level of RANKL and RANKL/OPG ratio were statistically and significantly lower in postmenopausal women.

Serum concentrations of OPG, RANKL and RANKL/OPG ratios were not related to BMDs at each site after adjustment for age and menopause. There was a weak but significantly positive correlation between serum level of OPG and urinary excretion of NTx ($r = 0.1453$, $P = 0.006$). Serum RANKL level was negatively correlated with serum concentration of OC ($r = -0.1928$, $P < 0.001$). RANKL/OPG ratio showed a trend to negatively correlate with NTx ($r = -0.1026$, $P = 0.051$), but was significantly negatively associated with serum level of OC ($r = -0.1303$, $P = 0.013$).

When the postmenopausal women were divided into normal, osteopenic and osteoporotic groups according to WHO criteria [18], it was found that age, years-since-menopause (YSM), NTx and BMDs at lumbar spine and proximal femur were different among these 3 groups (Table 2). But serum concentrations of OPG, RANKL and RANKL/OPG ratio showed no difference among the groups.

Multiple regression analysis was performed to evaluate the contribution of age, menopause, NTx, OC, BMDs to serum levels of OPG, RANKL and RANKL/OPG ratio. The results revealed that menopause ($r^2 = 0.181$, $\beta = -0.200$), age ($r^2 = 0.023$, $\beta = 0.267$) and urinary NTx ($r^2 = 0.016$, $\beta = 0.130$) were the independent contributors for serum OPG ($R^2 = 0.214$). Age ($r^2 = 0.069$, $\beta = -0.331$) and serum OC ($r^2 = 0.036$, $\beta = -0.203$) were the independent determinants for serum RANKL ($R^2 = 0.106$). Age ($r^2 = 0.201$), serum OC ($r^2 = 0.011$, $\beta = -0.118$) and urinary NTx ($r^2 = 0.012$, $\beta = -0.110$) were the major factors influencing RANKL/OPG ratio ($R^2 = 0.224$).

Table 1. Comparison of bone biochemical markers, BMDs, serum levels of OPG, RANKL and RANKL/OPG ratio in pre- and postmenopausal women

	Total	Premenopause	Postmenopause
N	504	282	222
Age (Yr)	46.5 ± 0.7	34.8 ± 0.5	61.3 ± 0.5*
Urinary NTx (nM BCE/nM)	38.0 ± 1.4	34.9 ± 1.4	45.6 ± 3.0*
Serum OC (ng/ml)	30.6 ± 1.1	35.6 ± 1.3	22.8 ± 1.9*
L2–4 (g/cm ²)	1.077 ± 0.008	1.172 ± 0.009	0.965 ± 0.010*
Femoral neck (g/cm ²)	0.894 ± 0.007	0.977 ± 0.008	0.795 ± 0.009*
Ward's (g/cm ²)	0.777 ± 0.009	0.894 ± 0.009	0.639 ± 0.009*
Trochanter (g/cm ²)	0.739 ± 0.006	0.792 ± 0.007	0.677 ± 0.007*
Total hip (g/cm ²)	0.931 ± 0.006	1.005 ± 0.007	0.845 ± 0.008*
Serum OPG (pg/ml)	83.5 ± 1.7	72.0 ± 1.8	107.6 ± 3.0*
Serum RANKL (pg/ml)	5.4 ± 0.2	5.8 ± 0.3	4.7 ± 0.4*
RANKL/OPG	0.083 ± 0.006	0.099 ± 0.008	0.045 ± 0.004*

*Compared with premenopausal women, $P < 0.001$

Table 2. Comparison of age, YSM, bone biochemical markers, BMDs serum concentrations of OPG, RANKL, and RANKL/OPG ratio among normal, osteopenic and osteoporotic postmenopausal women

	Normal	Osteopenia	Osteoporosis	P value
N	70	110	42	
Age (Yr)	58.0 ± 0.9	62.7 ± 0.6	63.5 ± 1.0	<0.001
YSM (Year)	10.6 ± 0.8	13.4 ± 0.6	13.2 ± 1.0	0.023
Urinary NTx (nM BCE/nM)	34.6 ± 3.8	56.1 ± 4.8	52.5 ± 8.6	0.002
OC (ng/ml)	27.2 ± 3.3	21.2 ± 2.5	17.6 ± 4.7	0.055
L2–4 (g/cm ²)	1.124 ± 0.012	0.943 ± 0.007	0.758 ± 0.008	<0.001
Femoral neck (g/cm ²)	0.888 ± 0.011	0.775 ± 0.008	0.694 ± 0.017	<0.001
Ward's (g/cm ²)	0.744 ± 0.015	0.616 ± 0.009	0.527 ± 0.017	<0.001
Trochanter (g/cm ²)	0.772 ± 0.009	0.659 ± 0.009	0.566 ± 0.014	<0.001
Total hip (g/cm ²)	0.953 ± 0.009	0.822 ± 0.009	0.723 ± 0.017	<0.001
Serum OPG (pg/ml)	102.2 ± 4.2	111.7 ± 5.2	111.7 ± 6.5	0.292
Serum RANKL (pg/ml)	4.3 ± 0.6	5.3 ± 0.8	4.1 ± 0.5	0.214
RANKL/OPG	0.044 ± 0.007	0.048 ± 0.007	0.041 ± 0.005	0.576

Discussion

In the present study, we have studied the serum levels of OPG and RANKL in 504 pre- and postmenopausal Chinese women. We found an increase of serum OPG with age, which was in accordance with the data from the Japanese population [19] and others [20]. It was also revealed that serum levels of OPG remained relatively constant in individuals younger than ~50 years [15], and were elevated sharply after 60 years of age [12]. Although we did not divide our samples according to age, the results also revealed a significant increase of OPG serum levels in postmenopausal women, suggesting that in addition to age, OPG serum levels might also be influenced by menopause. However, serum concentrations of OPG were also found to be age independent [21] and did not differ among premenopausal and postmenopausal women [22]. The differences in sample size, the opposing effects of estrogen deficiency and increased bone turnover on OPG production, as suggested by

Eghbali-Fatourehchi et al. [22], may be a cause for the discrepancies.

Serum RANKL concentration and RANKL/OPG ratio were found to be decreased with age, and the reduction was prominent in postmenopausal women in our cohort. But other studies did not observe the age-dependent [15] or menopause-related changes of RANKL serum level in healthy individuals. However, the common finding in these studies including ours, was the lack of correlation between OPG and RANKL, suggesting that different mechanisms are involved in the regulation of OPG and RANKL synthesis [15].

Since the OPG–RANKL system is very important for the regulation of bone remodeling, several studies analyzed the effects of serum OPG on BMDs and bone biochemical markers, but with inconsistent results [11–13, 20]. There have been even less data on the relationship between serum RANKL and BMDs until now.

The bone mineral densities in postmenopausal women were positively or negatively correlated, or not

related with serum OPG concentrations in various studies [11, 12, 23]. Since serum levels of OPG, RANKL and BMDs are all influenced by age and menopause, a partial correlation analysis was performed in our study after adjustment for these two factors to elucidate the associations between serum levels of OPG, RANKL, RANKL/OPG ratio and BMDs. None of these 3 parameters was correlated with BMDs at lumbar spine or proximal femur after adjustment for age and menopause. Serum levels of OPG, RANKL and RANKL/OPG ratio also showed no differences among normal, osteopenic and osteoporotic postmenopausal women. Yano et al. [19] reported an increase of OPG in osteoporotic versus nonosteoporotic women, but other studies do not confirm such a result [12, 23]. In Paget's disease, serum RANKL concentration and RANKL/OPG ratios were not different between patients and controls, although the mean OPG levels in patients were higher [14].

The mechanisms and pathophysiological role of high serum OPG levels and low RANKL concentrations in postmenopausal women are not clear. It is suggested that the rise of serum OPG levels with age and bone turnover [1, 24, 25], especially in osteoporotic women [20], and their decrease in Paget's disease patients with higher baseline values after antiresorptive treatment [14] may reflect a paracrine mechanism of the skeleton to compensate for increased bone resorption. The positive correlation of serum OPG levels with urinary NTx and higher concentrations of serum OPG in postmenopausal women in our study also support such a hypothesis. On the other hand, lower serum levels of OPG may present as a risk factor for osteoporotic fracture [20].

RANKL is an essential factor for osteoclasts' activities [1], but it is somewhat difficult to explain our results that serum levels of RANKL and OC were both reduced in postmenopausal women, and were negatively associated.

Many studies demonstrated higher serum OC levels in postmenopausal women than in premenopausal ones [26–28]. But similar to our study, some studies found lower serum levels of OC in osteoporotic postmenopausal women [29] and in women who had sustained a fracture [30]. Preanalytical variability of this marker may be one of the factors accounting for these discordant results [31, 32]. However, since the blood samples were drawn, stored, and tested with the same protocol and under the same conditions in our study, such factors, if any, should generally have an impact on all samples, not only to postmenopausal women, thus are unlikely to be the major attributable factor for this difference.

Although there is some new understanding for the function of circulating OC [33], it is generally considered to cause osteoblast activity [32] and its level showed no difference between serum and cancellous bone [34]. So,

the changes of serum OC levels in postmenopausal women may reflect a reduced bone formation. A negative association between serum concentration of RANKL and OC found in our study seems to indicate that the lower the RANKL (less osteoclastic activity), the higher the OC (more osteoblastic function). If this is true, then the suppressed level of RANKL may also be a limiting factor to impaired bone formation and excessive resorption in our postmenopausal women, as indicated by a lower concentration of serum OC and higher levels of urinary NTx: it will not further aggravate osteoclastic activities, and may even provide more possibilities for osteoblasts to restore their function. However, our hypothesis is only inferred from a marker of late osteoblastic activities, which may not be synthesized simultaneously with other bone formation parameters [35]. Measurement of a more stable and reproducible form of osteocalcin, such as N-terminal midpeptide, and/or other bone formation markers, and observing their changes longitudinally may further examine and modify this speculation.

It should also be noted that RANKL is made as a membrane anchored molecule, and can be cleaved from the cell surface as a soluble molecule [36]. The RANKL test kits available now are designed to detect soluble RANKL in biological fluids, such as serum. Whether the amount and activities of soluble RANKL are related to their membrane-bound forms, and whether the presence of OPG in serum will interfere with the detection of RANKL are still under investigation [2, 37].

In order to further explore the relative contribution of age, menopause, bone biochemical markers and BMDs to serum OPG, RANKL, and RANKL/OPG ratio, we performed multiple regression analyses. The results revealed that up to nearly 20% variance of OPG-RANKL system in peripheral circulation can be explained by age, menopause and bone biochemical markers, but bone mineral densities were not the independent determinants. These results suggested that although serum OPG and RANKL are not specific markers for bone mineral densities, several possibilities may account for this.

First, besides bone, a variety of other tissues, such as lung, kidney and heart all can express OPG mRNA [4]. Serum OPG has also been involved in vascular calcification [38], diabetic microvascular complications [39] and is associated with the cause of mortality in elderly women [23]. RANKL/OPG ratio is also a prognostic index for survival in MM patients [13]. So, although changes in the OPG-RANKL system may not be specific to bone metabolism, they might function as a compensatory self-protective mechanism against age-associated diseases [12].

Second, serum OPG and RANKL levels may not consistently reflect the local milieu in the bone. The changes of OPG-RANKL system in bone microenvi-

ronment seems to be different from those in peripheral circulation. For example, in contrast to the common findings of age-related increase of serum OPG, the expressions of OPG in human bone marrow cells decreased with age [40]. The OPG mRNA levels in mice osteoblast-like cells also declined with age, while the expressions of RANKL mRNA in adult and old mice were higher than those in the young mice [41]. The surface concentration of RANKL per bone marrow mononuclear cells was increased by 2–3 fold in early postmenopausal women over premenopausal and estrogen-treated postmenopausal women, and showed no correlation with its serum value [22]. These findings highlight the importance of assessing the levels of these factors directly in the bone microenvironment [22].

In conclusion, the present study showed that in Chinese women, age and menopause were the major contributors to serum OPG and RANKL concentrations; bone biochemical markers also had some effects. Although serum OPG and RANKL levels were unrelated to BMDs and were not different among normal, osteopenic and osteoporotic postmenopausal women, the age-dependent increase of serum OPG concentration is very likely a compensatory response for accelerated bone resorption. Whether the age-dependent decrease of serum RANKL level is also beneficial for restoring the reduced bone formation in postmenopausal women needs to be further studied in different populations, especially in longitudinal studies.

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