CLINICAL STUDY

Increased serum osteoprotegerin values in long-lived subjects: different effects of inflammation and bone metabolism

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Abstract

Aim: To evaluate serum osteoprotegerin (OPG) concentrations in relation to age-dependent changes in serum markers of bone metabolism and systemic inflammation.

Methods: Two-hundred and eighty-three healthy subjects were evaluated for plasma estimated creatinine clearance (Cr-clearance), C-reactive protein (CRP), bone alkaline phosphatase, C-telopeptides of type-1 collagen (CrossLaps), nuclear factor-kappaB ligand (RANKL) and OPG concentrations.

Results: In adult subjects (82 cases aged between 27 and 64 years) serum OPG concentrations were significantly and independently correlated with RANKL and Cr-clearance (R^2 : 0.29), but not with CRP and biochemical markers of bone metabolism. In old subjects who were between 65 and 84 years of age (52 cases) serum OPG concentrations were significantly higher as compared with the adult subjects and correlated independently and significantly with serum RANKL, Cr-clearance and CrossLaps values (R^2 : 0.63). The highest OPG values were found in the long-lived subjects (149 cases with ages between 85 and 110 years) who also showed increased serum CrossLaps and CRP concentrations as compared with the younger subjects. However, in the long-lived subjects serum OPG concentrations were significantly and independently correlated with Cr-clearance and CRP (R^2 : 0.45) but not with CrossLaps values.

Conclusions: These data would suggest that different factors might be responsible for the age-dependent enhancement of OPG production. Bone metabolism would seem to be the most important factor influencing serum OPG concentrations in old subjects under 85 years of age, whereas in long-lived subjects the circulating values of this cytokine seem to be mainly correlated with serum CRP which could be a marker of inflammation and cardiovascular risk.

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Introduction

The ageing process is associated with a progressive remodelling of immune, endocrine and metabolic systems that allows a resetting of body function in relation to advancing age (1). Particular attention has been paid to inflammation. It has been theorised that a global reduction in the capacity to cope with a variety of stressors and a concomitant progressive increase in proinflammatory status are major characteristics of the ageing process (2).

The last years have seen an enormous surge in research focused on inflammation and atherosclerotic heart disease (3). Markers of inflammation such as C-reactive protein (CRP) are becoming established as important additions in helping to define the risk of progressive vascular disease (4–7). Ageing is accompanied by two- to fourfold increases in plasma/serum levels of

inflammatory mediators (8, 9), and minimal acute-phase changes predict poor prognoses in many conditions and predict disability and mortality in the elderly (10).

Age remodelling is a very important process as it allows a resetting of body function in relation to advancing age (1). Indeed, such a phenomenon occurs in some individuals successfully and in others unsuccessfully. Healthy long-lived subjects represent the successful case since in their ageing remodelling occurs without problems and this population is an excellent model to investigate the mechanisms responsible for 'successful ageing' (1).

Osteoprotegerin (OPG) and its cognate ligand, receptor activator of nuclear factor-kappaB ligand (RANKL), have been identified as important factors involved in the regulation of bone metabolism mediating the paracrine signalling between osteoblast and osteoclast (11). OPG

acts as a decoy receptor, binding RANKL and thereby inhibiting the interaction between RANKL and its receptor on the osteoclast surface (12). In humans, OPG seems to exert a protective role in postmenopausal osteoporosis (13, 14) and other metabolic diseases characterised by bone loss (15, 16). The OPG/RANKL system has also been involved in the pathogenesis of cardiovascular diseases, thus underlining the possible link between vascular biology and bone metabolism (17, 18).

It is well known that OPG production increases with ageing in healthy populations (19), but the reasons for this positive trend have not been completely clarified. Although the age-dependent modifications in bone metabolism and structure may be important in determining the well-known increase in OPG production, other factors may be involved in this process.

To better clarify this aspect, we investigated serum OPG concentrations in relation to the age-dependent changes in serum markers of bone metabolism, energetic metabolism and systemic inflammation. Particular attention was paid to long-lived subjects, in order to have some information about the role of OPG in the successful age remodelling occurring in this frail population.

Materials and methods

We examined 283 subjects, out of whom 82 were adults (Group Ad (69 females, 13 males, aged between 27 and 64 years)), 52 were old, aged between 65 and 84 years (Group Old-I (34 females, 18 males)) and 149 were long-lived with ages ranging from 85 to 110 years (Group Old-II (114 females, 35 males)) (Table 1). The strategy to select long-lived people in

the population is based on evidence that the selection for longevity starts at about 85 years (20). Moreover, previous data showed that people beyond the age of 85 have peculiar metabolic/anthropometric parameters in comparison with the rest of the ageing population, suggesting that the former subjects have been highly selected by mortality forces (21, 22).

All subjects were contacted at home or in their institution and were examined by physicians previously trained to administer a questionnaire that included cognitive and depression tests. Moreover, a clinical and complete biochemical evaluation was performed in order to exclude acute and chronic disorders such as infection, inflammation, malignancy, cardiac insufficiency, dementia, diabetes, liver disease and moderate to severe renal insufficiency as assessed by an estimation of creatinine clearance. The latter parameter was calculated by the Cockcroft and Gault formula (23): $[(140 - \text{age}) \times \text{weight} / 72 \times \text{serum Cr}] \times (0.85 \text{ if female})$. None of the subjects used medications affecting body composition and bone metabolism. Finally, the confined-to-bed subjects were excluded from the study.

Premenopausal women had all measurements made during the follicular phase of the menstrual cycle while postmenopausal women were not receiving hormone replacement therapy.

After a clear explanation of the study, all subjects (as well as the relatives of centenarians) gave informed consent to participate in the study, which was approved by the Ethical Committee of our Institutions.

All subjects were evaluated for plasma concentrations of creatinine, glucose, calcium, cholesterol and triglycerides. The subjects were also evaluated for serum CRP, bone alkaline phosphatase (BALP), C-telopeptides of type-1 collagen (CrossLaps), RANKL

Table 1 Demographical (sex and age) and biochemical features (estimated creatinine (Cr) clearance, plasma glucose, cholesterol, triglycerides, calcium and serum C-reactive protein (CRP), calcium, bone alkaline phosphatase (BALP), C-telopeptides of type-1 collagen (CrossLaps), osteoprotegerin (OPG) and nuclear factor-kappaB ligand (RANKL) values) of 283 enrolled subjects (Group Ad (82 adults who were between 27 and 64 years aged), Group Old-I (52 old subjects who were between 65 and 84 years aged), Group Old-II (149 long-lived subjects who were between 85 and 110 years aged). Age, estimated Cr clearance, glucose, cholesterol, triglycerides, CRP and calcium values were presented as mean $\pm$ s.d., whereas BALP, CrossLaps, OPG and RANKL values were presented as median and ranges.

	Group Ad (<i>n</i> = 82)	Group Old-I (<i>n</i> = 52)	Group Old-II (<i>n</i> = 149)
Age (years)	47.9 $\pm$ 11.4	69.2 $\pm$ 4.1*	93.0 $\pm$ 5.5**
Sex (F/M)	69/13	34/18	114/35*
Cr-clearance (ml/min)	94.5 $\pm$ 7.4	69.4 $\pm$ 11.5*	54.1 $\pm$ 12.0**
Glucose (g/l)	0.80 $\pm$ 0.15	0.90 $\pm$ 0.30	0.95 $\pm$ 0.23
Cholesterol (mg/dl)	156 $\pm$ 25.5	175 $\pm$ 48.0*	183.5 $\pm$ 49.0*
Triglycerides (mg/dl)	95.0 $\pm$ 30.6	118.0 $\pm$ 47.2*	113.2 $\pm$ 45.6
CRP (mg/l)	0.89 $\pm$ 0.40	1.59 $\pm$ 0.63*	2.1 $\pm$ 0.90**
Calcium (mg/dl)	9.5 $\pm$ 0.3	9.0 $\pm$ 0.7	9.1 $\pm$ 0.6
Crosslaps (ng/ml)	0.28 (0.10–0.66)	0.41 (0.25–0.89)*	0.72 (0.18–3.0)**
BALP (μ g/l)	7.3 (1.0–14.8)	11.3 (4.8–21.5)*	9.4 (2.5–28.5)*
RANKL (pmol/l)	0.14 (<0.08–0.17)	0.21 (0.05–0.54)*	0.20 (<0.08–1.4)*
OPG (pmol/l)	2.2 (1.4–6.0)	3.5 (1.8–11.7)*	8.9 (2.6–50.0)**

The values of the three age-related groups of subjects were compared by ANOVA (for the mean) or Kruskal–Wallis test followed by Mann–Whitney test (for the median). Bonferroni correction was applied to the significance level.

*, Statistically significant vs. Group Ad; **, statistically significant vs. Group Ad and Group Old-I.

and OPG concentrations. Blood samples were collected according to the ethical guidelines between 0900 and 1100 h after a 12-h fast, and then frozen at -40°C . OPG and RANKL were assayed by enzyme immunoassays (BIOMEDICA GRUPPE, Wien, Austria). The detection limits of the assays were 0.14 pmol/l and 0.08 pmol/l for OPG and RANKL respectively. The mean intra- and interassay coefficients of variation of the OPG assay were 6.7% and 8.9% respectively. The mean intra-assay and interassay coefficients of variation of the RANKL assay were 4.0% and 7.5% respectively. Serum CrossLaps concentrations were assayed by an ELISA method (Nordic Bioscience Diagnostic A/S, Herlev, Denmark). The detection limit of the assays and the intra-assay and interassay variations expressed as coefficients of variation were: 0.01 ng/ml, 5.3% and 6.3% respectively. In our laboratory the reference ranges of CrossLaps values were: 0.05–0.45 ng/ml, 0.05–0.55 ng/ml and 0.1–0.80 ng/ml, for male, premenopausal and post-menopausal women respectively. Serum BALP was assayed by an immunoradiometric method (Tandem-T Ostase, Beckman Coulter, Milan, Italy). The detection limit, intra-assay and interassay coefficients of variation were 0.5 $\mu\text{g/l}$, 4.5% and 7.0% respectively. In our laboratory the reference ranges of BALP values were: 5.0–18.5 $\mu\text{g/l}$, 3.5–14.0 $\mu\text{g/l}$ and 7.0–22.0 $\mu\text{g/l}$, for male, pre-menopausal and post-menopausal women respectively.

Plasma creatinine, glucose, calcium, cholesterol, triglycerides and serum CRP concentrations were measured by commercial kits.

Statistical analysis

Data were presented as means $\pm$ standard deviation (s.d.), unless otherwise stated. The normal distribution of the variables was analysed using the Kolmogorov–Smirnov test. Logarithm transformation was applied to give data skew values of 0 ± 1 whenever possible. The values of the three groups of subjects were compared by ANOVA or Kruskal–Wallis test, followed by Mann–Whitney test, as appropriate. Bonferroni correction was applied to the significance level. Relationships between OPG and different variables were sought using Pearson's correlation coefficient and followed by multiple regression analysis with forward selection. The multiple regression analysis was performed only with the variables that were significantly correlated with OPG in the univariate analysis. The regression coefficient generated by this analysis indicated the slope of association among the dependent variable and the specified independent variables. Frequencies were compared using the Chi-square test, with Fisher's correction, when appropriate. Statistical significance was assumed when the probability (P) was less than or equal to 0.05, with the exceptions described above.

Results

In the whole population of subjects, serum OPG and RANKL values increased significantly with the age of the subjects (r : 0.47, $P < 0.001$; r : 0.16, $P = 0.008$ respectively). The correlation between age and serum OPG values was found in both females (r : 0.5, $P < 0.001$) and males (r : 0.37, $P = 0.002$). By contrast, the significant age-dependent increase in RANKL values occurred in females (r : 0.15, $P = 0.02$) but not in males (r : 0.12, $P = 0.3$).

Subjects of Group Old-II (149 cases) had significantly higher serum CRP ($P = 0.02$), CrossLaps ($P = 0.001$) and OPG ($P < 0.001$) values and lower estimated creatinine clearance ($P < 0.02$) as compared with the subjects of Group Old-I (52 cases), without any significant differences in glucose, cholesterol, triglycerides, calcium, BALP and RANKL values (Table 1). Both groups of old subjects showed significantly higher serum cholesterol, CRP, CrossLaps, BALP, OPG and RANKL and lower estimated creatinine clearance as compared with the adult young subjects (Group Ad) (Table 1).

Table 2 shows the correlation coefficients, as calculated by univariate analysis, between OPG and different demographical and biochemical variables in the three groups of subjects. In Group Ad, serum OPG was significantly correlated with RANKL and Cr-clearance. In Group Old-I, serum OPG values were correlated with RANKL, Cr-clearance, CRP, and Crosslaps values. In the Group Old-II, serum OPG values were still correlated with Cr-clearance, RANKL and CRP, whereas the correlation with Crosslaps disappeared. In all three groups of subjects we did not find any correlation between OPG

Table 2 Pearsons' coefficients, as calculated by univariate analysis, between serum osteoprotegerin (OPG) and different variables (sex, estimated creatinine (Cr) clearance, glucose, cholesterol, triglycerides, c-reactive protein (CRP), calcium, bone alkaline phosphatase (BALP), C-telopeptides of type-1 collagen (CrossLaps) and nuclear factor-kappaB ligand (RANKL)) in the 3 different age-related groups (Group Ad (82 adults who were between 27 and 64 years of age), Group Old-I (52 old subjects who were between 65 and 84 years of age), Group Old-II (149 long-lived subjects who were between 85 and 110 years of age))

Variable	Serum OPG (Pearson's coefficients of correlation)		
	Group Ad ($n = 82$)	Group Old-I ($n = 52$)	Group Old-II ($n = 149$)
Sex	0.08	−0.01	−0.09
Cr-clearance	0.26	0.46	0.34
Glucose	0.07	0.1	−0.12
Cholesterol	0.05	0.11	−0.19
Triglycerides	0.1	0.13	−0.06
CRP	0.09	0.32	0.59
Calcium	−0.1	0.05	0.07
Crosslaps	−0.002	0.47	0.1
BALP	0.33	−0.02	−0.2
RANKL	0.48	0.34	0.32

The values in bold are statistically significant in the univariate analysis.

and glucose, cholesterol, triglycerides, calcium and BALP values (Table 2).

A multivariate linear analysis was performed to investigate the independent effect of the variables above described on serum OPG values. Indeed, only the variables significantly correlated with OPG in the univariate analysis were considered in the linear regression models. In the adult subjects (Group Ad), serum RANKL and estimated creatinine clearance were independently correlated with serum OPG values (R^2 : 0.29). In the old subjects who were under 85 years aged (Group Old-I), serum RANKL, estimated creatinine clearance and CrossLaps values were independently correlated with OPG (R^2 : 0.63). However, in multivariate analysis performed in the long-lived subjects (Group Old-II), serum CRP and estimated creatinine clearance remained significantly correlated with OPG (R^2 : 0.45) whereas RANKL, which was found to be significantly correlated with OPG by the univariate analysis, did not attain significance when considered as a possible addition to the baseline model.

The above correlations were maintained in each group even after correction for the sex of the subjects

Discussion

This cross-sectional study shows that in the long-lived subjects serum OPG values were elevated regardless of high bone turnover, in close relationship with the pro-inflammatory status as assessed by the serum C-reactive protein measurement. This feature was different from that found in the old subjects who were under 85 years of age in whom bone turnover seemed to be the most important factor influencing circulating OPG values.

OPG is synthesised by skeletal and extraskeletal tissues and is regulated by a variety of cytokines and hormones (24). Even considering the limitations that are inherent to all studies assessing circulating concentrations of paracrine cytokines (24), the increase in serum OPG values in our series would reflect a true increase in cytokine production. The progressive reduction in renal capacity throughout the aging process may have contributed to the increase in serum OPG values in our old and long-lived subjects. However, the renal function was not so impaired in any subjects as to justify completely the huge increase in serum OPG values in our old and long-lived subjects. Moreover, the multivariate analysis demonstrated that serum OPG values were correlated independently with different biochemical parameters in addition to the renal function, providing convincing evidence that other factors in addition to the age-related decrease in renal capacity may have been responsible for the progressive increase in serum OPG values throughout ageing.

Previous studies suggested that the age-related increase in OPG production likely reflects a compensatory self-defence mechanism against age-associated diseases such as osteoporosis (19). In our

study, the correlation between OPG and bone resorption was found in the old subjects who were under 85 years of age but not in the long-lived subjects. This finding was really unexpected, since several studies had already demonstrated that in the presence of osteoporosis, as found in long-lived subjects (25), serum OPG values should be strictly correlated with bone turnover (13–16). Although we did not evaluate the bone structure in our subjects, the huge increase in serum bone resorption markers in long-lived subjects would suggest that the bone was impaired in this population, in agreement with previous observations (25). Another interesting finding was the disappearance of a correlation between OPG and RANKL in long-lived subjects when the statistical analysis was performed by the multivariate instead of the univariate approach. Since its characterization, OPG has been identified as a decoy receptor, binding RANKL and thereby inhibiting the interaction between RANKL and its receptor on the osteoclast surface (12). Our findings would lead us to hypothesise that in long-lived subjects OPG may increase regardless of the change in RANKL production.

The novelty deriving from our data is the close correlation between OPG and CRP in long-lived subjects. To the best of our knowledge this is the first demonstration of a correlation between OPG and inflammation in long-lived subjects, such as has already been demonstrated by others in patients with coronary artery disease (26). Over recent years, several studies have demonstrated that the ageing process is accompanied by an increase in serum markers of inflammation and this trend has been considered, although with some critical appraisal (27, 28), as an expression of risk to develop cardiovascular disease (4–7). It has been theorised that the beneficial effects of inflammation devoted to the neutralisation of harmful agents early in life and in adulthood become detrimental late in life, according to the antagonistic pleiotrophy theory of ageing (1). However, even if the longevity is associated with an increase in risk factors for cardiovascular diseases (29, 30), the long-lived subjects are unexpectedly free from these disorders. It has been pointed out that unknown protective factors guarantee the well-being in the oldest-old even in the presence of important risk factors (2). It is noteworthy that none of our subjects, adult or old or very old, had clinical evidence of important cardiovascular disease. In this context, the high OPG concentrations demonstrated in long-lived subjects and their strong correlation with markers of inflammation may reflect a physiological remodelling of the organism which occurs with ageing in response to internal and external damaging agents (29, 30).

In conclusion, our data lead us to suggest that different factors, in addition to the expected modifications of renal function, are responsible for the age-dependent enhancement of OPG production. Bone metabolism would be the most important factor influencing serum OPG concentrations in old subjects under 85 years of age, whereas in the long-lived subjects

the circulating values of this cytokine seem to be mainly correlated with the pro-inflammatory state, which could be a marker of cardiovascular risk. These results would be a start-point for future investigations on the eventual role of OPG as a protective factor against the age-related diseases in the context of the so-called antagonistic pleiotropy of ageing.

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