EXPERIMENTAL STUDY

Serum parathyroid hormone, but not menopausal status, is associated with the expression of osteoprotegerin and RANKL mRNA in human bone samples

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Abstract

Objective: Osteoprotegerin (OPG) and its ligand 'receptor activator of NF- κ B ligand' (RANKL) are important regulators of bone metabolism. RANKL, expressed in osteoblasts, activates osteoclast differentiation and osteoclast function by binding the 'receptor activator of NF- κ B' (RANK), expressed in osteoclast precursors and mature osteoclasts. The effect is prevented by OPG, a soluble receptor of RANKL. *In vitro* studies have suggested that estrogen stimulates OPG, whereas parathyroid hormone (PTH) inhibits OPG expression and stimulates the expression of RANKL.

Design: In the present study, we examined the relationship between the menopause, serum PTH and the expression of OPG and RANKL in human bone tissue *in vivo*.

Methods: To address this question, we established a 5'-nuclease assay to quantify the mRNA copies of human OPG and RANKL, normalized to the number of copies of β -actin mRNA in 169 women (mean age: 52.4 ± 11.6 years), who underwent surgery for early breast cancer. Intact serum PTH was measured by chemoluminescence in 61 women.

Results: We found no significant difference in the expression of OPG and RANKL between postmenopausal women and premenopausal women. Also, the ratio of RANKL to OPG was unchanged in relation to the menopausal status. Serum PTH was negatively associated with the expression of OPG ($r = -0.33$, $P = 0.01$), but also, surprisingly, with the expression of RANKL ($r = -0.28$, $P = 0.03$).

Conclusion: We failed to observe the expected changes in the expression of OPG and RANKL in human bone samples at menopause. High *in vivo* levels of circulating PTH are accompanied by low levels of expression of the two transcripts in human bone tissue.

European Journal of Endocrinology 145 199–205

Introduction

Recent discoveries of members of the tumor necrosis factor (TNF) receptor and ligand family have potential implications for the understanding of osteoclast differentiation and activation.

The receptor activator of NF- κ B (RANK) is a type 1 transmembrane protein expressed in a variety of tissues. Among bone cells, RANK is found only in osteoclast-lineage cells (1). The importance of activation of NF- κ B is highlighted by the observation that double-knockout mice deficient in both NF- κ B1 and NF- κ B2 had impaired osteoclastogenesis and osteopetrosis (2).

The natural ligand for RANK, RANKL (RANKL) was identified independently by several groups (3–6). The transmembrane protein is expressed in various stromal cell lines, in osteosarcoma cell lines,

and in primary murine osteoblasts (7). In bone, RANKL stimulates osteoclast differentiation (5, 6, 8), enhances the activity of mature osteoclasts (5, 9, 10), and inhibits osteoclast apoptosis (9).

Osteoprotegerin (OPG) (11, 12) acts as a secreted receptor for RANKL/OPG-ligand that prevents it from binding to, and activating, RANK on the osteoclast surface (13). Therefore, the biological effects on bone cells are the opposite of that of RANKL/OPG-ligand, including inhibition of the terminal stages of osteoclast differentiation (11, 14–17), suppression of the activation of mature osteoclasts (5, 10, 18), and induction of apoptosis (19).

On the other hand, it is well known that the bone-resorption pathway is influenced by a variety of osteotropic factors, including estrogens and parathyroid hormone (PTH). Estrogen deficiency, in particular, is associated with a strong increase in bone loss.

Bone loss in this situation is mainly induced by the activation of osteoclasts (20). In relation to the new members of the TNF receptor and ligand family, it could be demonstrated that estrogen raises the production of OPG by mature osteoblasts (21), suggesting that the RANKL/OPG system is involved in the antiresorptive capacity of estrogens. *In vivo* data concerning the relationship between the menopause and RANKL/OPG in human bone are still lacking.

For PTH also, a relationship to the RANKL/OPG system could be demonstrated *in vitro*: in murine bone-marrow cultures, PTH stimulates RANKL and inhibits OPG expression (22–24). In addition, Onyia *et al.* recently demonstrated the inhibition of OPG expression in bone by treatment of rats with human PTH (1–38) (25). On the other hand, there is evidence that PTH does not affect OPG levels in osteoblastic cells such as MC3T3 E-1 cells and SaOS-2 cells (7).

In the present study, we examined the relationship between the menopause, serum PTH, and the expression of OPG and RANKL in human bone samples.

Study population and methods

Study population

Jamshidi bone biopsies from the anterior–superior iliac crest were consecutively obtained from 169 women during surgery for early breast cancer at the Gynecology Department of the University of Heidelberg. The mean length of the jamshidi biopsies was 1–2.5 cm. Samples were immediately stored in liquid nitrogen until further preparation. The biopsies were part of a prospective study examining the impact of local bone-growth factors on the development of bone metastases. This study was approved by the local human-studies committee.

For the present study, we analyzed bone samples only from women at an early tumor stage (T1, T2) and who had no metastases at the time of surgery, normal serum calcium concentrations and no other evidence of systemic tumor disease.

Preparation of RNA

Total RNA was extracted by the guanidinium thiocyanate method described by Chromczynski & Sacchi (26). The quality of the RNA preparation method and the samples was assessed in 20 samples by electrophoresis through denaturing agarose gels, staining with ethidium bromide, and visualization of the 18S and 28S RNA bands under UV illumination. The extraction yield was quantified spectrophotometrically. The yield was 1–4 µg.

Complementary DNA synthesis

Reverse transcription of 1 µg RNA was performed in a final volume of 20 µl containing 5 mM MgCl₂, 1 × PCR

Buffer II (PE Applied Biosystems, Langen, Germany), 1 mM each dNTP (Boehringer Mannheim, Mannheim, Germany), 1 U/µl RNase inhibitor (PE Applied Biosystems), murine leukemia virus (MuLV) reverse transcriptase (PE Applied Biosystems) and 2.5 µM Oligo d(T)₁₆ primer (PE Applied Biosystems). Reaction conditions were as follows: 10 min incubation at room temperature, 42 °C for 60 min, followed by 5 min at 95 °C for inactivation of MuLV. The usual reverse transcriptase mixture was used for all RNA samples included in the study. The complementary DNAs (cDNA) were diluted 1:10 in nuclease-free H₂O.

Determination of messenger RNA levels using real-time PCR

We developed a real-time PCR assay based on the 5'-nuclease assay first described by Holland *et al.* (27). Oligonucleotide primers and TaqMan probes were designed using the computer program PRIMER EXPRESS (PE Applied Biosystems). Sequences were obtained from the GenBank databases as described in Table 1. Oligonucleotides, primers and probes were purchased from Perkin Elmer/PE Applied Biosystems. The nucleotide sequences of both primers and probes are given in Table 1.

The PCR reaction was performed with 96 sample tubes per assay, using a cDNA equivalent of 2 µl of the cDNA solution of each sample described above. Each sample was analyzed in duplicate. The TaqMan PCR core reagent kit (Perkin Elmer/PE Applied Biosystems) was used according to the manufacturer's instructions: 1 × Buffer A, 5 mmol/l MgCl₂, 200 µmol/l dATP/dCTP/dGTP 400 µmol/l dUTP, 1.25 U AmpliTaq Gold DNA polymerase, 2.5 U uracil N-glycosylase, 200 mmol/l probe and 300 mmol/l each primer. For each target, a unique master mix aliquoted for use in 5 different assays necessary to measure all 169 samples was prepared. The PCR was developed on an ABI Prism 7700 Sequence Detector (Perkin Elmer/PE Applied Biosystems). The PCRs involved 40 cycles of two-step PCR, including 15 s of denaturation at 95 °C and 1 min annealing–elongation at 59 °C, using the standard protocol of the manufacturer. To generate a standard curve for each target, the sequence of interest was cloned in the pCR-Script SK(+) plasmid, according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The cloned sequence was checked by using a PCR followed by direct sequencing of the PCR products. After plasmid preparation (plasmid extraction mini kit; Qiagen, Hilden, Germany) the number of plasmid copies was determined spectrophotometrically. A standard curve (50, 100, 500, 1000, 5000 and 10 000 copies) and controls (100 and 5000 copies) were generated for each transcript, aliquoted, and used in all TaqMan assays. To normalize differences in the amount of total, intact mRNA added to the reaction, quantification of the β-actin mRNA copies

Table 1 Primer and probes used for TaqMan-PCR. GenBank numbers: β -actin, M10278; osteocalcin, X53698; OPG, AB002146; RANKL, AF053712.

Transcript	Forward primer	Probe	Reverse primer	Amplicon length
β -Actin	ATTGCCGACAGGATGCAGAA	TCATTGCTCCTCCTGAGCGCAAGT	GCTGATCCACATCTGCTGGAA	150
Osteocalcin	CTCCTGAAGCCGATGTGGT	AGCCATTGATAGAGGTAGCGCCTGGG	GGCAGCCGAGGTAGTGAAGA	151
OPG	AGGCATCTTCAGGTTTGCTG	TACAAAGTTACGCCCTAACTGGCTTAGTGCTCTGGT	GCCTTTACTTTGGTGCCA	91
RANKL	TGCCAACATTTGCTTTTCGAC	TTCAGGAGACCTAGCTACAGAGTATCTTCAACTAATGGTG	TTGGGATTTTGATGCTGGTTT	101

in each sample was performed; the amount of each transcript is given as copies per 100 000 copies of β -actin mRNA. With the 5-nuclease assays in a 96-well format we were able to measure 39 samples in the same assay. The intra-assay coefficient of variation (CV) was 8.4% and the interassay CV was 8.8%. The highest variation is introduced by the process of RNA preparation and the reverse transcription. In our assay we found, for completely different RNA preparations (reverse transcription reactions and 5'-nuclease assays), a CV of 15–20% depending on the transcript.

Measurement of serum intact PTH

Measurement of serum intact PTH levels was performed by using a chemoluminescence assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The sensitivity is 2 pg/ml, the intra-assay variance was 6.2%, and the interassay variance was 5.6%.

Statistical analysis

Descriptive statistics and simple and partial Pearson correlations were obtained using the STATISTICAL ANALYSIS SYSTEM software program (SAS Institute, Cary, NC, USA). Differences between groups were analyzed by using the general linear model. The distribution of most variables was skewed to the left, and these measurements were log-transformed before analysis to achieve normal distribution.

Results

Characteristics of the study population

The characteristics of the study population are given in Table 2. In 48 women (28.4%), the exact menopausal status was not determinable. The women classified as 'postmenopausal with HRT' stopped taking hormone-replacement therapy (HRT) on the day of admission to the clinic, and surgery and bone biopsies were normally performed 1–2 days after admission.

In a substantial proportion of the women, menopausal age could be determined exactly; thus, we were able to classify the women as 'less than five years since menopause' ($n = 23$), 'less than ten years since menopause' ($n = 28$), and 'less than 15 years since menopause' ($n = 36$). Women with a history of HRT were not considered in this classification.

The expression of OPG and RANKL was calculated as copies per 100 000 copies of β -actin. The expression of OPG was higher than the expression of RANKL in bone biopsies. After log transformation, both parameters showed a normal distribution. We found no significant association between age or body mass index with OPG and RANKL; the ratio of OPG to RANKL showed a tendency for a negative association with age ($r = -0.11$; $P = 0.17$) (data not shown).

Table 2 Characteristics of the study population. Data were derived from 169 women; PTH levels could be determined in $n = 61$ women.

Characteristic	Mean $\pm$ s.d.
Age (years)	52.4 $\pm$ 11.6
Body mass index (kg/m ²)	25.9 $\pm$ 5.3
Menopausal status:	
No exact information available	28.4% ($n = 48$)
Premenopausal	25.4% ($n = 43$)
Perimenopausal	4.1% ($n = 7$)
Postmenopausal, with HRT	10.1% ($n = 17$)
Postmenopausal, without HRT	32.0% ($n = 54$)
PTH (ng/ml)	30.4 $\pm$ 13.1
Copies osteocalcin mRNA per 100 000 copies β -actin	545.3 $\pm$ 822.9
Copies osteoprotegerin mRNA per 100 000 copies β -actin	2942.1 $\pm$ 5594.8
Copies RANKL mRNA per 100 000 copies β -actin	960.7 $\pm$ 1707.4
Ratio of osteoprotegerin mRNA to over RANKL mRNA	4.3 $\pm$ 6.6

Expression of OPG and RANKL and menopausal status

Figure 1 shows the expression of OPG, RANKL, and the OPG/RANKL ratio in premenopausal women, postmenopausal women without HRT, and postmenopausal women with HRT before admission. No significant differences between the groups could be observed. Table 3 summarizes the analysis of the expression of OPG and RANKL stratified for menopausal status. To test the hypothesis that the changes in OPG/RANKL expression after the menopause may be limited to the first 5–10 years, we analyzed the data by stratification for early menopausal women ('less than five years postmenopausal') and women 'less than 10 or 15 years

postmenopausal'. Again, no significant differences could be observed. Even use of the osteocalcin expression level to normalize the osteoblastic cell fraction in the bone samples produced no significant differences between the groups (data not shown).

Expression of OPG and RANKL and association with serum PTH

PTH measurements were available for 61 women. To evaluate the relationship between serum PTH levels and the expression of OPG and RANKL in the bone biopsies, we carried out a linear regression analysis. The result of the linear association is given in Fig. 2. We found a significant negative association between the expression of OPG and serum PTH, and there was also a significant negative association between the expression of RANKL and serum PTH. This was primarily due to low OPG and RANKL expression with high-normal or slightly elevated PTH serum levels. The OPG/RANKL ratio was not significantly associated with serum PTH levels. Adjustment of the mentioned association for age and body mass index resulted in a persistent, significant association (Table 4). The use of osteocalcin instead of β -actin to normalize the osteoblastic cell fraction in the biopsies also resulted in a negative association of osteoprotegerin and RANKL with serum PTH ($r = -0.27$, $P = 0.04$; $r = -0.29$, $P = 0.03$, respectively).

Discussion

Our understanding of differentiation and activation of cells of the osteoclastic lineage has been extended by recent discoveries of members of the TNF receptor and ligand families. The development of active osteoclasts *in vitro* requires intimate contact between osteoblast/

Figure 1 Expression of OPG, RANKL, and the ratio of OPG to RANKL in premenopausal women ($n = 43$), postmenopausal women without HRT ($n = 54$), and postmenopausal women with HRT ($n = 17$) before admission to the clinic. Data are means $\pm$ S.E.M. No significant differences could be observed when tested using the general linear model.

Table 3 Menopausal status and expression of OPG- and RANKL-mRNA. No significant differences were observed.

	OPG (mean±S.E.M.)	RANKL (mean±S.E.M.)	OPG/RANKL ratio (mean±S.E.M.)
Premenopausal women (n = 43)	6.97±0.23	5.77±0.25	1.21±0.10
Postmenopausal women without HRT (n = 54)	6.73±0.20	5.57±0.22	1.16±0.09
Less than 5 years since menopause (n = 23)	6.54±0.31	5.31±0.35	1.23±0.15
Less than 10 years since menopause (n = 28)	6.77±0.28	5.58±0.32	1.19±0.13
Less than 15 years since menopause (n = 36)	6.67±0.24	5.48±0.28	1.19±0.12

stromal cells and osteoclast precursors, and RANKL has been shown to mediate this interaction (7). It binds to receptors on osteoclast precursors (RANK), promoting an event leading to osteoclast formation. This process can be blocked by OPG, a decoy receptor. Recently, several *in vitro* studies revealed that the expression of RANKL and OPG is affected by known osteotropic hormones such PTH, estrogens, and vitamin D (13). *In vivo* data relating to this issue remain limited.

One aim of the present study was the analysis of expression of RANKL and OPG in human bone samples in relation to menopausal status. We found no significant differences in the expression of OPG and RANKL between pre- and postmenopausal women.

The molecular mechanisms of estrogen action on bone are not understood completely. Estrogens act directly on osteoblastic and osteoclastic cells through high-affinity receptors (28). The principal effect on bone is a decrease in bone resorption. Recent reports suggest that OPG is involved in the paracrine mediation of estrogen effects on bone: human osteoblastic cells demonstrate stimulated expression of OPG *in vitro* (21). On the other hand, Yano *et al.* (29) reported a threefold increase in serum OPG levels with age in postmenopausal women. We found no substantial differences between premenopausal women, postmenopausal women on HRT, and postmenopausal women without HRT in terms of the expression of OPG and RANKL in bone biopsies.

Our study has some limitations: first, given the number of bone samples in our study, we are able to distinguish twofold (or more) differences in the expression of the transcripts of interest when comparing pre- and postmenopausal women. We cannot exclude the

possibility of significant differences below this detection limit; the biological impact that would be exerted by such differences is unclear. The power is strongly related to the precision of the measurement. In comparison with other methods of RNA quantification, the 5'-nuclease assay allows quantitative measurement of mRNA that compares favorably with the best non-PCR method, namely the RNase protection assay (30).

A second limitation of our study is that we were only able to measure gene expression; the amounts of OPG and RANKL protein in bone biopsies, however, also could be regulated at the translational level. *In vitro* examinations clearly demonstrated regulation at the transcriptional level for OPG, but the *in vitro* studies could not exclude the possibility that OPG is regulated at both the transcriptional level and the translational level (21). In addition, the present analysis gives no information about the cellular origin of the transcripts examined. We cannot exclude the possibility of significant differences limited to a cellular subset in the bone biopsies. To test the hypothesis that the changes might be limited to the osteoblastic cells in the bone samples, we normalized the OPG and RANKL expression levels to osteocalcin; again, no significant differences could be observed. Recently, the presence of pre-B-cells (31) – which are present in normal bone marrow, express RANKL, and are able to promote osteoclastogenesis – was published. However, although we cannot exclude the possibility of a significant change in RANKL expression in these cells at the menopause, the RANKL expression of these cells also contributes to the total RANKL mRNA measured in our study. On the other hand, analysis of total RNA extracted from bone has an advantage over bone-cell cultures in that paracrine mediators can be measured

Figure 2 Unadjusted linear Pearson correlation between the expression of OPG in human bone biopsies and serum PTH (a), between RANKL and serum PTH (b), and the ratio of OPG over RANKL and serum PTH (c). The correlation coefficients are given in each graph. *Expression per 100 000 copies of β -actin.

Table 4 Correlation coefficients of the association of PTH with OPG/RANKL.

	OPG	RANKL	OPG/RANKL ratio
Serum PTH	-0.33 ^a	-0.28 ^b	0.09
Partial age and body mass index	-0.33 ^b	-0.30 ^b	-0.02

^a*P* < 0.01; ^b*P* < 0.05.

with their local regulatory mechanisms intact. That the samples were obtained from women with breast cancer could mean that there is a potential bias in the study. It is known that these women have a slightly higher bone mineral density than women without breast cancer, because of higher estrogen exposure. Since the regulatory mechanism of the RANK/RANKL system is intact in these women, this should not limit the results of our analysis.

A second objective of our study was to examine the relationship between serum PTH and OPG and RANKL expression in human bone *in vivo*. In bone cell cultures (5, 22, 23) and in rodents (25), PTH has been shown to reduce OPG mRNA expression. Our data are compatible with an inhibitory effect of PTH on OPG expression in human bone, as we observed a negative association with circulating PTH levels. However, in contrast to bone-cell culture, in which PTH has been shown to stimulate RANKL expression (5, 22, 23), an inverse association was observed by us between circulating PTH levels and RANKL expression. The reasons for this paradoxical *in vivo* findings remain unclear. Breast cancer cells are capable of producing PTH-related peptide, which may have affected the relationship between PTH and RANKL in our particular study sample of women with breast cancer. However, all samples were from women with early-stage breast cancer, normal serum calcium levels and undetectable circulating PTH-related peptide (according to radio-immunoassay). A possible explanation for the inverse association is that, as RANKL expression *in vitro* is affected by various hormones and cytokines (13), one or more inhibitory determinants of RANKL expression may be linked to high levels of circulating PTH *in vivo*. For example, vitamin D₃ has been shown to stimulate both OPG and RANKL expression in bone cells (13). Decreases in vitamin D, resulting in secondary increases in PTH, would therefore have additive inhibitory effects on OPG expression *in vivo*, but might neutralize the stimulatory effects of PTH on RANKL expression. In fact, low levels of RANKL expression in our bone samples were observed particularly at high-normal or slightly elevated PTH levels in the presence of normal serum calcium – compatible with mild secondary hyperparathyroidism. Alternatively, PTH might change the composition of RANKL- and OPG-producing cells in bone tissue – a possibility that is difficult to examine only in *in vitro* studies. Unfortunately, the small amounts of blood samples and bone

tissue available to us in the present study (for ethical reasons) restricted further analysis.

In conclusion, in the present study we examined OPG and RANKL expression in relation to estrogen status and circulating PTH, using a large number of human bone samples. We failed to observe any effect of the menopause on OPG and RANKL expression in human bone samples. Both OPG and RANKL expression were inversely correlated with circulating PTH levels, indicating that it is difficult to predict the net effects of bone-cell regulators *in vivo* on the expression of these osteoclast regulators from their effects *in vitro*.

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Received 14 February 2001

Accepted 27 April 2001