Increased bone resorption in HD patients: is it caused by elevated RANKL synthesis?

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Abstract

Background. The receptor activator of nuclear factor κB ligand (RANKL), produced by osteoblasts/stromal cells, is a member of the RANK/RANKL/OPG system, which regulates bone resorption by osteoclasts. Since RANKL and osteoprotegerin (OPG) production in bone is influenced by parathyroid hormone (PTH), we measured serum RANKL and OPG concentrations in haemodialysis (HD) patients, who commonly hypersecrete PTH. We aimed to determine if clinically demonstrated PTH-enhanced bone resorption is a consequence of increased RANKL synthesis.

Methods. RANKL, OPG, osteocalcin, intact PTH, bone-specific alkaline phosphatase, tartrate-resistant acid phosphatase 5b and β-CrossLaps (CTx) were measured in blood samples from 80 HD patients and 50 age-matched controls. HD patients were stratified to tertiles according to their serum PTH levels: 29.3–103.0, 109.7–263.0 and 262.0–1700.0 pg/ml in the first, second and third tertiles, respectively.

Results. Mean serum RANKL levels were 1.6 times higher in HD patients than in age-matched controls (1.36±0.39 vs 0.83±0.70 pmol/l; P<0.001). All the measured bone markers significantly differed between patients and controls (P<0.001). Spearman’s tests of correlation showed a statistically significant association of RANKL with PTH, osteocalcin and CTx (r=0.322, P=0.004; r=0.231, P=0.039; and r=0.230, P=0.040, respectively). Mean serum RANKL levels were significantly different between PTH tertiles (P=0.003), but serum OPG levels were not (P=0.144). The highest RANKL levels were measured in the upper PTH tertile (1.54±0.39 pmol/l) and were significantly higher than in the middle or lower tertiles (1.27±0.42 and 1.23±0.26 pmol/l, respectively; P=0.003). Both of the measured bone-resorption markers, tartrate-resistant acid phosphatase 5b and CTx, as well as both bone formation markers, osteocalcin and bone-specific alkaline phosphatase were also significantly higher in the upper tertile, indicating that whole-bone remodelling is activated at high PTH and RANKL levels.

Conclusions. Serum RANKL levels were significantly higher in HD patients than in healthy age-matched controls. Moreover, RANKL levels were significantly higher in the upper PTH tertile, indicating enhanced RANKL synthesis in a PTH-dependent fashion. Thus, our clinical findings clearly support published in vitro studies that demonstrated a stimulating effect of PTH on RANKL synthesis. Therefore, the hypothesis that PTH increases bone resorption in HD patients through RANKL appears valid.

Keywords: bone biochemical markers; bone turnover; osteoprotegerin ligand; parathyroid hormone; renal osteodystrophy; RANKL

Introduction

Progressive reduction in kidney function leads to reduction in the synthesis of calcitriol and to changes in parathyroid hormone (PTH) secretion. These alterations are the key contributors to the pathogenesis of renal osteodystrophy (ROD) [1]. In bone remodeling, osteoblastic bone formation and osteoclastic bone resorption occur in a precise and quantitative manner. These two processes are balanced in young adults; however, in persons >35 years of age, bone resorption exceeds bone formation. The process of coordinated resorption and formation of bone in ROD may be up- or down-regulated by systemic hormones (PTH, calcitriol) or local factors [interleukins (IL-1, IL-6) and growth factors (tumour necrosis factor-α, IGF)] [2,3].
There is evidence that PTH enhances the synthesis of a special cytokine, receptor activator of nuclear factor κB ligand (RANKL), in osteoblasts [4,5]. RANKL (also known as TRANCE/OPGL/ODF) plays a pivotal role in osteoclastogenesis by providing an essential signal to osteoclast progenitors through the membrane-anchored receptor RANK. Signal transduction through RANK leads to osteoclast differentiation and functional activation. This signalling pathway can be modified by a naturally occurring decoy receptor for RANKL, termed osteoprotegerin (OPG). It blocks interaction between RANKL and RANK. Thus, by binding to RANKL, OPG inhibits bone resorption and protects bone tissue against extensive bone deterioration. Bone marrow osteoblasts/stromal cells secrete both RANKL and OPG; thus, osteoclast formation is determined principally by the ratio of RANKL to OPG in bone [4–9].

The recent in vitro study by Huang et al. [5] confirmed that PTH significantly up-regulates RANKL mRNA in bone marrow stromal osteoblasts and inhibits OPG gene expression at all stages of osteoblast differentiation. The importance of circulating OPG levels in ROD has been reported already [10–12]. Little is known presently about the effects of PTH on RANKL synthesis in uraemia and no study has examined RANKL serum levels in HD patients nor evaluated the relationships between RANKL and bone markers in serum. The aim of our work was to find out whether or not clinically demonstrated PTH-enhanced bone resorption is a consequence of increased RANKL synthesis.

### Subjects and methods

#### Patients

We enrolled 80 haemodialysis (HD) patients in this study. The subjects’ end-stage renal failures were caused by various kidney diseases, including chronic glomerulonephritis (n = 15), hypertensive nephrosclerosis (n = 15), analgetic nephropathy (n = 6), diabetic nephropathy (n = 8), polycystic kidney disease (n = 8), pyelonephritis (n = 5) and other causes (n = 23). The patients were treated three times a week for 4–6 h with bicarbonate- or acetate-free dialysis solutions for 35 months (range: 6–240 months). Their mean calcium and phosphate levels were maintained within tolerable ranges (2.39 ± 0.24 and 1.66 ± 0.39 mmol/l, respectively). All patients were free of infections; four patients had undergone parathyroidectomy (two total and two subtotal); two patients had undergone renal transplantation 11 and 15 months previously; and 71 patients had been receiving phosphate-binding agents [calcium carbonate (n = 47), aluminium hydroxide (n = 19) and both binders (n = 5)]. Patients receiving vitamin-D therapy within 2 months prior to joining the study were excluded.

#### Control group

Serum PTH, RANKL, OPG and bone markers were measured in 50 healthy individuals of similar age and sex distribution as the patients (Table 1). The medical records of the controls were reviewed to ensure that they had no evidence of bone and renal disease and that they were not taking any medications that could alter normal bone turnover. The study protocol was approved by our local ethics committee and informed consent was obtained from all participants in the study.

#### Blood sampling

Venous blood was drawn into Vacutainer tubes (Becton Dickinson, Rutherford, USA) in the morning, prior to dialysis after an overnight fast. The samples were allowed to clot for 30 min at room temperature, after which they were centrifuged and sera were aliquoted and stored at −70°C for ≤3 months.

#### Measurement of biochemical markers

Biochemical parameters were measured: RANKL, OPG, tartrate-resistance acid phosphatase 5b (TRAP-5b), β-CrossLaps (CTx), bone-specific alkaline phosphatase (bALP), osteocalcin and intact PTH.

**Receptor activator of nuclear factor κB ligand.** RANKL was measured by immunoassay (Biomedica Medizinprodukte, Vienna, Austria). The method is designed to determine soluble free human RANKL directly in serum. Intra- and interassay coefficients of variation (CV) of the assay were <10% and its detection limit was 0.08 pmol/l.

**Osteoprotegerin.** OPG was determined using an enzyme-linked immunosorbent assay (Biovendor, Czech Republic) that detects both monomer (5%) and dimer forms (95%). The detection limit of this assay was 2 pg/ml and its intra- and interassay CVs were <10%.

**Tartarate-resistant acid phosphatase, isoenzyme 5b.** TRAP-5b was measured by solid-phase immunoaffixed enzyme activity assay (bone TRAP assay; SBA, Oulu, Finland), whose detection limit was 0.2 U/l and intra- and interassay CVs were <6% and <8%, respectively.

### Table 1. The baseline characteristics and measured parameters of HD patients and controls

<table>
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<tr>
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<th>HD patients (n = 80)</th>
<th>Controls (n = 50)</th>
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<tr>
<td><strong>Baseline characterisitics</strong></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>59.7 ± 13.3</td>
<td>62.0 ± 17.1</td>
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<tr>
<td>Male/female (%)</td>
<td>38 (47.5)/42 (58.5)</td>
<td>26 (0.52)/24 (0.48)</td>
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<td>HD (months)</td>
<td>35 (6–240)</td>
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<tr>
<td><strong>Biochemical parameters</strong></td>
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<tr>
<td>Phosphate (mmol/l)</td>
<td>1.66 ± 0.39</td>
<td>1.22 ± 0.16</td>
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<tr>
<td>Calcium (mmol/l)</td>
<td>2.39 ± 0.24</td>
<td>2.34 ± 0.13</td>
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<tr>
<td>RANKL (pmol/l)</td>
<td>1.36 ± 0.39</td>
<td>0.83 ± 0.70</td>
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<tr>
<td>OPG (pg/ml)</td>
<td>804.0 (342.0–3144.0)</td>
<td>271.5 (109.0–552.0)</td>
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<tr>
<td>BALP (U/l)</td>
<td>0.51 (0.13–2.98)</td>
<td>0.39 (0.18–0.57)</td>
</tr>
<tr>
<td>Osteocalcin (μg/l)</td>
<td>18.6 (1.0–195.8)</td>
<td>7.6 (3.1–12.1)</td>
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<tr>
<td>TRAP-5b (U/l)</td>
<td>4.04 ± 1.35</td>
<td>2.98 ± 0.89</td>
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<tr>
<td>CTx (ng/ml)</td>
<td>1.79 (0.43–6.85)</td>
<td>0.50 (0.39–0.66)</td>
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</table>

Data are expressed as means ± SD, median (range) or number (%), as appropriate.

Except calcium, all measured biochemical parameters differed significantly between patients and controls (P ≤ 0.001).
Bone-specific alkaline phosphatase. BALP was determined by an immunoassay (Alkphase-B; Metra Biosystems, Mountain View, CA, USA), whose sensitivity was 0.7 U/l and intra- and interassay CVs were <6% and <8%, respectively.

β-CrossLaps. CTx quantifies cross-linked isomerized collagen degradation fragments that contain the octa-peptide EKAHD-β-GGR. To quantify it, we used the electrochemiluminescence immunoassay ECLIA on a Roche Elecsys 2010 analyser (Roche Diagnostics GmbH, Manheim, Germany). Interassay CV was <4.6%.

**PTH and osteocalcin.** These were measured on an Immulite DPC analyser (Los Angeles, USA) according to the manufacturer’s instructions. The assays recognize intact PTH, intact osteocalcin and large fragments of both molecules. The CVs of intra- and interassay were <8% for both tests.

**Statistical analysis**

All data are presented as means ± SD unless indicated otherwise. Since none of the serum markers, with the exception of TRAP-5b, were distributed normally, the differences between the groups were analysed using the Kruskal–Wallis test and relationships between markers were assessed by Spearman’s correlation method. A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using the statistical software package SPSS 12.0 for Windows® (SPSS Inc., Chicago, IL, USA).

**Results**

The baseline characteristics and the measured biochemical parameters of patients and controls are given in Table 1. There were no significant differences in gender and age between patients and controls. Mean serum RANKL levels were 1.6 times higher in the patients than in healthy controls (1.36 ± 0.39 vs 0.83 ± 0.70 pmol/l; P < 0.001) (Figure 1). The Kruskal–Wallis test showed that all measured bone markers differed significantly between patients and controls (P < 0.001). Spearman’s tests of correlation showed statistically significant but weak correlations of RANKL with PTH, osteocalcin and CTx (r = 0.322, P = 0.004; r = 0.231, P = 0.039; and r = 0.230, P = 0.040, respectively).

To evaluate the influence of PTH on RANKL levels, we stratified HD patients into tertiles according to plasma PTH levels, which ranged as follows: 29.3–103.0, 109.7–263.0 and 262.0–1700.0 pg/ml, in the lower, middle and upper tertiles, respectively. Mean serum RANKL levels were significantly different between tertiles, as assessed by the Kruskal–Wallis test (P = 0.003) (Table 2, Figure 2). The highest RANKL levels were obtained in the upper PTH tertile (1.54 ± 0.39 pmol/l) and they were significantly higher than in the middle or lower tertiles (1.27 ± 0.42 and 1.23 ± 0.26 pmol/l, respectively; P = 0.003). While serum RANKL levels differed among tertiles, serum OPG levels did not (P = 0.144). Both of the measured bone-resorption markers, TRAP-5b and CTx, as well as both bone formation markers, osteocalcin and bALP, were also significantly higher in the upper tertile (Table 2). Diuresis as a measure of residual renal function persisted in 27 patients (312.9 ± 260.9 ml) and was not different between PTH tertiles.

**Discussion**

This is the first report of serum RANKL levels in HD patients. In our study, mean serum RANKL levels were 1.6 times higher in HD patients than in matched healthy controls. More importantly, RANKL levels were significantly higher in the upper PTH tertile, indicating the enhanced synthesis of RANKL in a PTH-dependent fashion. RANKL correlates significantly with PTH, osteocalcin (a bone formation marker) and with CTx (a bone resorption marker). Similar studies have been done in Paget’s disease [13], multiple myeloma [14] and rheumatoid arthritis [15]. Those reports indicated that RANKL played a key role in bone disorders characterized by excessive resorption. In any case, the pathogenesis of the bone diseases mentioned above is different and our results support these findings. It seems that local bone-regulating factors, such as RANK/RANKL/OPG, might modulate the progress of ROD, probably because they are somehow influenced by PTH. To date, findings explaining the effect of PTH on RANKL synthesis are scarce. Yasuda and colleagues [4] demonstrated that PTH stimulated the expression of RANKL on the surface of osteoblasts; Rubin and Bilezikian [16] tried to explain the mechanism by which PTH increased the production of RANKL; and Huang et al. [5] demonstrated that PTH differentially regulated the
expression of RANKL and OPG mRNA in osteo-
blasts. Our results, which show a 1.6 times greater
increase of RANKL in HD patients, support these
findings. Moreover, the differences in RANKL levels
between the upper and lower PTH tertiles indicate
enhanced RANKL synthesis in a PTH-dependent
fashion. RANKL, which binds to RANK on pre-
and mature osteoclasts, stimulates the maturation and
activity of osteoclasts and increases bone resorption in
hyperparathyroidism. Both of the measured bone-
resorption markers, i.e. TRAP-5b and CTx, increased
with RANKL, but the correlation was significant only
with CTx. The simultaneous significant increases of
bone formation markers, osteocalcin and bALP are not
surprising, because bone formation is ‘coupled’ with
bone resorption in the biological process of bone
turnover.

In any case, it does not seem probable that in our
study circulating RANKL levels might be enhanced
due to decreased renal clearance. The retention of the
38 kDa protein of RANKL might have contributed,
theoretically, to the increased RANKL levels in HD
patients only when compared with the healthy controls.

Nevertheless, the differences in RANKL levels between
HD patients with almost the same diuresis (as a
measure of residual renal function) could not be a
consequence of RANKL retention. The comparison of
mean diuresis between HD patients stratified to tertile
groups (analysis of variance; data not shown) shows no
significant differences ($P = 0.611$). Furthermore, in
the upper tertile both RANKL level and diuresis were the
highest. We conclude that retention could not be an
important factor influencing RANKL levels in HD
patients.

In conclusion, serum RANKL levels are elevated in
HD patients when compared with healthy controls. The
differences in RANKL levels between HD patients
indicate enhanced RANKL synthesis in a PTH-
dependent fashion. Owing to this, our clinical findings
clearly support published $in vitro$ studies that demon-
strated the stimulating effect of PTH on RANKL
synthesis [4,5,16]. Thus, the hypothesis that PTH
increases bone resorption in HD patients through the
RANKL could be validated.

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Conflict of interest statement. None declared.

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