

*Advances in Brief***Serum Osteoprotegerin Levels Are Increased in Patients with Advanced Prostate Cancer¹**

Julie M. Brown,² Robert L. Vessella,
Paul J. Kostenuik, Colin R. Dunstan,
Paul H. Lange, and Eva Corey

Department of Urology, University of Washington, Seattle, Washington 98195 [J. M. B., R. L. V., P. H. L., E. C.], and Amgen, Inc., Thousand Oaks, California 91320 [P. J. K., C. R. D.]

Abstract

Purpose: Osteoprotegerin (OPG) is a soluble osteoclastogenesis inhibitor that regulates bone turnover. We reported recently that OPG protein expression is significantly increased in prostate cancer (CaP) cells present in bone metastases. The aim of this study was to determine serum OPG levels in patients at different stages of CaP and correlate the results with disease status.

Experimental Design: OPG levels were examined in patients with benign prostatic hyperplasia, clinically localized CaP, early recurrence of CaP, and advanced CaP and evidence of bone metastases. Serum OPG levels were measured by sandwich ELISA assays. The serum Crosslaps (sCTX) assay was used to quantify bone resorption in the advanced CaP group.

Results: Serum OPG levels were increased significantly in the advanced CaP group *versus* all other groups. There was no significant correlation between serum OPG levels and PSA levels either in the advanced CaP group or within any of three treatment subclasses of this group: no Tx, those not treated; Tx, those treated; and R, those treated with resorption blockers. Levels of OPG were negatively correlated with sCTX levels only in the advanced CaP Tx group. sCTX levels correlated with prostate-specific antigen levels in the advanced CaP Tx and R groups but not in the no-Tx group.

Conclusions: Our data show that serum OPG levels are increased with advanced CaP. We hypothesize that OPG levels are related to CaP progression and suggest that further studies of the biological effects of OPG on CaP metastases are warranted.

Introduction

Metastasis of CaP³ to bone stimulates an overall increase in both bone remodeling and bone volume (1). This is the most critical complication of advanced CaP, often resulting in severe pain, morbidity, and mortality (2, 3). Normal bone turnover is a tightly controlled, coupled process wherein the regulated destruction of mineralized extracellular matrix (resorption) is followed by a formative phase. The cells responsible for resorption are large multinucleated osteoclasts, which are formed by the fusion of mononuclear hematopoietic precursors (reviewed in Ref. 4). Osteoblasts, the cells responsible for new bone formation, regulate osteoclastogenesis and osteoclast activity by interacting with osteoclast precursors (5, 6), but until recently the molecular regulators of this interaction were unknown.

Recently, two osteoblast-expressed proteins, OPG and RANKL, were shown to regulate osteoclastogenesis directly and therefore bone resorption. Acquisition and activation of the osteoclast phenotype depends on the interaction between RANKL (7) and RANK, which is expressed on the cell surface of osteoclast precursors (7, 8). The absolute requirement of this interaction for osteoclastogenesis *in vivo* was demonstrated by the generation of transgenic *rankl* $-/-$ and *rank* $-/-$ mice that developed severely hyperdense bones (osteopetrosis) because of an absence of osteoclasts (9, 10). Furthermore, administration of soluble extracellular RANKL to mice resulted in hypercalcemia and reduced bone volume, concomitant with a doubling of osteoclast size (8). In normal bone, excessive bone resorption is controlled by OPG (11). This glycoprotein acts as a soluble decoy receptor for RANKL, thereby neutralizing its interaction with RANK and abrogating osteoclast formation, activation, and survival *in vitro* (7, 12) and *in vivo* (8). The crucial role of OPG in bone remodeling was further shown using transgenic *opg* $-/-$ mice, which demonstrated uncontrolled bone resorption and severe osteoporosis (13). These studies suggest that the balance between RANKL and OPG determines the extent of bone resorption in that a relative decrease in OPG results in excessive resorption, and a relative increase in OPG inhibits resorption.

Bone cancers such as osteoclastomas (giant cell tumors of bone) that destroy bone locally were shown to express relatively high levels of RANKL when compared with OPG (14), suggesting that these proteins may be involved in tumor-mediated bone destruction. Breast cancer metastasis to bone also results in increased osteolysis, but this is regulated by a more indirect mechanism, whereby tumor-produced parathyroid hormone-related protein stimulates osteoblast expression of RANKL and decreases osteoblast-expressed OPG levels (15). Although the

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² To whom requests for reprints should be addressed, at Oncology Research Centre, School of Clinical Medicine, Prince of Wales Hospital, Level 2, Clinical Sciences Building, Barker Street, Randwick, NSW 2031, Australia. Phone: 61-2-9382-2610; Fax: 61-2-9382-2629.

³ The abbreviations used are: CaP, prostate cancer; OPG, osteoprotegerin; RANKL, RANK ligand; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; sCTX, serum type I collagen telopeptide(s).

Table 1 Patient data by disease stage

Disease groups	No. of patients	Median age (range)	Median PSA ng/ml (range)	Median OPG	
				Assay 1 in pg/ml (range)	Assay 2 in pg/ml (range)
Normal	6	32 ^a (23–50)	Not recorded	25.97 (19.73–60.68)	22.39 (15.95–57.47)
BPH	25	65 (55–75)	5.84 (1.35–17.31)	31.08 (15.09–68.64)	34.48 (21.74–77.06)
Primary CaP	25	59 (53–73)	3.81 (<0.06–39.3)	23.19 (7.56–81.26)	22.43 (3.31–82.98)
Recurrent CaP	25	65 (51–82)	0.34 (0.13–1.49)	37.01 (15.73–67.97)	30.71 (12.11–74.50)
Advanced CaP	24	71 (52–88)	436.2 (112.2–4817.5)	54.52 (16.32–1858.95)	43.24 (14.81–961.20)

^a The age of one subject was not recorded.

bone metastases produced by CaP are commonly osteoblastic or mixed, there is a lytic component indicative of progression (16), suggesting a role for RANKL and OPG in regulating the bone response in CaP bone metastasis. In a recent study, we showed that expression of OPG and RANKL is significantly increased in CaP cells present in bone compared with primary CaP or nonosseous metastases (17). Our hypothesis in undertaking the current study was that serum OPG levels would be increased in patients with CaP bone metastases over patients at earlier stages of prostatic disease.

Materials and Methods

Samples. Blood samples were collected by venipuncture from patients at the University of Washington Medical Center and the Veteran's Administration Medical Center in Seattle after informed consent and were processed as described previously (18). Ninety-nine patients who gave consent were identified. Group 1 consisted of 25 patients diagnosed with BPH. The diagnosis of BPH was based on symptoms of bladder obstruction, digital rectal examination, and pathological examination of needle biopsy. Group 2 consisted of 25 patients with organ-confined prostate cancer (primary CaP). The diagnosis was determined based on pathological examination and stage. The Gleason score of these patients ranged from 5 to 7 with median 6; 7 patients were staged as T_{2a} and 18 as T_{2c}. Group 3 was 25 patients with early recurrent CaP as defined by biochemical relapse (PSA >0.2 ng/ml). Group 4 was 24 patients with advanced CaP, and bone imaging confirmed bone metastases in 22 of 24 of these patients, with the remaining two being found positive for bone metastases within 1 year of serum sampling. Patient demographics are presented in Table 1. The normal range of OPG was determined using a group of 6 normal controls.

Immunoassays. Serum OPG levels were measured by R&D Systems (Minneapolis, MN) using two sandwich ELISA assays of different formats. RANKL (assay 1) or an antihuman OPG monoclonal antibody (assay 2) was used to capture OPG from serum. Captured OPG was detected with an horseradish peroxidase-conjugated antihuman OPG antibody (monoclonal for assay 1, polyclonal for assay 2) and with luminol and hydrogen peroxide substrate. Five quality controls were run in duplicate or triplicate on 2 days. The intra-run, between-run, and total coefficients of variation were shown to be 4.84, 2.09, and 3.19% or less, respectively, using assay 1, and 2.97, 3.99, and 4.40% or less, respectively, using assay 2.

The serum levels of type I collagen COOH-terminal cross-

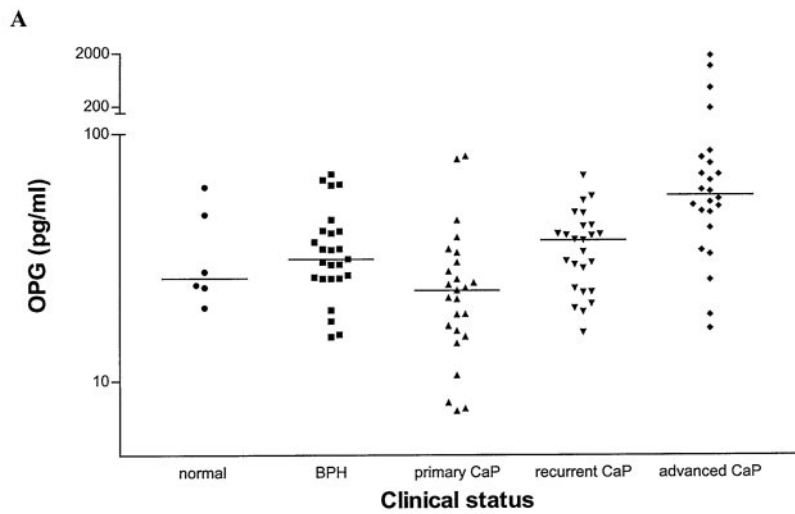
linked telopeptides (sCTX) were measured using the serum Crosslaps One Step ELISA (Osteometer Biotech, Herlev, Denmark) according to the manufacturer's instructions. Intra-assay variation was 4.285% or less, inter-assay variation was 5.02%, and the total coefficient of variation was 4.75%. The levels of total PSA were evaluated using the IMx total PSA assay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's protocol.

Statistical Methods. The logarithms of OPG levels in serum were used for statistical analysis. The data were analyzed using a single factor (disease status) ANOVA. All pairwise comparisons were made using the least significant difference without adjustment for multiple comparisons. The SAS System Version 8 General Linear Model procedure was used for the analyses. Comparisons between age and serum PSA levels were analyzed using Mann-Whitney *U* tests. Associations between OPG, PSA, and sCTX levels in the advanced CaP patient group were compared by Spearman rank correlation using GraphPad Prism software.

Results

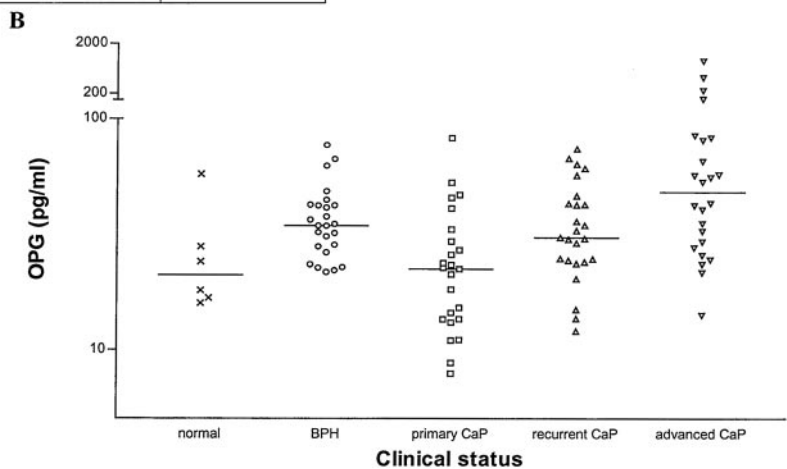
We found that serum OPG levels varied significantly among the patient groups (Fig. 1 and Table 1). Specifically, the levels of serum OPG in patients with advanced CaP were significantly increased using assay 1 (median value, 54.52 pg/ml; range, 16.32–1858.95 pg/ml) or assay 2 (median value, 43.24 pg/ml; range, 14.81–961.20 pg/ml) compared with any of the other patient groups. Excluding the advanced group from the analysis, we compared the levels of serum OPG between normal controls, patients with BPH, primary CaP, and early recurrent CaP and found that OPG levels in patients with primary CaP were significantly lower than those in patients with BPH (assay 1, *P* = 0.0062; assay 2, *P* = 0.0004) or early recurrent CaP (assay 1, *P* = 0.0037; assay 2, *P* = 0.0022). We found no significant differences between the serum OPG levels of patients with Gleason grades 5–7.

Analysis of the potential differences among advanced CaP patients was performed by stratifying this group into three subgroups: patients that had had no treatment for CaP (no Tx); those that had had intervention (Tx: prostatectomy, orchiectomy, hormonal therapy, radiotherapy, chemotherapy in any combination); and those that had one or more of these interventions plus bone resorption blockers [R: matrix metalloproteinase inhibitors (Ref. 2; Agouron trial) or bisphosphonates zoledronate (2), alendronate (2), and/or pamidronate (5)]. PSA levels were not significantly different among these three groups. These



	Normal	BPH	Primary CaP	Recurrent CaP
Advanced CaP	0.0071	<0.0001	<0.0001	0.0001
Recurrent CaP	0.8037	0.9005	0.0399	
Primary CaP	0.2980	0.0531		
BPH	0.8644			

Fig. 1 Scatterplot of serum OPG levels against clinical status for all patients tested, as measured using assay 1 (A) and assay 2 (B). Horizontal bars, median values. Tables show *P*s following ANOVA on log-transformed data. Shaded boxes, significant values.

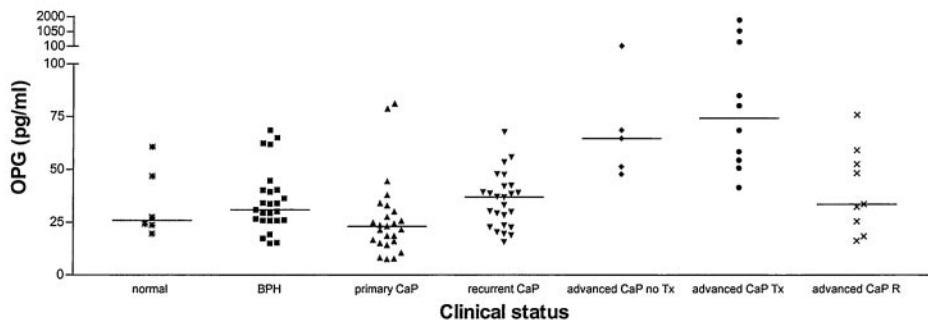


	Normal	BPH	Primary CaP	Recurrent CaP
Advanced CaP	0.0053	0.0135	<0.0001	0.0038
Recurrent CaP	0.3156	0.6570	0.0133	
Primary CaP	0.5773	0.0038		
BPH	0.2015			

subgroups were then compared with BPH, primary CaP, and recurrent CaP groups to determine differences in the levels of serum OPG (Fig. 2). Using assay 1 (Fig. 2A), we found that OPG levels were significantly lower in primary CaP than in BPH ($P = 0.0340$), recurrent CaP ($P = 0.0243$), or in any of the

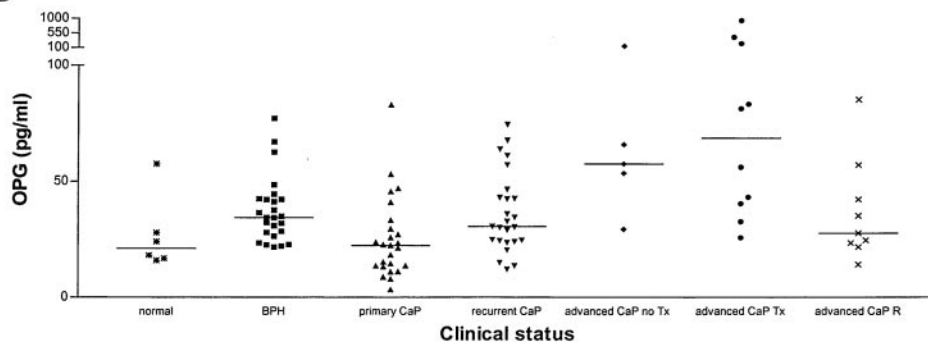
advanced CaP subgroups (no Tx, $P = 0.0002$; Tx, $P < 0.0001$; R, $P = 0.0499$). These comparisons held true after analysis of serum OPG levels measured using assay 2 (Fig. 2B), with the exception of the difference between the primary CaP and the advanced CaP R subgroup, which failed significance using

A



	Normal	BPH	Primary CaP	Recurrent CaP	Advanced CaP no Tx	Advanced CaP Tx
Advanced CaP R	0.6668	0.6748	0.0499	0.7489	0.0461	0.0001
Advanced CaP Tx	<0.0001	<0.0001	<0.0001	<0.0001	0.0581	
Advanced CaP no Tx	0.0276	0.0098	0.0002	0.0122		
Recurrent CaP	0.8216	0.8912	0.0243			
Primary CaP	0.2343	0.0340				
BPH	0.8883					

B



	Normal	BPH	Primary CaP	Recurrent CaP	Advanced CaP no Tx	Advanced CaP Tx
Advanced CaP R	0.3771	0.6983	0.0610	0.9636	0.0576	0.0003
Advanced CaP Tx	<0.0001	<0.0001	<0.0001	<0.0001	0.2329	
Advanced CaP no Tx	0.0126	0.0632	0.0004	0.0339		
Recurrent CaP	0.2883	0.6384	0.0089			
Primary CaP	0.5552	0.0022				
BPH	0.1766					

Fig. 2 Scatterplot of serum OPG levels in patients with BPH, primary CaP, recurrent CaP, advanced CaP without treatment (no Tx), advanced CaP with treatments (Tx), and advanced CaP with treatments including bone resorption blockers (R). For each subgroup, serum OPG levels were measured using assay 1 (A) and assay 2 (B). Measurements were log-transformed prior to analysis by one-way ANOVA. Shaded boxes, significant values.

assay 2, but with a result similar to that seen with assay 1 ($P = 0.0610$). Using assay 1, advanced CaP no Tx and Tx patients also had significantly higher levels of serum OPG than any of the other groups, including the advanced CaP R subgroup,

although no difference was found when the no Tx and Tx subgroups were compared with each other. Using assay 2 (Fig. 2B), advanced CaP Tx patients displayed significantly higher serum levels of OPG than any of the other groups, except *versus*

Table 2 Correlation of serum OPG and CTX levels in patients with advanced CaP

The correlation of serum OPG levels, as measured by RANKL capture (assay 1) or OPG antibody capture (assay 2), with serum CTX levels in the same serum sample was assessed using the Spearman rank correlation test for statistical significance. Significant results are shown in boldface.

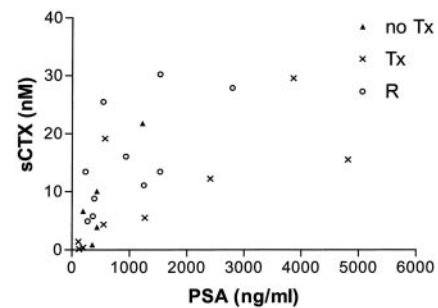
	<i>n</i>	Assay 1 (<i>r</i>)	Assay 1 (<i>P</i>)	Assay 2 (<i>r</i>)	Assay 2 (<i>P</i>)
Advanced CaP	24	-0.0209	0.9229	-0.1657	0.4391
Advanced CaP no Tx	5	0.9000	0.0833	0.9000	0.0833
Advanced CaP Tx	10	-0.6809	0.0347	-0.6687	0.0390
Advanced CaP R	9	-0.3333	0.3853	-0.4833	0.1938

advanced CaP no Tx. Advanced CaP no Tx patients had significantly higher serum OPG levels than did patients with primary CaP ($P = 0.0004$) or recurrent CaP ($P = 0.0339$); differences between advanced CaP no Tx patients and BPH, advanced CaP Tx, or advanced CaP R subgroups were not significant.

The relationship between clinical stage and serum PSA levels was shown to be statistically significant when all groups were compared ($P < 0.0001$), although serum PSA levels in patients with BPH were not significantly different from those in patients with primary CaP. Significant differences were observed between patients with BPH *versus* those with recurrent CaP; BPH *versus* advanced CaP; primary CaP *versus* recurrent CaP; primary CaP *versus* advanced CaP; and recurrent CaP *versus* advanced CaP (all comparisons, $P < 0.001$). No significant correlation was found between Gleason grades (5–7) and PSA levels. Age was a significant variable ($P = 0.0040$); patients with advanced CaP were significantly older than those with primary CaP ($P < 0.01$), but there was no difference between patients with advanced CaP and those with BPH or those with recurrent CaP. Serum OPG and PSA levels were not correlated in patients with advanced CaP when this group was analyzed as a whole or when the group was stratified into three treatment subgroups.

Because advanced CaP patients with bone metastases had increased levels of OPG, we examined these samples for indications of bone resorption by measuring sCTX levels. We found that there was increased sCTX in advanced CaP patients (median value, 10.06 nM; range, 0.14–30.24 nM) compared with published normal ranges (2.81–5.51 nM). However, we found no significant correlation between serum OPG and sCTX levels in the advanced CaP patient group when all advanced CaP patients were analyzed together (Table 2). However, when the advanced group was separated into the three subgroups, we found a significant negative correlation with both assays between OPG and sCTX in the advanced CaP Tx group. A negative correlation that did not reach significance was observed in the advanced CaP R group. There was no correlation between OPG and sCTX levels in the advanced CaP no Tx group.

We found a significant correlation between sCTX and PSA levels in advanced CaP patients (Fig. 3). When we separated the advanced CaP patients into no Tx, Tx, and R subgroups, we found a significant correlation between sCTX and PSA levels in the advanced CaP Tx patients and in the advanced CaP R subgroup but not in those that had no treatment.



	r value	p value
Advanced CaP	0.7141	<0.0001
Advanced CaP no Tx	0.5000	0.4500
Advanced CaP Tx	0.6930	0.0306
Advanced CaP R	0.8167	0.0108

Fig. 3 Scatterplot of sCTX levels against serum PSA levels in subgroups of patients with advanced CaP. Parameters were compared using the Spearman rank correlation test. There was a significant correlation between sCTX and PSA in the advanced CaP group overall and in the advanced CaP Tx and R subgroups after stratification. Shaded boxes, significant values.

Discussion

CaP metastasis to bone results in the establishment and development of lesions that are typically osteoblastic; excess woven bone is produced that compromises the integrity of the bone, leading to pathological fractures and compressions. This formative component of the metastatic process is accompanied by an increase in bone resorption through which the structure of established bone is weakened. In normal adult bone, the master regulators RANK, RANKL, and OPG control the resorptive process (19, 20). Our previous work has demonstrated that CaP cells that have metastasized to bone express significantly greater levels of OPG than primary CaP cells (17), and because OPG is a secreted protein, we hypothesized that patients with CaP bone metastases would exhibit higher serum OPG levels. We used two assays to test for serum OPG levels; assay 1, using RANKL as capture, was more sensitive than assay 2 using an anti-OPG antibody. The different sensitivities of these assays may be attributable to different affinities of RANKL *versus* the anti-OPG antibody for OPG, or possibly to binding to different forms of OPG. Our data showed that advanced CaP patients have higher levels of serum OPG than patients at other stages of prostatic disease. *In vitro* studies have shown that prostate cancer cells can stimulate human (21, 22) and rat (23) osteoblasts. Therefore, in addition to the increased expression of OPG by CaP cells in bone metastases, the observed increases in serum OPG levels may be partly attributable to increased osteoblastic activity in response to CaP cells present in the bone milieu.

Interestingly, we found that serum OPG levels were significantly lower in patients with primary CaP than in those with BPH. These results are in agreement with our previous study in

which we showed by immunohistochemistry that although normal prostatic epithelial cells express OPG protein, this expression is lost upon transformation (17). Also in agreement with our previous study, the finding that OPG levels were significantly increased in patients with advanced disease over any of the other patient groups suggests that expression of OPG is associated with progression of CaP to bone.

We analyzed the advanced group further by stratification according to treatment provided: no Tx, Tx, and R subgroups. The differences between the subgroups suggest that treatments may affect the levels of OPG in advanced CaP patients; for example, hormonal therapy was reported previously to affect the levels of several bone biochemical markers (24). Alternatively, OPG expression may cause or reflect some pathological aspect of advanced CaP that triggers the physician to initiate treatments. A recent study showed that OPG promotes the survival of endothelial cells (25), which may in turn support the growth of prostate cancer metastases. A prospective study in which serum OPG levels of patients with different stages of CaP are tested before and after treatment would be required to provide the data necessary to determine definitively whether serum OPG is influenced by the treatment modalities.

Because OPG is involved in regulation of bone resorption, we examined the levels of bone resorption in advanced CaP patients by measuring a degradation product of type I collagen in serum (sCTX; Refs. 26–28) that is a sensitive marker of increased bone resorption (29–31). One would expect that increased levels of OPG would inhibit bone resorption and result in lower levels of sCTX. We have shown that the levels of sCTX were higher in the advanced group than published levels of sCTX in controls. Increased bone resorption despite the presence of increased antiresorptive OPG is consistent with the idea that CaP in bone dysregulates normal bone resorption. Prevailing evidence indicates that the OPG:RANKL ratio is critical in determining the level of osteolysis. We have reported recently that the levels of RANKL, the major regulator of osteoclast development and function and therefore of bone resorption, were increased in CaP cells present in bone metastases (17). Also Zhang *et al.* (32) published recently that prostate cancer cells *in vitro* produce RANKL and stimulate the formation of osteoclasts. Therefore, a possible explanation of the apparent disparity between higher levels of the resorption inhibitor OPG and higher bone resorption in advanced CaP is that the increased expression of OPG and RANKL reflect increased bone turnover. An alternative hypothesis is that other mechanisms not yet identified partially regulate bone resorption in CaP bone metastases.

There was no significant correlation between OPG and sCTX in the advanced group; however, we found that there was a significant negative correlation between sCTX and OPG in the advanced CaP Tx subgroup, whereas the R group exhibited a negative correlation that did not reach significance, and the advanced CaP no Tx subgroup exhibited a positive correlative trend between these parameters. The negative correlation observed in the Tx and R groups suggest either that the treated patients represent a different phenotype of advanced CaP disease that is more effectively regulated in terms of bone resorption, or that the treatments themselves are playing a role in the

restoration of the balance between the levels of OPG and the amount of bone lysis.

Our results show that PSA levels in the advanced CaP group were significantly correlated with sCTX levels, as has been reported previously by Revilla *et al.* (33), suggesting that progression of CaP and increased bone resorption are closely associated. Among the three advanced subgroups there were significant positive correlations between PSA and sCTX in the Tx and R groups, whereas there was no significant correlation between PSA and sCTX in the advanced CaP no Tx subgroup. These observations suggest that the treatments provided to the patients may have influenced the relationship between PSA and sCTX, but larger studies are needed to confirm these observations.

Although OPG is higher in CaP patients with established bone metastasis, it may not become a clinically useful marker of early bone metastasis in the near future. PSA is a superior early marker of recurrent disease, although not necessarily indicative of bone metastases. Upon further study, OPG levels may be found to be clinically informative in patients with CaP bone metastases.

In conclusion, the results of this study suggest that the antiresorptive protein OPG is associated with progression of CaP. The implications of the involvement of this protein in skeletal homeostasis warrant further investigation of the biological effects of OPG on CaP cells and CaP metastases in bone.

Acknowledgments

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