

# Osteoprotegerin (OPG) Is a Survival Factor for Human Prostate Cancer Cells<sup>1</sup>

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## Abstract

Factors that aid survival of prostate cancer cells in the presence of the various categories of cytotoxic cytokines present in tumors *in vivo* are largely unknown. Osteoprotegerin (OPG) is a decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that inhibits TRAIL-induced apoptosis. In relation to this activity, we hypothesized that the ability to produce OPG by prostate cancer cells would confer a survival advantage on these cells. In this study we have demonstrated that high levels of OPG are produced by the hormone-insensitive prostate cancer cell lines PC3 and Du145, whereas the hormone-sensitive cell line LNCaP produced 10–20-fold less OPG under the same conditions. A strong negative correlation was observed between levels of endogenously produced OPG in the medium and the capacity of TRAIL to induce apoptosis in cells that produced high levels of OPG. The antiapoptotic effect of OPG was reversed by coadministration of 100-fold molar excess of receptor-activator of nuclear factor- $\kappa$ B ligand, another protein that selectively binds OPG. These observations suggest that prostate cancer-derived OPG may be an important survival factor in hormone-resistant prostate cancer cells.

## Introduction

Prostate cancer metastasizes preferentially to bone in ~70% of patients with advanced disease. These lesions exhibit mixed bone resorption and formation activities with osteoblastic reactions predominating. This implies a shift in the balance of bone turnover in these tumors favoring the laying down of new bone involving the activities of both osteoclasts and osteoblasts.

Osteoclast differentiation has been shown recently to be positively and negatively regulated by a complex signaling system involving RANK,<sup>3</sup> OPG, and RANKL. The key interactions involving these factors and osteoclastogenesis are: (a) induction of osteoclastogenesis and activation of bone resorption by binding of RANK on the surfaces of osteoclast progenitors to RANKL on osteoblasts during direct cell contact; and (b) suppression of osteoclastogenesis by interference with RANK/RANKL interactions by binding of the soluble protein OPG to RANKL (1).

Whereas much of the current interest in OPG in prostate cancer (2, 3) is focused on its ability to inhibit osteoclastogenesis, this protein was originally identified as a member of the TNF receptor family and has been shown to be a soluble receptor for TRAIL (4). TRAIL is present in tumors *in vivo* being produced by monocytes in response to IFN- $\gamma$  or - $\alpha$  and is the principle mediator of acquired tumor killing

activity in these cells, which are themselves resistant to TRAIL (5). TRAIL mediates its effects through two classes of membrane-bound receptors carrying so called death domains (DR4 and DR5), but its activity is also modulated by nonproductive associations with decoy receptors that do not carry death domains (6). Whereas two of the latter class of TNF receptors are membrane bound and confer TRAIL resistance on expressing cells, the third, OPG, is soluble and competes for TRAIL binding to death-activating receptor species in sensitive cells, providing a mechanism for protection against apoptosis in the presence of TRAIL. The prostate cancer cell lines PC3 and Du145 used in the present study are sensitive to TRAIL and express receptors carrying death domains (7). Therefore, expression of OPG could be an appropriate strategy for these cells to avoid TRAIL-induced apoptosis.

In the present study we investigated OPG production by the human prostate cancer cell lines PC3, Du145, and LNCaP, and tested the hypothesis that OPG facilitates survival of prostate cancer cells *in vitro*.

## Materials and Methods

**Cell Lines and Tissue Culture.** The human prostatic cancer cell lines PC3, Du145, and LNCaP were obtained from American Type Culture Collection, Manassas, VA. LNCaP has been widely demonstrated to be androgen sensitive, and PC3 and Du145 unresponsive to androgens. The cell lines were routinely maintained in DMEM supplemented with antibiotics and fetal bovine serum (10%). For generation of conditioned medium, PC3 and Du145 cells were seeded into 24-well plates (~2 cm<sup>2</sup> culture surface) and grown for 4 days. Conditioned medium (0.3 ml/well) was collected over these cultures from 1 to 4 days after this time and accumulated OPG levels measured. LNCaP were seeded at the same density as the other cell lines but in 25 cm<sup>2</sup> flasks. All of the medium/reagents and plastics were from Life Technologies, Inc., Paisley, Scotland, United Kingdom, or Costar.

**Challenge of Cells with TRAIL.** Cultures of PC3 cells were generated in 24-well plates and grown for a total of 8 days. On day 8, cells were challenged with TRAIL (R&D Systems, Abingdon, United Kingdom) either in fresh medium or in medium in which they had been growing for up to 4 days. The effects of TRAIL on apoptosis were examined 24 h after challenge. Cells were also treated with TRAIL in fresh medium supplemented with recombinant OPG-Fc (Gift of Dr Colin Dunstan, Amgen plc, Thousand Oaks, Ca; 1–1000 ng/ml). In an additional series of experiments, cells were challenged with TRAIL (50 ng/ml) in either fresh medium containing 10 ng/ml recombinant OPG or in medium in which the cells had been growing for 4 days in the presence and absence of 100-fold molar excess of soluble RANKL (sRANKL) (R&D Systems). At the end of each experiment the samples were spun down, supernatant transferred to a separate tube, and frozen at –80°C. The cell pellet was resuspended in 10  $\mu$ l PBS and fixed in 4% formaldehyde.

In other experiments, OPG present in conditioned medium was removed by immunoadsorption using antibodies raised to OPG and adsorption of OPG-antibody complexes onto protein A-Sepharose. Briefly, medium was conditioned over near confluent cultures of PC3 cells (9 ml/T-75 flask) for 4 days and collected medium centrifuged and filtered to remove cells. The conditioned medium was then divided into aliquots for immediate freezing (untreated) or for removal of OPG (OPG-depleted). Two different mouse monoclonal antibodies (R&D and Alexis Corp., San Diego, CA) were incubated (1  $\mu$ l stock antibody/ml conditioned medium) with 4-day conditioned medium for 2 h at 4°C. Prehydrated protein A-Sepharose was suspended in 50 mM Tris-HCl

Received 7/30/01; accepted 1/31/02.

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<sup>3</sup> The abbreviations used are: RANK, receptor activator of nuclear factor nuclear factor  $\kappa$ B; OPG, osteoprotegerin; TRAIL, TNF-related apoptosis-inducing ligand; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; TNF, tumor necrosis factor; DAPI, 4',6-diamidino-2-phenylindole.

buffer (pH 7; 200 mg/ml), added to the conditioned medium-antibody solution (40  $\mu$ l/ml of solution), and stirred overnight at 4°C. The suspension was then centrifuged at 12,000  $\times$  g and supernatant collected and sterile filtered. Untreated and OPG-depleted medium was evaluated for the presence of OPG and tested for effects on TRAIL-induced apoptosis in experiments similar to those described.

**Counting of Apoptotic Cells.** Fixed cells were cytocentrifuged onto glass and stained using DAPI (Sigma Chemical Co.) at 1  $\mu$ g/ml. The samples were mounted using Citiflour (Agar Scientific), and the preparations were examined using a  $\times$ 40 objective on a Leica DMIBR microscope. Images were recorded using a cooled digital Spot camera and processed using Spot-32 software (Diagnostic Instruments Inc., Sterling Heights, Michigan). Only cells displaying classical apoptotic morphology with fragmented nuclei were counted as apoptotic.

**PCR.** Total RNA was extracted using TRIzol (Life Technologies, Inc.), and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). PCR was performed using AmpliTaqGold (Applied Biosystems) and the following primers: OPG forward 5' GAA ACC TCT CAT CAG TG 3' and OPG reverse 5' GCT GCA CAT TGA CAC GTA 3'. PCR conditions were 94°C for 12 min followed by 35 cycles of 94°C for 30 s/50°C for 1 min/72°C for 1 min, and 72°C for 12 min using a Perkin-Elmer Gene Amp 9700. PCR products were purified and sequenced to confirm their identity.

**ELISA.** The concentration of OPG in the culture medium was determined using an ELISA method. Briefly, 96-well plates were coated with 2  $\mu$ g/ml mouse monoclonal antihuman OPG (R&D Systems). The OPG standard curve was generated using recombinant human OPG (R&D Systems) at concentrations from 2000 to 31.25 pg/ml. The secondary antibody was biotinylated antihuman OPG (R&D Systems) at 200 ng/ml, and detection was done using streptavidin-horseradish peroxidase (R&D Systems) in combination with a 3,3', 5,5'-tetramethylbenzidine substrate (Sigma Chemical Co.). The reaction was stopped after 5–20 min incubation in the dark by addition of 50  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub>. The plate was read at 450 nm on a Dynatech plate reader using Revelation software.

**Statistics.** All of the experiments were repeated at least twice. Unless otherwise stated results obtained in one representative experiment are shown. Data are shown as mean  $\pm$  SE for quadruplicate samples; comparison between groups was done using Student's *t* test.

## Results

**Prostate Cancer Cell Lines Produce Different Amounts of OPG.** All three of the cell lines expressed OPG as measured by reverse transcription-PCR (Fig. 1A). These analyses, while being

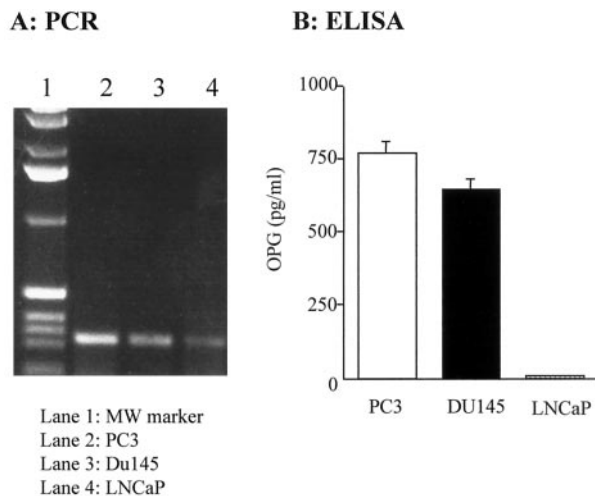


Fig. 1. Expression of OPG in prostate cancer cell lines determined by PCR (A) and ELISA (B). PCR was performed as described in "Materials and Methods" using primers corresponding to a 310-bp fragment of OPG. For the ELISA each cell line was grown for 24 h and conditioned medium collected. Medium (100  $\mu$ l) was assayed in quadruplicate, and the data are expressed as mean; bars,  $\pm$  SE.

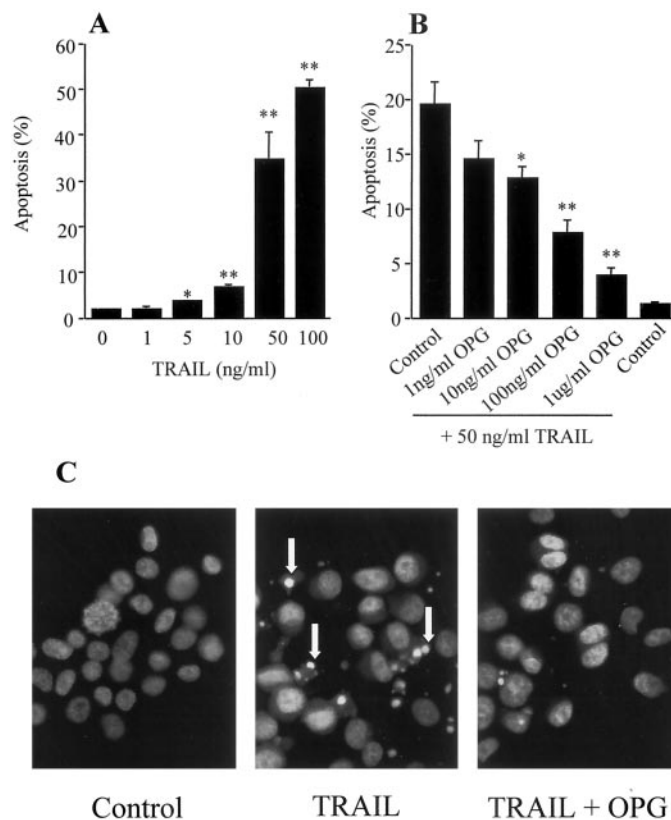


Fig. 2. TRAIL induces apoptosis in PC3 cells, and this can be reversed by addition of recombinant OPG. A, PC3 cells were grown for 4 days, transferred to fresh medium, and challenged with increasing concentrations of TRAIL as indicated. Apoptosis was assessed 24 h later by visualization of nuclear morphology. B, PC3 cells were grown as stated above and treated with the given concentrations of OPG. Apoptosis was assessed 24 h later by visualization of nuclear morphology. Apoptosis is expressed as percentage of total cell number counted. C, microscopic images ( $\times$ 40) of PC3 cells from each of the following treatment groups: left panel: Control; middle panel, 50 ng/ml TRAIL; right panel, 50 ng/ml TRAIL and 1  $\mu$ g/ml OPG. Data are expressed as mean of quadruplicate determinations; bars,  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  compared with absence of TRAIL (A) or absence of OPG with (left control) or without TRAIL (right control; B).

essentially qualitative, suggested lower levels of OPG reverse transcription-PCR product in reactions using LNCaP RNA than in those using material derived from the other cell lines. ELISA analyses demonstrated that the cell lines secreted OPG at very different levels. PC3 and DU145 produced between 750 and 600 pg/ml/24 h of OPG (Fig. 1B), whereas LNCaP produced OPG at levels that were near the lower limit of the detection of the system (<30 pg/ml) but were nevertheless measurable. Over 4 days, OPG levels rose in the PC3 cultures up to 2.5 ng/ml compared to 750–600 pg/ml produced in the first 24 h (Fig. 3A).

**TRAIL Induces Apoptosis in PC3 Cells.** Challenge of PC3 cells with TRAIL across a concentration range of 1–100 ng/ml demonstrated that induction of apoptosis could be obtained at doses as low as 10 ng/ml but that 50 ng/ml reliably induced up to  $\sim$ 30% apoptosis in these cells (Fig. 2A) at 24 h after treatment. This concentration was used in subsequent experiments. Addition of recombinant OPG at concentrations between 1 ng/ml and 1  $\mu$ g/ml to PC3 cells treated with 50 ng/ml of TRAIL in fresh medium reduced the levels of TRAIL-induced apoptosis in a dose-dependent manner (Fig. 2B). Clear inhibition of apoptosis was observed at doses  $>$ 10 ng/ml of OPG. At 1  $\mu$ g/ml of OPG, levels of apoptosis approached those observed in the absence of TRAIL. Analysis of apoptosis was based on DAPI staining of cells. Untreated PC3 cells were found to have normal nuclear morphology with smooth edges (Fig. 2, C, left panel), whereas treatment for 24 h with 50 ng/ml TRAIL caused a substantial proportion

of the cells to undergo apoptosis characterized by condensation of chromatin and the presence of apoptotic bodies (Fig. 2, C, middle panel). When PC3 cells were treated for 24 h with 50 ng/ml TRAIL and also 1  $\mu$ g/ml OPG, the cells appeared to maintain a normal nuclear morphology; however a few apoptotic cells could still be identified (Fig. 2, C, right panel).

**Accumulated OPG Reduces the Levels of TRAIL-induced Apoptosis in PC3.** Analysis of endogenous OPG levels in medium conditioned over PC3 cells for up to 4 days indicated that OPG production increased with time reaching 2.5 ng/ml after 4 days (Fig. 3A). Cultures from which these mediums were collected were simultaneously challenged with 50 ng/ml TRAIL (Fig. 3B). Analysis of apoptosis in these cells demonstrated an inverse correlation between the level of OPG in the medium and the level of apoptosis present (Fig. 3, A and B) with around half the level of apoptosis being observed in cells challenged with TRAIL in 4-day conditioned medium containing 2.5 ng/ml OPG than that observed in cultures challenged with TRAIL in fresh medium in which OPG levels were much lower (500–600 pg/ml).

**The Antiapoptotic Effect of Recombinant OPG and Conditioned Medium Can Be Inhibited by sRANKL and Removed by Antibodies for OPG.** When PC3 cells were treated with 50 ng/ml TRAIL in the presence of 10 ng/ml recombinant OPG or 4-day conditioned medium, the addition of sRANKL completely reversed the protective effect of exogenous or endogenous OPG on TRAIL-induced apoptosis (Fig. 4, A and B). Soluble RANKL did not affect the levels of apoptosis in the absence of TRAIL in either fresh or conditioned medium. Levels of apoptosis in cultures treated with sRANKL in the presence of TRAIL were higher than those observed in cultures treated with TRAIL alone in fresh medium (Fig. 4, A and B).

Endogenous OPG was completely removed from conditioned medium by treatment with antibodies and immunoadsorption (confirmed by ELISA pre- and post-antibody treatment). TRAIL-induced apoptosis in antibody-treated medium (OPG-depleted) was higher (mean = 141%  $\pm$  12.8%;  $n$  = 4) compared with untreated conditioned medium (100%). Addition of OPG (5 ng/ml) to OPG-depleted medium restored the protective effect to slightly below (mean = 94%  $\pm$  9.6%;  $n$  = 4) that observed with untreated conditioned medium.

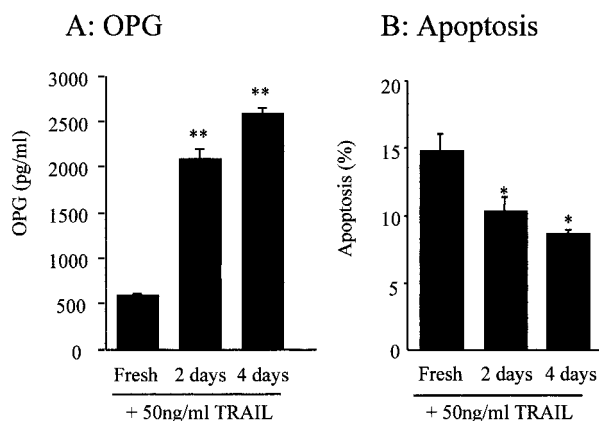


Fig. 3. OPG accumulation correlates with decreased apoptosis in PC3. A, PC3 cells were grown for 0, 2, and 4 days in the presence of 50 ng/ml TRAIL, and the level of OPG produced by the cells was determined by ELISA. B, the cells from A were fixed after 0, 2, and 4 days in culture, and apoptosis was determined by visualization of nuclear morphology after DAPI staining. Apoptosis is expressed as percentage of total cell number counted. Data are expressed as mean of quadruplicate determinations; bars,  $\pm$  SE. \*,  $P$  < 0.05; \*\*,  $P$  < 0.001 compared with appropriate control.

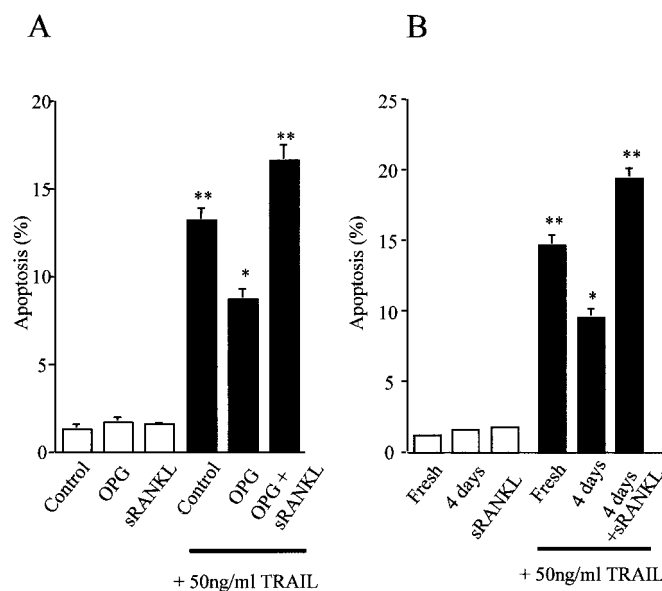


Fig. 4. The antiapoptotic effect of OPG can be reversed by sRANKL. A, PC3 cells were challenged with 50 ng/ml TRAIL in fresh medium  $\pm$  10 ng/ml OPG-fc  $\pm$  sRANKL (100  $\times$  molar excess compare OPG). Cells were fixed and apoptosis determined by visualization of nuclear morphology after DAPI staining. B, PC3 cells were grown for 3 days and subsequently challenged with 50 ng/ml TRAIL in the presence or absence of sRANKL ( $\sim$ 100  $\times$  molar excess compare OPG measured in conditioned medium after 4 days). Cells were fixed, and apoptosis was determined by visualization of nuclear morphology after DAPI staining. Data are expressed as mean of quadruplicate determinations; bars,  $\pm$  SE. \*,  $P$  < 0.05; \*\*,  $P$  < 0.001 compared with appropriate control.

## Discussion

In the present study we have demonstrated the expression of OPG by prostatic cancer cells *in vitro* and the release of biologically active quantities of this molecule into culture medium. Two androgen-insensitive cell lines, PC3 and DU145, accumulated OPG up to a concentration of 600–750 pg/ml in 24 h and up to 2.5 ng/ml over 4 days. In contrast, the androgen-sensitive cell line LNCaP produced very low levels of OPG, never exceeding 100 pg/ml during the same time period. Under conditions in which endogenous OPG levels were depleted, we found that recombinant OPG doses of  $\geq$ 10 ng/ml were able to significantly protect PC3 cells against TRAIL-induced apoptosis. However, inhibition of apoptosis was also observed at 1 ng/ml (Fig. 2B), suggesting that inhibition of TRAIL-induced apoptosis by OPG achieves significance between the concentrations of 1 and 10 ng/ml. This is supported by later experiments where TRAIL-induced apoptosis was inhibited in cultures challenged in conditioned medium containing approximately 2–2.5 ng/ml of endogenously produced OPG (Fig. 3). It is worth noting that during secretion of endogenous OPG, the concentrations in close proximity to producing cells would be expected to be slightly higher than the measured levels in the total medium volume. The relative concentrations of recombinant and endogenous OPG, shown to be present in conditioned medium, are, therefore, sufficiently close to being consistent with the proposition that OPG was the antiapoptotic factor present in the latter. This was tested in later experiments.

The antiapoptotic effect of OPG could be completely reversed by cotreatment with sRANKL, a molecule known to bind to OPG with high affinity. sRANKL alone had no effect on apoptosis levels in these cells. Cultures treated with TRAIL in the presence of sRANKL showed consistently higher levels of apoptosis than those treated with TRAIL alone. This effect may be because of suppression of residual levels of endogenous OPG shown to be present in these cultures. These data suggest that the observed protective effect of medium conditioned over cultures before challenge with TRAIL was mainly

mediated by accumulated OPG. Our findings also indicate that binding of OPG to sRANKL alters its ability to bind and inhibit the activities of TRAIL. The effect of removal of OPG using antibody immunoadsorption confirmed that endogenous OPG was active in suppressing TRAIL-induced apoptosis.

OPG, RANK, and RANKL are recently discovered key regulators of osteoclast biology (1). RANKL, a member of the TNF family expressed mainly on undifferentiated stromal cells and osteoblasts, binds to RANK on osteoclasts/osteoclast precursors and stimulates differentiation, activation, and survival of these cells. OPG, a member of the TNF receptor superfamily, is a soluble decoy receptor capable of binding to RANKL and preventing its association with RANK, thereby inhibiting osteoclast formation and activity. OPG is expressed by a variety of cell types, in bone mainly by osteoblasts. Prostate cancer cells have been shown to produce both OPG and RANKL (3, 8; present study), making them capable of influencing bone metabolism both through inhibition (OPG) and stimulation (RANKL) of osteoclastogenesis. Recent immunohistochemical studies have suggested that both OPG and RANKL are present in prostatic cancers *in vivo*, with elevated levels of OPG detected in bone metastases compared with primary tumors and metastases from nonosseous sites (8). The present study is the first to show that prostate cancer cells express and secrete high levels of OPG *in vitro*. This activity may have implications for tumor-associated bone remodeling in bony metastases of these tumors *in vivo*, and OPG can now be added to the growing list of factors affecting bone turnover shown to be produced by prostate cancer cells. However, the potential involvement of OPG in suppressing apoptotic responses to TRAIL may be of greater significance for the survival and progression of prostate cancer than the bone-related effects of OPG. Interestingly, TRAIL has been shown to be highly expressed in the prostate (9), but a function of TRAIL has yet to be established in this tissue.

Two of the cell lines used in the present study, PC3 and DU145, have been shown previously to be sensitive to TRAIL over similar concentration ranges to those used here, but in the published studies the cells were always challenged with TRAIL in fresh medium (*i.e.*, without accumulated OPG; Ref. 7). These studies suggested that TRAIL responses were mediated by the DR4 receptor and downstream caspases. We have confirmed that the cell lines we used also expressed DR4 receptors (data not shown). Interestingly, we found that LNCaP produced 10–20-fold less OPG than either PC3 or DU145 under identical control conditions. This observation suggests that if OPG production was the only mechanism for inhibition of TRAIL-induced apoptosis in cancer cells, LNCaP might be expected to be more sensitive to challenge with TRAIL than DU145/PC3. However, recent studies showed that LNCaP cells were resistant to TRAIL (10). The authors suggested this insensitivity to TRAIL involves altered intracellular signaling responses, specifically failure in the cleavage of the proapoptotic protein Bid. Cleavage of Bid is part of the death response cascade and triggers the release of mitochondrial cytochrome *c*, an important step in induction of apoptosis. In other tumor types TRAIL resistance has also been shown to be mediated through down-regulation of the DR4 receptor (5, 6), mutations in the death domain of DR4 (6), and also by increased expression of caspase inhibitors (6). Therefore, there are a variety of mechanisms used by tumor cells to avoid the induction of apoptosis by TRAIL. The involvement of OPG in resistance to TRAIL in LNCaP may be less important than other mechanisms, but it remains possible that OPG production is induced by challenge with TRAIL in this cell line, a proposition not addressed in the present study. Increases in OPG-levels in response to challenge with TNFs and other cytokines have been demonstrated in other cell types (11), and it may be that OPG

production in LNCaP is tightly regulated as opposed to the apparent constitutive expression observed in DU145/PC3.

In our study there was variability in levels of TRAIL-induced apoptosis in fresh medium between experimental series (14%–37%). Because TRAIL-induced apoptosis has been shown elsewhere (10, 12) to be affected by other signaling systems, it is perhaps not surprising that there would be difficulty in obtaining the same level of apoptosis over large numbers of separate experiments where interacting factors would be difficult to control. The studies presented here did not aim to compare the levels of apoptosis in different experiments. Despite the noted variability there was consistency between experiments in the pattern of response. For example, apoptosis was always inhibited in cultures challenged with TRAIL in either conditioned medium or in medium containing 10 ng/ml OPG compared with cultures challenged in fresh medium (compare Figs. 3B *versus* 4B and 2B and 4A, respectively). As stated above, a number of *in vitro* studies have suggested that apoptotic responses are regulated by a balance between pro- and antiapoptotic stimuli. In particular elevated IP3 and Akt kinase (10) activity have been shown to counteract induction of apoptosis by DR4-mediated signaling. An additional difference between LNCaP and PC3/DU145 is that the latter two cell lines are either p53 null or mutant for p53, whereas LNCaP cells are wild type for this gene. A recent study has suggested that active p53 is involved in TNF- $\alpha$  responses in LNCaP (12). Together, the above studies suggest that cellular modulation of apoptotic responses to members of the TNF family are complex, cell type-specific, and interactive with antiapoptotic signals associated with the microenvironment to which tumor cells are exposed.

The present study has shown that OPG is expressed at biologically active levels in poorly differentiated, androgen-independent prostatic cancer cell lines, whereas an androgen-dependent cell line produced near undetectable levels of OPG under the same conditions. The significance of these observations in regard to androgen dependence has yet to be determined, but it is worth noting that the androgen receptor expressed by LNCaP is mutated, so that this cell line may not be the best model to examine the impact of androgens on OPG expression and or action. Nevertheless, high levels of OPG expression may still be a marker of poor differentiation and disease progression, and as such might be expected to be associated with high Gleason grades, loss of androgen dependence, and poor prognosis. The immunohistochemical study referred to above suggested that expression of OPG was higher in metastatic prostate cancer than in primary tumors, but the series was too small to come to any definitive conclusions regarding associations between OPG expression in tissues and tumor characteristics such as Gleason grade (8). Associations of OPG expression with loss of hormone sensitivity was not attempted in the latter study. This is an important area for future work related to the present study. A recent study has suggested that serum OPG levels were elevated in a small series of prostate cancer patients (13), but the study showed large interpatient variation and did not stratify patients with regard to androgen sensitivity/resistance or survival. In summary, the present study has identified OPG as a potential survival factor for androgen-independent prostatic cell lines, protecting them from TRAIL-induced apoptosis. TRAIL is a major inducer of apoptosis produced by monocytes in and around primary and metastatic lesions in response to tumors, and is highly expressed in normal prostate. At the present time, the therapeutic use of recombinant TRAIL as a potential antitumor agent represents an exciting prospect for the treatment of a number of cancers because its activities are largely tumor specific. However, the usefulness of this strategy will depend on the capacity of tumor cells to inhibit TRAIL-induced apoptosis. The present study suggests that tumor cells *in vitro* produce OPG at levels that effectively inhibit TRAIL-induced apoptosis, a factor that may be taken into consideration when developing TRAIL as an antitumor agent.

## Acknowledgments

We thank Dr. Gareth Evans for his help in generating the microscopic images.

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*Cancer Res* 2002;62:1619-1623.

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