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Osteoprotegerin secretion from prostate cancer is stimulated by cytokines, in vitro

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Abstract

Osteoprotegerin (OPG), a member of the tumor necrosis receptor family, is produced by various tissues and inhibits osteoclast differentiation and activity. Since the metastasis of prostate cancer to bone often induces osteosclerosis, the possibility that these tumor cells secrete OPG is of interest. We have investigated whether the prostate cancer cell lines LNCaP, PC-3, and DU-145 produce and secrete OPG in vitro and if the production might be regulated by cytokines involved in remodeling of bone. OPG transcripts were detected by RT-PCR in all cell lines. OPG in culture media was analyzed by ELISA. In all three lineages, treatment with tumor necrosis factor-α and interleukin-1β dose dependently (5–5000 pM) stimulated the OPG secretion. Treatment with tumor necrosis factor-β in increasing concentrations (1–1000 pM) stimulated OPG secretion in PC-3 but had no effect on the DU-145 and LNCaP cells. Dexamethasone (100 pM) had a small, but not significant, inhibitory effect on OPG secretion from DU-145 and LNCaP. In human non-malignant prostate cells, used as controls, there was no effect of IL-1 or TNFs on the secretion rate of OPG.

Keywords: OPG; Prostate cancer; IL-1; TNF; LNCaP; PC-3; DU-145; Dexamethasone

In the latter part of the 1990s osteoprotegerin (OPG) was independently identified by different research groups. By sequence homology, OPG was found to be a novel member of the TNF receptor superfamily [1]. This new glycoprotein was named by the different groups, osteoclastogenesis inhibitory factor [2], TNF receptor-like molecule 1 [3], follicular dendritic cell (FDC)-like cell line (FDC-1) receptor-1 [4], and osteoprotegerin [1]. Recently the American Society for Bone and Mineral Research President's Committee on Nomenclature has recommended the name osteoprotegerin (OPG) [5].

OPG is produced and secreted by osteoblasts and bone marrow stromal cells, but is also expressed in a wide variety of other cells and tissues. In humans OPG mRNA is expressed in fetal tissues of the lung, the kidneys, and bone. In adults OPG mRNA has been detected in heart, lung, kidneys, bone, placenta, liver, thyroid, spinal cord, brain, in immune and hematopoietic tissues, and in mesenchymal organs [1,3,6,7]. Other cells that express OPG mRNA are osteosarcoma cell lines MG-63 and SaOS-2, various normal human osteoblastic cells line (hFOB, hFOB/ER-9), primary endothelial cells, human aorta smooth muscle cells, primary fibroblasts, monocytic leukemia line THP-1, ovarian cancer cell line CAOV-3, breast cancer cell line MCF7, and several lymphoma cell lines [3,7–11]. In the immune system, OPG/OCIF expression is restricted to lymphoid cells and is most abundant in B cells, DC, and follicular DC-enriched fractions.

The role of OPG in bone remodeling is to act as a decoy receptor for receptor activator of NF-κB ligand (RANKL), that is expressed on osteoblast surfaces thereby preventing it to bind the receptor activator of NF-κB (RANK) on the preosteoclast surface [12,13]. The interruption of RANK/RANK ligand interaction causes inhibition of osteoclastic differentiation and activity resulting in osteosclerosis [1]. Transgenic mice

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overexpressing OPG suffer from osteopetrosis caused by a reduction in the osteoclast number. OPG also protects against bone loss in ovariectomized rats [1].

Most malignant tumors metastasizing to bone cause osteolysis while others, for instance, metastases from prostate cancer, cause sclerosis. Recent studies, using histochemistry, have shown that OPG is expressed in bone metastases of prostate cancer [14]. Clinical studies have also shown that serum levels of OPG are elevated in prostate cancer patients with bone metastasis [15,16]. In this study we have therefore investigated whether prostate cancer cell lines secrete OPG, in vitro, and if this secretion might be regulated by cytokines believed to be involved in bone remodeling.

Materials and methods

Materials. RPMI 1640 was purchased from BioWhittaker Europe, Verviers, Belgium. Alpha modification of Eagle's medium (α-MEM), polyethylene sorbitan monolaurate (Tween20), tumor necrosis factor β (TNF-β) as well as dexamethasone, and fetal calf serum (FCS) were purchased from Sigma Chemical, St. Louis, MO, USA. Penicillin, streptomycin, L-glutamine, trypsin–ethylenediaminetetraacetate (trypsin–EDTA), phosphate buffered saline (PBS), tetramethylbenzidine, and 0.1 M potassium citrate containing H_2O_2 were purchased from SVA, Uppsala, Sweden. The mouse anti-human OPG capture antibody, goat anti-human OPG detecting antibody, and recombinant human OPG (rhOPG) were purchased from R&D Systems, Abingdon, UK. Streptavidin horseradish peroxidase (streptavidin-HRP) was purchased from Vector Laboratories, Burlingham, CA, USA. The Maxisorb microtiter plates were purchased from NUNC, Roskilde, Denmark. DNA-polymerase AmpliTaq Gold, PCR-buffer, and $MgCl_2$ were purchased from Roche Molecular Systems, Branchburg, NJ, USA. Keratinocyte-SFM, F-12K Nutrient Mixture (Kaighn's Modification), Trizol Reagent, Superscript II for RT-PCR, and DNA Taq-polymerase were purchased from GIBCO, Life Technologies AB, Täby, Sweden. Human immortalized prostate cell lines, PZ-HPV-7, were purchased from ATCC, Manassas, VA, USA.

Cell culture. LNCaP and DU-145 cells were cultured in RPMI-1640 and PC-3 cells in F-12K, both media containing 10% fetal calf serum (FCS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml amphotericin-B), and 2 mM L-glutamine. The human non-malignant prostate cell line PZ-HPV-7 was cultured in keratinocyte-SFM supplemented with antibiotics. The cells were incubated in a humidified CO_2 incubator at 37 °C and the medium was changed twice weekly. At confluence, the cells were trypsinated and seeded into 24-well plates to be used for the different experiments.

RT-PCR. Total ribonucleic acid from the prostate cancer cell lines was isolated according to the method described by Chomczynski and Sacchi [17]. Briefly, confluent prostate cancer cells in 75 cm^2 culture flasks were washed in ice cold PBS and lysed in Trizol Reagent. The lysate was subjected to acid phenol/chloroform extraction. The RNA was precipitated with isopropanol and subsequently dissolved in H_2O. The RNA was treated with DNAse I for 60 min followed by proteinase K digestion and another phenol/chloroform extraction. The purified RNA was analyzed by agarose gel electrophoresis and quantified spectrophotometrically. Total RNA from the prostate cancer cell lines was converted to cDNA by reverse transcriptase (Superscript II) and the cDNA content was verified by PCR with DNA Taq-polymerase using primers for the house-keeping gene GAPDH. For PCR amplification standard reagents and Taq-Gold polymerase were used together with custom-made primers. A 220 bp cDNA fragment of the human OPG cDNA gene was amplified from RNA by RT-PCR using the following primer pair 5′-AAG AAT CCC TCC TCA CAC AGG-3′ and 5′-GAA CCC CAG AGC GAA ATA CAG-3′. The amplification profile for OPG consisted of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 90 s for 40 cycles. The PCR-product was cloned into a vector (pPCR-Script Amp $SK^{(+)}$) and verified by sequencing on an ABI-877 Integrated Thermal Cycler using ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Norwalk, CT, USA) followed by sequencing on a DNA automated fluorescence sequencer, ABI PRISM 310 Genetic Analyzer (Perkin–Elmer, Norwalk, CT, USA).

Measurement of osteoprotegerin secretion. The cells were seeded in 24-well culture plates at a density of 50,000 cells/well and left to adhere. LNCaP and DU-145 cells were cultured in RPMI-1640 and PC-3 in F-12K supplemented with 10% FCS, antibiotics (PEST), and L-glutamin. PZ-HPV-7 in keratinocyte-SFM supplemented with antibiotics (PEST). After 48 h the cells were starved for 24 h then the test substances were added and the cells were incubated for another 48 h. OPG secretion was measured by ELISA [18]. Briefly a MaxiSorb microtiter plate was coated with mouse anti-human OPG capture antibody for 24 h at 4 °C. The plate was then blocked with 1% bovine serum albumin, 0.05% NaN_3, and 5% sucrose in PBS overnight at 4 °C. Samples or the standard rhOPG were added and incubated for 2 h at 37 °C. Biotinylated goat anti-human OPG detecting antibody was supplied followed by another 2 h incubation. Then streptavidin horseradish peroxidase was added to the plates for 30 min before development using 1 mM tetramethylbenzidine in 0.1 M potassium citrate, pH 4.25, containing H_2O_2. The reaction was terminated with 0.9 M H_2SO_4 and the plates were read at 450 nm in a microtiter plate reader (Labsystems iEMS reader MF, Stockholm, Sweden). Between each step of the ELISA, the plates were washed three times with PBS containing 0.05% Tween20.

Results

The expression of OPG mRNA was detected by RT-PCR in all three prostate cancer cell lines (PC-3, DU-145, and LNCaP) (Fig. 1). PCR products were confirmed by nucleotide sequencing. The OPG levels in the culture media of prostate cancer cell lines were investigated using an OPG ELISA method described by Brandstrom et al. [18]. The cell count in the wells after treatment with cytokines did not show any corre-

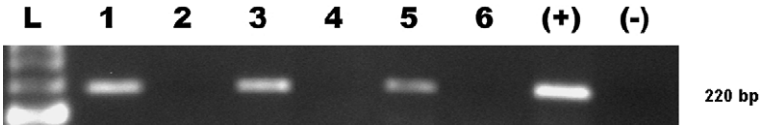

Fig. 1. Constitutive expression of OPG mRNA in PC-3, DU-145, and LNCaP evaluated by RT-PCR. PCR products were confirmed by nucleotide sequencing. L = ladder 123 bp/step. 1, PC-3; 2, PC-3 without addition of Superscript II (−Superscript II); 3, DU-145; 4, DU-145 − Superscript II; 5, LNCaP; 6, LNCaP − Superscript II. (+) Human osteoblasts used as positive control. (−) H_2O used as negative control.

sponding increase when there was increase in the OPG production (data not shown).

The PC-3, LNCaP, and DU-145 cells were treated with various doses of TNF-α (5–5000 pM), IL-1β (5–5000 pM), TNF-β (1–1000 pM), and dexamethasone (0.1–100 nM) for 48 h.

PC-3 cell line increased the OPG production by 1316% ± 411 SD with TNF-α (5000 pM), 516% ± 44 SD with IL-1β (5000 pM), and 234% ± 45 SD with TNF-β (1000 pM) (Figs. 2a,b, and c). DU-145 cell line increased OPG production by 182% ± 31 SD with TNF-α (5000 pM) and 132% ± 3.8 SD with IL-1β (5000 pM), but treatment with TNF-β (1000 pM) 106% ± 21 SD showed no effect (Figs. 2a,b, and c).

LNCaP cell line increased the OPG production by 319% ± 133 SD with TNF-α (5000 pM) and 1121% ± 43 SD with IL-1β (5000 pM), but treatment with TNF-β (1000 pM) 106% ± 29 SD showed no effect (Figs. 2a,b, and c).

Treatment with dexamethasone (0.1–100 nM) known to influence OPG secretion in other cell systems slightly lowered levels in PC-3 and LNCaP medium and halved the OPG levels compared with control in DU-145 medium (Fig. 2d).

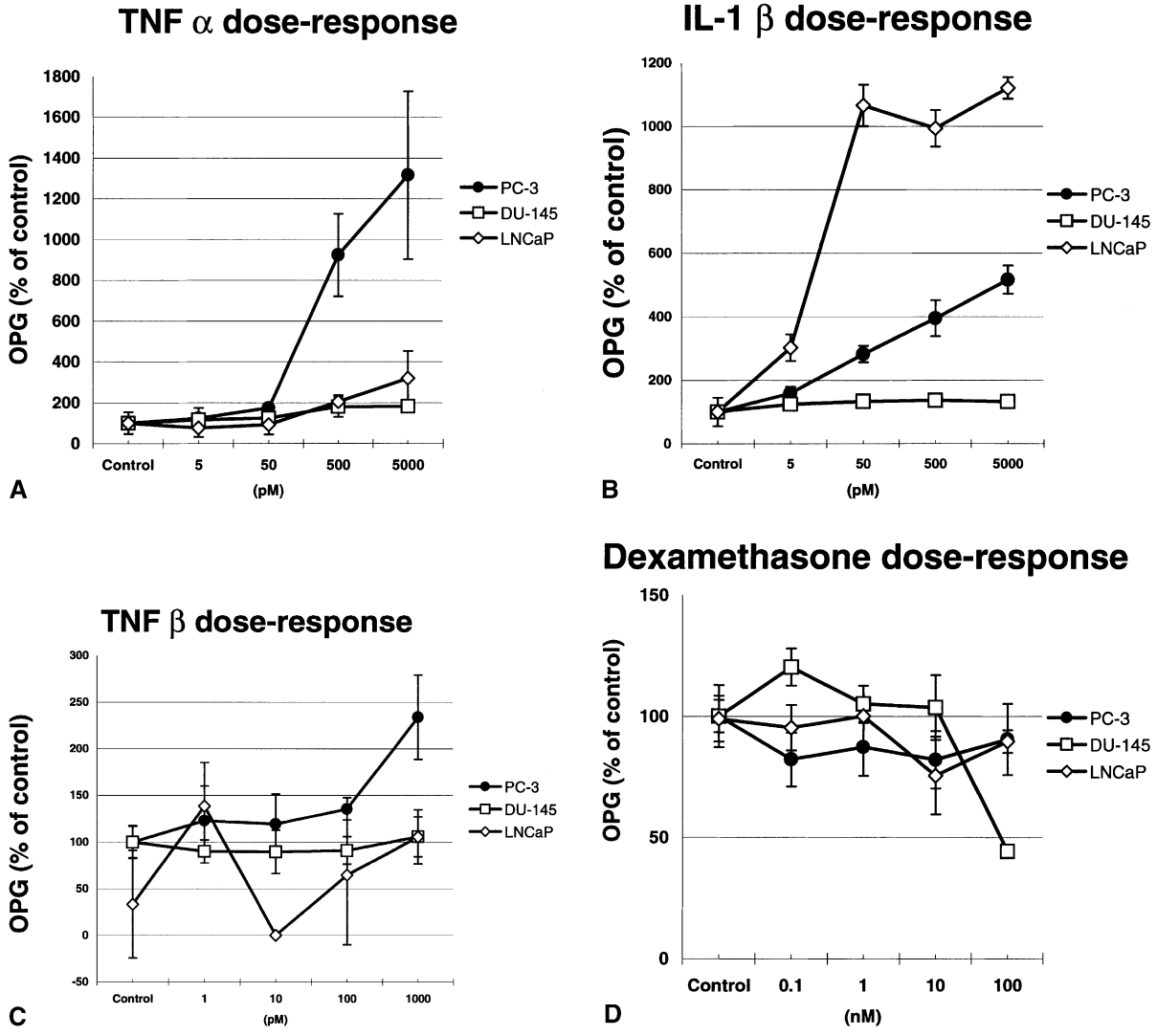

Fig. 2. OPG secretion in prostate cancer cell lines. PC-3, DU-145, and LNCaP cells were treated for 48 h with different doses of TNF-α (5–5000 pM), IL-1β (5–5000 pM), TNF-β (1–1000 pM), and dexamethasone (0.1–100 nM). OPG levels in media were measured with ELISA. After treatment with TNF-α (5–5000 pM) there was an increase in OPG levels compared with control of 1316% ± 411 SD, 182% ± 31 SD, and 319% ± 133 SD in PC-3, DU-145, and LNCaP, respectively, at maximum dose (a). When treated with IL-1β (5–5000 pM) the OPG levels compared with control were 516% ± 44 SD, 132% ± 3.8 SD, and 1121% ± 43 SD in PC-3, DU-145, and LNCaP, respectively, at maximum dose (b). Treatment with TNF-β (1–1000 pM) gave OPG levels as compared with control of 234% ± 45 SD, 106% ± 21 SD, and 106% ± 29 SD in PC-3, DU-145, and LNCaP, respectively, at maximum dose (c). Dexamethasone (0.1–100 nM) treatment decreased the OPG levels compared with control in DU-145 medium at maximum dose, 44% ± 2.2 SD, and slightly lowered levels in LNCaP and PC-3 media (d).

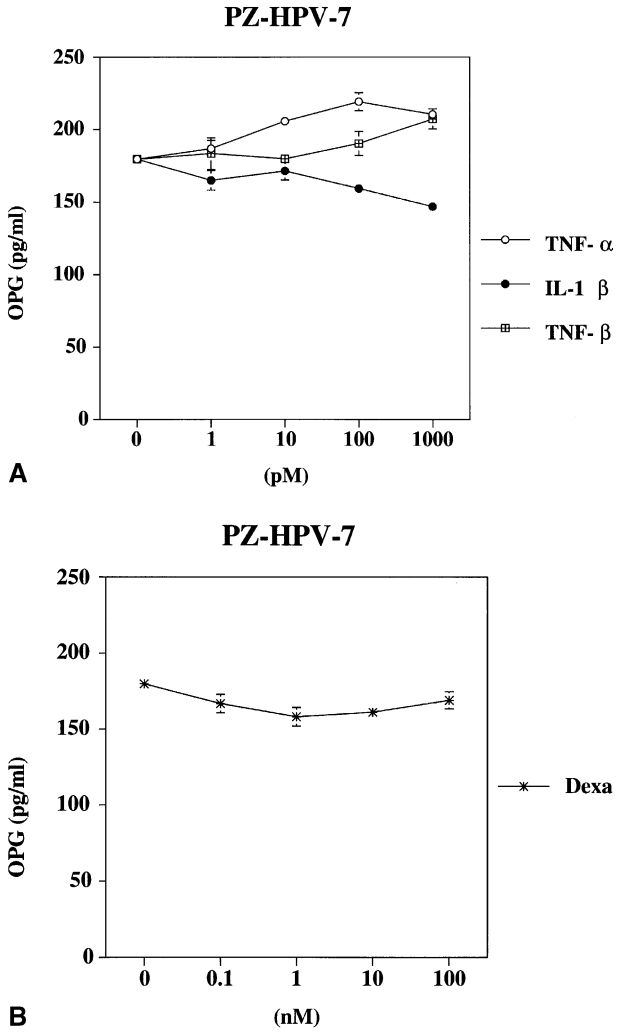

Fig. 3. Human immortalized prostate cell lines, PZ-HPV-7, treated with TNF-α (5–5000 pM), IL-1β (5–5000 pM), TNF-β (1–1000 pM) (a), and dexamethasone (0.1–100 nM) (b), show no significant changes of OPG levels measured with ELISA.

As a control, human immortalized prostate cell lines were used. Also in these cell cultures OPG was detected in the media, but only very slight effects on the levels could be seen with treatment with TNF-α/β, IL-β, or dexamethasone (Figs. 3a and b).

Discussion

Tumor invasion into skeletal tissues is a serious clinical problem. Most malignancies have the capacity to cause metastatic bone lesions. These lesions are often lytic and may lead to severe morbidity involving pain, fractures, and hypercalcemia. Examples are malignant tumors from breast, thyroid, lung, or kidney. Further, it has been speculated that the ability to interact with bone remodeling is one prerequisite to metastasis. Such "homing" mechanisms may explain the preference for bone, characteristic of many tumors. It is believed that cytokines and growth factors secreted by these tumor cells exert effects on the bone cells interrupting the naturally occurring constant remodeling of bone. In the lytic lesions factors believed to induce resorption are parathyroid hormone related peptide, interleukin-6, and tumor necrosis factor. Some tumors, however, cause sclerotic lesions. Most common is that seen in the case of prostate cancer. Far less is known about the putative factors that might cause sclerosis in bone adjacent to tumor cells.

With the discovery of OPG as a potent inhibitor of resorption, a new field in bone biology evolved. Clearly it is tempting to speculate on a role for OPG in the development of sclerotic lesions. Recently two reports found evidence that OPG is involved in prostate cancer induced sclerosis. In one of the studies [14] OPG expression was found in prostate tumor metastases in vivo. Two other studies [14,16] found elevated levels of OPG in serum from patients with prostate cancer. Our data support and strengthen the view that OPG is mediating sclerosis adjacent to prostate cancer cells. We detected OPG secretion from prostate tumor cells in vitro, and, the secretion of OPG is potently upregulated by IL-1 and TNFs. In contrast, non-malignant prostate cells do have a secretion rate of OPG, but this is not affected by the inflammatory cytokines. Thus, we suggest that prostate cells produce and secrete OPG. Also malignant prostate cancer cells produce OPG. The fact that this secretion is under potent regulation by cytokines, believed to be involved in the remodeling of bone, suggests that the prostate cancer cells in the bone microenvironment secrete OPG in such a substantial amount that the osteoclastic activity in the vicinity is altered. This might then be the cause of severe osteosclerosis seen sometimes in prostate cancer. Further studies will evaluate whether serum levels of OPG might reflect the severity of the disease or even predict tumor progression.

Conclusion

OPG is secreted from prostate cancer cells in vitro and this secretion is further stimulated by IL-1 and TNFs. We suggest that OPG originating from tumor cells is involved in the pathogenesis of the sclerotic lesions seen in bone metastasis from prostate cancer.

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