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On the Role of Osteoprotegerin/ RANK/RANKL System in the Interaction between Prostate Cancer and Bone

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Abstract

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Metastases to bone are observed in around 80% of prostate cancer patients and represent the most critical complication of advanced prostate cancer. Unlike other solid tumors that are associated with osteolytic bone metastases, prostate cancer bone metastases stimulate osteoblastic activity with sclerosis in the bone lesions as a consequence. Osteoprotegerin (OPG) is part of a system with three proteins that play a key role in bone remodeling; namely OPG, RANK and RANKL. RANKL regulates osteoclast activity by binding to RANK on the osteoclasts surface, and this interaction is interrupted by OPG. OPG also plays a role in the lifecycle of tumor cells by blocking TNF-related apoptosis-inducing ligand (TRAIL) making it possible for them to evade cell death. The aim of this thesis was to investigate the interaction between the OPG/RANK/RANKL system and prostate cancer.

Data showed that there was production of OPG from prostate cancer cell lines *in vitro*. This expression was under the influence of cytokines that are present in the microenvironment of bone. Further, there was documented a previously unnoticed cell surface expression of RANKL. Co-culturing the prostate cancer with human osteoblasts increased the expression of RANKL.

To connect these findings with *in vivo* studies, OPG-gene single nucleotide polymorphisms (SNP) were investigated. To evaluate OPG SNPs association with bone, a cohort of elderly men was used. OPG SNPs was shown to be correlated to bone mineral density at hip and spine. There was also an association to fragility fractures. Then there was examined the association of the same SNPs to the incidence of prostate cancer but after a four-year follow-up there was no association to the genetic variants.

To summarize this research, we hereby present data that the OPG/RANK/RANKL system might be relevant for prostate cancer growth in bone, and for the skeletal related morbidity in this disease. Future *in vitro* and *in vivo* studies will demonstrate the relative importance of this crosstalk, and whether pharmacological interference with the system might be used as a therapeutic tool aiming to decrease skeletal morbidity and possibly also prolong survival in prostate cancer.

Keywords: metastases, bone, prostate cancer, osteoprotegerin, RANKL, osteoporosis, genetics, polymorphisms

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Penno H., Silfverswärd C-J., Frost A., Brändström H., Nilsson O., Ljunggren Ö. (2002) Osteoprotegerin secretion from prostate cancer is stimulated by cytokines, in vitro. *Biochemical and Biophysical Research Communications*, 293:451-5
- II Penno H., Nilsson O., Brändström H., Winqvist O., Ljunggren Ö. (2009) Expression of RANK-ligand in prostate cancer cell lines. *The Scandinavian Journal of Clinical & Laboratory Investigation*, 69(1):151-5
- III Penno H., Grundberg E., Mallmin H., Pastinen T., Olsson C., Mellström D., Karlsson M.K., Ljunggren Ö., Kindmark A. Polymorphic variations in the gene for osteoprotegerin are associated with bone mineral density and predict fractures in elderly men: Data from MrOS Sweden. *Manuscript*
- IV Penno H., Grundberg E., Pastinen T., Mallmin H., Ohlsson C., Karlsson M.K., Kindmark A., Damber J.E., Mellström D., Ljunggren Ö. Polymorphic variations in the gene for osteoprotegerin do not predict prostate cancer incidence: Data from MrOS Sweden. *Manuscript*

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Abbreviations

BMD	Bone mineral density
BMP	Bone morphogenetic protein
DNA	Deoxy ribonucleic acid
DR4,DR5	Death receptors 4 and 5
DU-145	Human prostate cancer cell line
DXA	Dual-emission X-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GWAS	Genome-wide association study
hOB	Human osteoblast like cells
IGF	Insulin-like growth factor
IL	Interleukin
LNCaP	Human prostate cancer cell line
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence index
OPG	Osteoprotegerin
PC-3	Human prostate cancer cell line
PDGF	Platelet-derived growth factor
PSA	Prostate-specific antigen
PZ-HPV-7	Human non-malignant prostate cell line
RANK	Receptor activator of nuclear factor- κ B
RANKL	Receptor activator of nuclear factor- κ B ligand
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFRSF11B	TNF receptor superfamily member 11B
TRAIL	TNF-related apoptosis-inducing ligand
UTR	Untranslated region

Introduction

Human skeleton

Overview

The human skeleton is a constantly remodeling organ. In the first year of life 100% of the skeleton is replaced and in adults the remodeling proceeds at a rate of 5-10% per year. The remodeling process is in a positive balance up until the age of 20-30 years (1, 2) when the bone has its maximum quality and mass. From the age of 30-40 years, the balance becomes negative, where the amount of resorbed bone exceeds the amount of newly formed bone. Thereafter the peak bone mass diminishes by approximately 1-2% per year.

In this formation and resorption of bone the key players on a cellular level are osteoclasts and osteoblasts. The balance between these two cell types determines the amount and quality of bone.

The osteoclast, the bone-resorbing cell, originates from the hematopoietic stem cells in the bone marrow where the preosteoclasts fuse into a multinuclear osteoclast (Figure 1.). The osteoclast attaches to the bone surface once the single layer of lining cells withdraws and presents an opening (3). To attach they use specialized actin-rich podosomes, by which they form tight seals with the underlying bone matrix in roughly circular extensions of their cytoplasm. Within these sealed zones they form ruffled border membranes; this ruffling is made to increase the area of the cell membrane for secretion. Now this cell starts its resorbing task secreting different proteolytic enzymes, i.e. cathepsin K and hydrochloric acid that have the ability to dissolve mineralized bone (4-6). In their trace the osteoclasts leave clear holes or troughs exposing raw bone surface and collagen fibers (see the picture on the cover of the book).

Next the osteoblast, the bone-forming cell, enters the scene (Figure 1). This cell type originates from the osteoprogenitor cells (7) located in the periosteum and bone marrow. The osteoblast produces an organic matrix and a mineral phase. The organic matrix is composed of collagen to 90% and of amorphous ground substance to 10%. On average the organic/mineral ratio in human cortical bone is 1:3 by weight (8, 9). About every tenth osteoblast becomes embedded in the osteoid and is from then on called an osteocyte. This star-shaped cell is thought to function in a paracrine manner and to be

mechanosensory (10). They are networked with each other through small canals or canaliculi, and can probably control each other and the osteoblasts on the bone surface. Osteocytes are also in contact (11) with osteoclasts and are thus in a key position in the remodeling process.

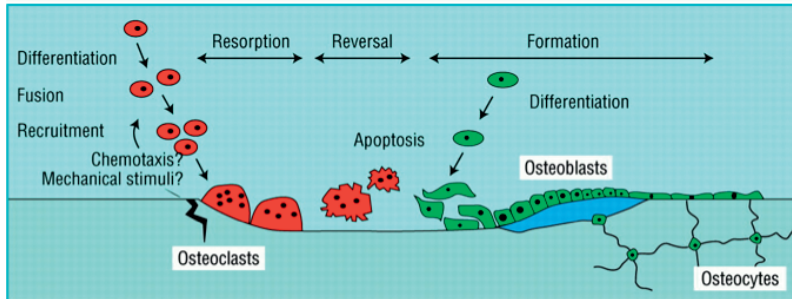


Figure 1 Bone remodeling. Remodeling begins with attraction of osteoclasts to the target site, probably triggered by mechanical stimuli or release of chemotactic substances from areas of microdamage in bone. Mature osteoclasts remove bone during the phase of bone resorption. After resorption is complete, osteoclasts undergo apoptosis during the reversal phase, and bone formation begins as osteoblasts are recruited to the site. Some osteoblasts become trapped within bone matrix and differentiate to form osteocytes, which act as sensors of mechanical strain in bone. From Ralston S., BMJ, 1997

Clearly, when the body grows there is a need for the remodeling process, but there is also a need for remodeling soon after that growth stops. The body constantly remodels to meet altered physical demands (e.g., prolonged bed rest or space flight) (12-14) lessens the bone mass, while physical activities are osteogenic (15-18). The remodeling is also needed for normal physiological processes (e.g., in calcium homeostasis).

Osteoporosis

The internationally agreed description of osteoporosis is “a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures” (19). To define low bone mass the term “bone mineral density” (BMD) has been coined and it refers to the amount of matter per cubic centimetre of bones. It is measured by a procedure called densitometry, e.g. using dual energy X-ray absorptiometry (DXA) that is a radiological method. The World Health Organization (WHO) defines the disease osteoporosis as a bone mineral density (BMD) of 2.5 standard deviations (SD) or more below the young average value in women. Severe osteoporosis uses the same threshold but includes the presence of fragility fractures (20). The diagnosis

of osteoporosis by bone mineral measurements is at the same time an assessment of a risk factor for clinical outcome of fracture. (21) Osteoporosis causes a significant morbidity in society and affects millions of people. It is therefore recognized as a serious health problem. Because of its complications with fragility fractures it leads to great suffering as well as enormous costs for the public health systems (22, 23). Between 30 and 50% of women and 13 and 30% of men worldwide will suffer from a fracture related to osteoporosis in their lifetime (24). The number of hip fractures estimated in 1990 was 1.66 million, a figure that is predicted to rise to 4.5–6.26 million by the year 2050 (25, 26).

Primary osteoporosis, i.e. when there is now external cause, is mainly influenced by sex and age (27). The disease is most common in postmenopausal women, with one out of three women between the age of 70 and 79 years suffering from it (28). Because of this trend it is understandable that most of the research has been done on female populations. Nonetheless, the disease is not uncommon among men, where one of five men suffers from osteoporosis (29). In the annual incidence of 9 million fragility fractures, 39% were in men (23). As previously reported in women (30), there are clear data that the risk for fracture in men is markedly increased after low energy trauma (31).

Family history of fractures is included among the established risk factors for osteoporotic fractures, indicating the importance of the genetic background of osteoporosis. Twin studies also support the heritability of BMD (32), which is the prime indicator of bone strength. The heritability is in the range of 0.46 to 0.84 (32, 33). However the disease does not follow simple inheritance patterns but is polygenic which means there are multiple genes that contribute to osteoporosis. Approaches for the pathogenesis of diseases can be classified as deductive or inductive (34). Analyses about the roles of known substances or genes would be classified into deductive approaches and belong to the genetic approach. On the other hand, recent availability of whole genome information has made the inductive approach possible, which is named a genome-wide association study (GWAS). GWAS is an examination of all or most of the genes of different individuals to see how much the genes vary from individual to individual. Different variations are then associated with different traits, such as diseases. If genetic variations are more frequent in people with the disease, the variations are said to be "associated" with the disease (35). The GWAS studies should have two "arms", the detection arm and the confirmation arm, in which the most significant findings from the detection arm are followed up (36). In the case of osteoporosis these studies have been able to pinpoint 20 genes that are associated with BMD (37).

Because of osteoporosis is more common in the female sex the majority of subjects in the studies have been female (38). For instance, in an important study from 2008 (39) in the search for genes causing osteoporosis the

subjects were 87% women in the detection population and 60-100% in the confirmation population. The study showed five variants associated with BMD; RANKL, OPG, estrogen receptor 1 (ESR1), the zinc finger and BTB domain-containing 40 gene (ZBT40) and the major histocompatibility complex region. The study also found that these genes combined only accounted for approximately 3% of the BMD variation. Because the genetic studies on BMD have been done with female subjects, there was a study made to examine whether the genes determining BMD were gender-specific (40). In this study it was shown that three genes were male-specific, and three were not gender-specific. Other studies in humans also support the presence of gender-specific genes regulating areal BMD (41-44). It is therefore of importance to perform studies in male cohorts to be able to find genes that are male-specific. When it comes to other groups (e.g. populations), it has also been shown that there is no convincing heterogeneity between genetic association in white European and East Asian populations (45).

Prostate cancer

Overview

Prostatic cancer is the second most common malignancy in men worldwide (46). In the United States there were an estimated 186,300 new diagnoses of prostate cancer and 28,700 prostate cancer deaths in 2008, representing 25% of new cancer cases and 10% of male cancer deaths (47). These figures make prostate cancer the second leading cause of cancer death in men. Although the disease can potentially be cured when localized, metastatic prostate cancer remains incurable. Treatment of localized prostate cancer is usually centered on surgery, radiation therapy, or both. However, even after definitive local therapy, approximately 30–50% of patients will have a local or distant recurrence (48).

Metastases to the bone in prostate cancer are predominantly osteoblastic and cause a state of high bone turnover. Levels of bone resorption markers are high (49) showing a high activation of osteoclast and the formation of sclerotic bone shows a high activation of osteoblasts. This woven bone is radiographically dense but structurally weak and bears with it an increased risk of fracture (50, 51).



Figure 2 Multiple osteoblastic metastases to the pelvis and both hips from carcinoma of the prostate. The metastases are the sclerotic areas diffusely spread in the skeleton. With permission from theRadiologyAssistant.nl

Metastatic bone disease

Metastasis is defined as the process by which a malignant cell leaves the primary tumor, travels to a distant site via the circulatory system and establishes a secondary tumor. This is a multi-step process, which consists of a series of sequential events involving complex interactions between the cancer cell and its surroundings (52). Bone metastasis is a frequent complication of cancer. After the liver and lung, bone is the third most common site of hematogenous spread of tumor metastasis (53, 54). In patients with advanced breast or prostate cancer up to 70% have bone metastasis (55). Bone metastases are often classified as osteolytic or osteoblastic, meaning that they either predominately resorb bone or form new bone. Probably these are the extremes of a continuum and patients can have both lytic and osteoblastic lesions. The mechanism responsible for each one is based on the balance between bone resorption and formation. In osteolytic metastasis it is not the tumor cell that does the destruction but it is rather mediated by osteoclasts (Figure 3) (56, 57). However, the activation of the osteoclast depends on the tumor and differs between different tumors. In myeloma there is also a dysfunction in the osteoblast so there is no new bone formation (58, 59).

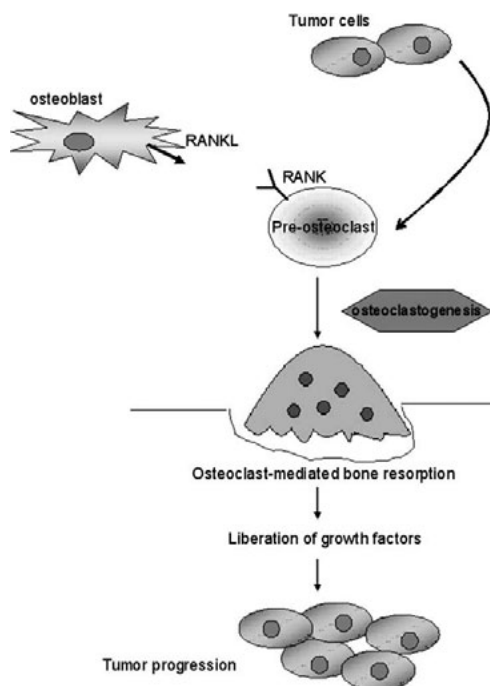


Figure 3 Osteoclastogenesis as the first step of establishment of micrometastasis. Tumor cells chemotactically stimulate osteoclast precursor cells (pre-osteoclasts) to fuse and form mature osteoclasts. This osteoclastogenesis process is mediated via the OPG/RANK/RANKL system and results in osteoclast-mediated bone resorption. Osteolysis leads to the release of growth factors, resulting in tumor progression. From Fili et al., Cancer Letters, 2009.

In the osteoblastic metastasis the factors involved are less known. Endothelin-1 has been implicated in osteoblastic metastasis and has been shown to stimulate bone formation. In addition, there are increased levels in patients with osteoblastic metastasis from prostate cancer (60-62). In addition to endothelin-1, there is involvement of PDGF, urokinase and PSA (Figure 4).

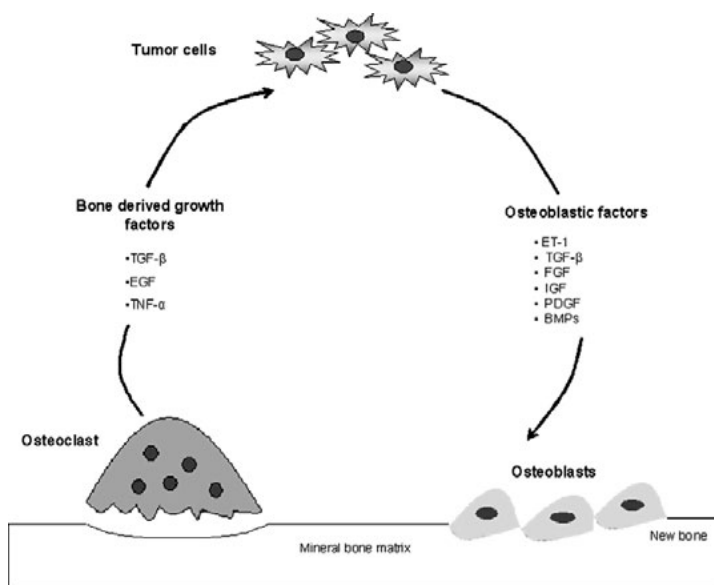


Figure 4 Mechanisms of osteoblastic lesions in cancer. Tumor cells directly contribute to osteoblastic lesions by producing ET-1 (endothelin-1), transforming growth factor β (TGF- β), fibroblast growth factor (FGF), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF) and bone morphogenetic proteins (BMPs). From Fili et al., Cancer Letters, 2009.

Bone metastases are infrequently silent and the consequences are often devastating. Osteolytic metastases cause severe pain, pathologic fractures, life threatening hypercalcemia, spinal cord compression and other nerve-compression syndromes. Osteoblastic metastases have bone pain and pathologic fractures because of the poor quality of bone produced by the osteoblasts. The question why metastases have such high avidity to bone is of course an important one. It might be that it is an organ with high blood flow but other highly vascularized organs that do not receive that many metastases. There might therefore be factors in the bone microenvironment that provide cancer cells with the ability to survive and proliferate. In recent time this interaction, or crosstalk, between the tumor cells and the bone microenvironment has become an area of interest. Research has shown that bone is a great source of growth factors (e.g., TGF- β , IGF-I, IGF-II, FGF, PDGF and BMP) (63, 64). Moreover, a vicious cycle has been described in which tumor cells produce factors that induce the formation of osteoclasts and then the resorption of the bone releases growth factors that stimulate tumor growth and bone destruction (65).

Prostatic cancer homing

Not all cancer cells have the ability to metastasize. Additionally, it has been shown that the process is inefficient, with only a very small number of cells leaving the primary organ having the potential to survive and establish colonies in secondary sites and eventually proliferating into clinically detectable metastatic neoplasms (66). There is increasing support for the “homing” theory to explain the directional cell migration of malignant cells to specific organs, which exhibit peak expression of chemoattractant molecules. Extracts from bone promote chemotaxis and invasion of prostate cancer cells (67). Further, one of the active chemoattractant factors has been shown to be the bone matrix protein osteonectin. Moreover TGF β -1 secreted by osteoblasts(68) and epidermal growth factor (EGF) (69) in medullary bone stimulate chemotaxis. Additionally insulin-like growth factors 1 and 2 (IGF-1,IGF-2) (70) are known to be produced by bone cells and type 1 collagen peptides found in the stroma of bone marrow attract prostate cancer cell lines (71).

OPG/RANK/RANKL

Overview

OPG

Osteoprotegerin (OPG) was given its name because of its protective effects to bone (Latin: *os* bone, *protegere* to protect). It was identified by sequence homology to the tumor necrosis factor receptor (TNFR) family during a rat intestine complementary deoxy ribonucleic acid (cDNA) sequencing project (72). The full-length rat OPG gene was overexpressed in its native soluble form in transgenic mice. These mice were born with high bone mass and marked reductions in osteoclast numbers and activity. Other scientists isolated a protein that had an ability to suppress osteoclastogenesis *in vitro* and named it osteoclastogenesis inhibitory factor (OCIF) (73). OCIF was later shown to be identical to OPG. At the 1998 TNF Conference OPG was given the name tumor necrosis factor receptor superfamily member 11B (TNFRSF11B), but fortunately the nomenclature committee of the American Society of Bone and Mineral Research (ASBMR) understood the disadvantages of such a complex nomenclature and the name osteoprotegerin, or OPG, was officially adopted (74). Composed of 401 amino acids (aa), human and murine OPGs consist of four cysteine-rich pseudo repeats located in the N-terminal, two death domains, a heparin-binding site located in the C-terminal and a 21 aa signal peptide (Figure 5) (75).

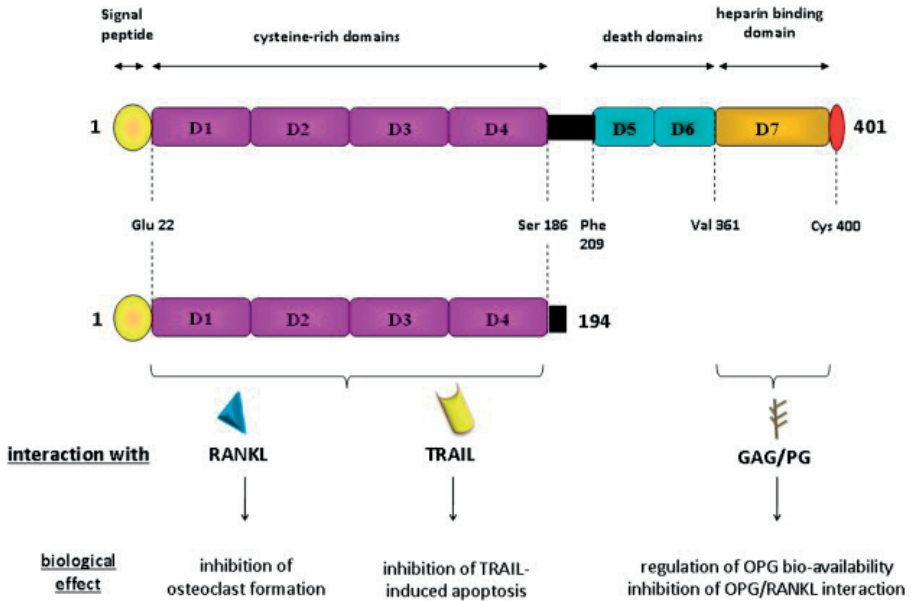


Figure 5. Comparison of the OPG 1–401 and OPG 1–194 functional domains. The molecular structure of OPG allows its binding with different ligands, such as RANKL or TRAIL on the cysteine-rich domains and GAG/PG on the heparin-binding domain. OPG: osteoprotegerin; RANKL: receptor activator of NF- κ B ligand; TRAIL: TNF-related apoptosis inducing ligand; GAG: glycosaminoglycan; PG: proteoglycan. From Lamoureaux, F., et al, *BBA- Reviews on cancer*, 2010.

OPG is an atypical member of the TNF receptor family in that it is a secreted protein with no transmembrane domain and no direct signaling properties (76). There are monomeric and homodimeric forms of which the dimeric form of OPG is probably more potent (77, 78). OPG is produced by osteoblasts, vascular smooth muscle cells, endothelial cells and other cells (79-85). The closeness of the osteoblast to the osteoclast during the remodeling process gives it an opportunity to directly regulate the absorption. There are also significant levels of OPG in circulating blood produced by, for instance, vascular cells to suppress differentiation of circulating preosteoclasts (86) (Figure 6). B-cells contribute to 65% of the bone marrow production of OPG (87) and consistently B-cell knockout (KO) mice are found to be osteoporotic and deficient in bone marrow OPG. The osteo-protective role of OPG in human bone has been supported by the findings of homozygous partial deletion of OPG gene in patients with juvenile Paget's disease. This disease is characterized by rapidly remodeling woven bone, osteopenia, fractures, and progressive skeletal deformity (88). Further, patients with idiopathic hyperphosphatasia, an autosomal recessive bone disease characterized by deformities of long bones, kyphosis and acetabular protrusion, show inframe deletion in exon 3 of the OPG gene (89).

RANKL

The reason why OPG prevents bone resorption and increases bone mass is its ability to bind via its TNF receptor domains to TNF domains within its natural ligand (77), receptor activator of nuclear factor- κ B ligand (RANKL). RANKL is a tumor necrosis factor (TNF)-related cytokine coded for by a single gene. However, alternative splicing results in the expression of three isoforms (90), two of which possess a transmembrane domain of either 317 or 270 aa and a third isoform of only 243 aa that lacks both the transmembrane and cytoplasmic domains and acts as a soluble ligand (sRANKL). Production of RANKL has been shown in osteoblasts, activated T cells, B-lymphocytes and megacaryocytes (91-96). RANKL is involved in numerous aspects of osteoclast differentiation and function and has been implicated in the fusion of osteoclast precursors into multinucleated cells, their differentiation into mature osteoclasts, their attachment to bone surfaces, their activation to resorb bone and their continued survival by avoiding apoptosis (97-100). RANKL has been shown to be reliant on M-CSF as a cofactor for its function in most cases (101, 102), but there is evidence that it can even function without it (103). Without RANKL, there can be no bone resorption, which shows RANKL's important position. Although the exact explanations for various isoforms are unknown, one possibility is that membrane-bound RANKL ensures cell-cell contact with osteoclasts and their precursors whereas sRANKL allows for diffusion to activate target cells. Membrane RANKL has been suggested to be somewhat more potent than soluble RANKL in stimulating osteoclastogenesis *in vitro* (104, 105). Osteoblasts express RANKL on their surface and facilitate osteoclastogenesis *in vitro* via cell-cell contact with osteoclast precursors (79). T cells express both soluble and membrane-bound forms of RANKL (94, 106); both forms are implicated in focal bone erosions associated with inflammatory arthritis (94, 107).

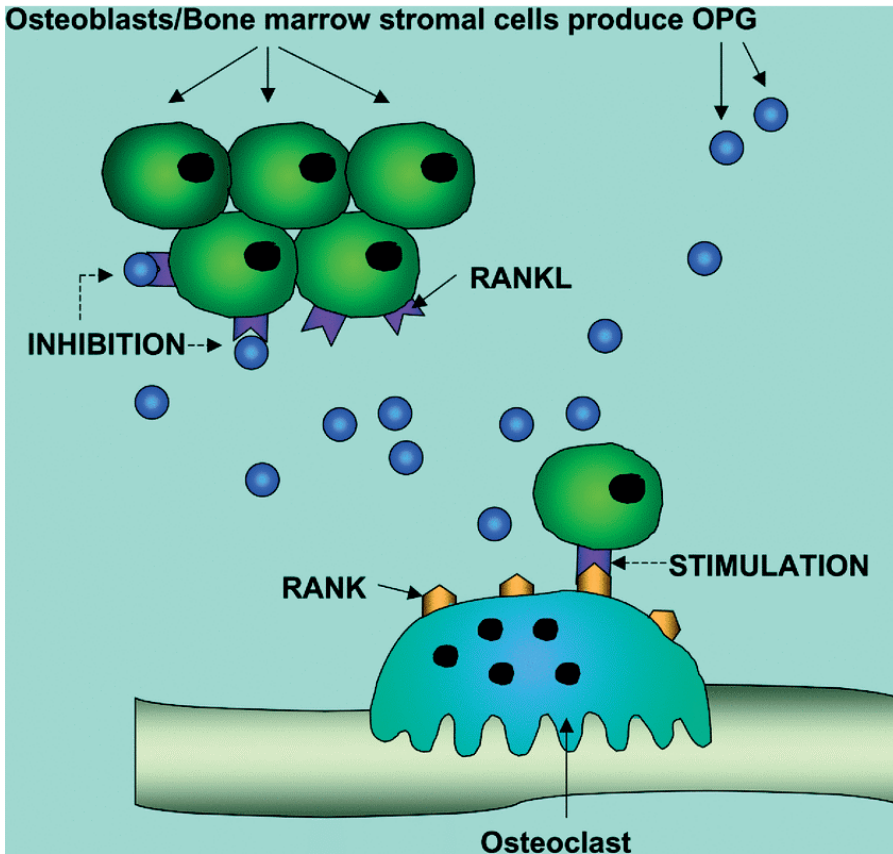


Figure 6 OPG produced by osteoblasts/BMSCs binds RANKL and prevents the association of RANK–RANKL required for osteoclast maturation and activity. From Holen, I., Shipman, C., *Clinical Science*, 2006.

RANK

The receptor for RANKL and OPG is called receptor activator of nuclear factor κ B (RANK). Human RANK is a transmembrane glycoprotein that consists of 616 aa (75, 108). The RANK gene is found on chromosome 18q22 and maps to the same region as does familial expansile osteolysis (FEO) and to one form of familial Paget's disease of bone (PDB) (88, 109). The binding and activation of RANK is thought to cause oligomerization of RANK and the subsequent activation of several signal transduction pathways (77, 110). When RANK binds to its ligand it recruits one of three adapter proteins known as TNF receptor associated factor 2, 5 and 6 (TRAF 2, 5 and 6), with TRAF6 being the most important. They relay, as second messengers, the RANK stimulation signal and activate downstream pathways including nuclear factor κ B (NF- κ B), c-jun N-terminal kinase (JNK) or Src pathways (111-114). Activated NF- κ B translocates to the nucleus and up-regulates the expression of c-fos, which then interacts with the nuclear

factor of activated T cells (NFAT)-c1 to induce the transcription of osteoclastogenic genes. RANK mRNA is expressed in bone, bone marrow, spleen, skeletal muscle, brain, heart, liver, lung, mammary tissue and skin and at the cellular level by osteoclasts, osteoclast precursors, dendritic cells, chondrocytes, endothelial cells, fibroblasts, macrophages and other cells (75, 115-118).

In summary OPG functions as a soluble decoy receptor to RANKL, and by binding to it prevents RANK from being activated. This event stops the osteoclast formation, attachment to bone, activation and survival (Figure 6).

OPG/RANK/RANKL system and prostate cancer

In addition to being central to regulating RANK–RANKL interactions in bone metabolism, OPG can stimulate cell survival by acting as a receptor for TNF-related apoptosis-inducing ligand (TRAIL) (119-121). TRAIL is a member of the tumor necrosis factor superfamily produced by immune cells within the tumor microenvironment such as monocytes in response to interferon- α and γ . This is capable of inducing tumor cell apoptosis through the death receptors DR4 and DR5, which contain cytoplasmic death domains that allow activation of apoptotic signaling pathways. In contrast, OPG acts as a soluble decoy receptor, binding TRAIL and preventing its interaction with the functional death receptors, which would allow cells to escape cell death (Figure 7).

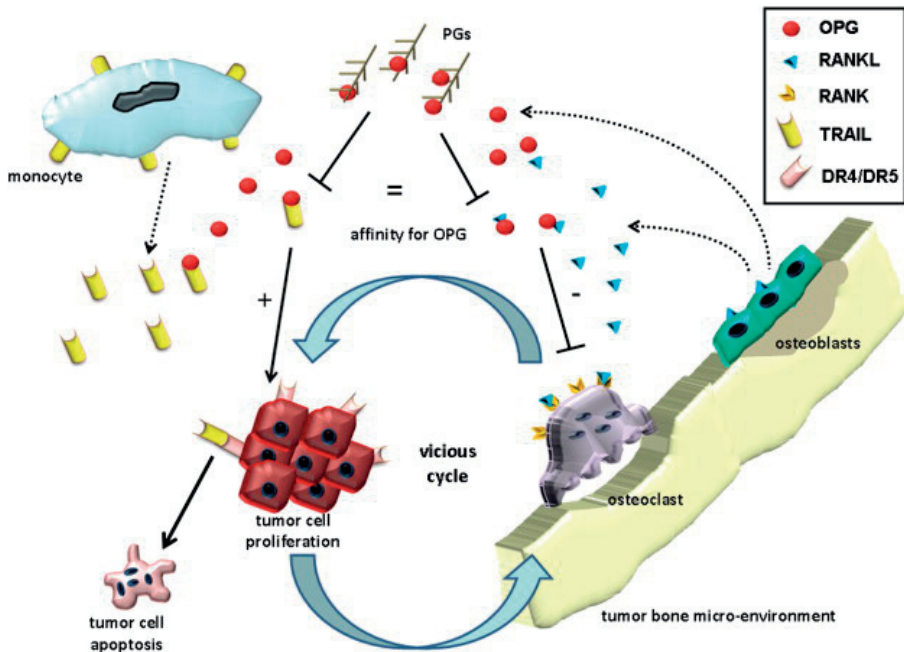


Figure 7 Regulation of OPG activity by the bone tumor microenvironment. The relative concentration of each OPG ligand (RANKL, TRAIL or GAG/PG) within the tumor-bone microenvironment will determine its anti- or pro-tumoral activity. From Lamoureaux, F., et al. *BBA- Reviews on cancer*, 2010.

Indeed, OPG has been shown to be a survival factor for prostate cancer, breast cancer and multiple myeloma cells; this effect works via the inhibition of TRAIL-induced apoptosis. OPG may be involved in survival of a number of tumor cell types via this mechanism. This could be of particular importance regarding the ability of tumor cells to evade cell death in that host immune cells present in the tumor microenvironment produce TRAIL. *In vivo* data suggest this to be important in mediating anti-tumor activity. As such, release of OPG by tumor cells is a potential mechanism of resistance by these cells to TRAIL-induced apoptosis. The biological importance of OPG–TRAIL interactions is underscored by recent findings that at physiological conditions, OPG can bind TRAIL with an affinity similar to that of RANKL (122).

Present study

Aims of the investigation

The general aim of the thesis was to see in what way the OPG/RANK/RANKL system is involved in the interaction between prostate cancer and the skeleton.

The specific aims of the thesis were to:

- Investigate whether prostate cancer cell lines express osteoprotegerin *in vitro*.
- Determine whether OPG expression from prostate cancer cells is influenced by cytokines normally present in the bone environment *in vitro*.
- Study whether prostate cancer cells express RANKL *in vitro*.
- Examine whether RANKL expression in prostate cancer cell lines can be affected by factors from human bone cells *in vitro*.
- Establish whether genetic polymorphisms in the gene encoding for osteoprotegerin is affecting bone tissue *in vivo*.
- Study whether genetic polymorphism in the osteoprotegerin gene influences the incidence of fractures in general and fragility fractures in particular *in vivo*.
- Determine whether genetic polymorphisms of the OPG gene influence the incidence of prostate cancer *in vivo*.

Materials and methods

In vitro studies

Cell culture (Paper I and II)

Prostate cancer cell lines

To study the behavior of prostatic cancer cells *in vitro* we used LnCaP, PC-3 and DU-145 cell lines. These cell lines are well proven for use in prostatic cancer research and have been used for over 30 years(123-125). To compare these to normal prostate tissue we use a non-malignant, immortalized, prostate cell line. Cell lines are commercially available and were purchased from American Type Culture Collection (ATCC). 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 CO₂ 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.

Osteoblast-like cells

Primary cultures of hOBs were isolated from cancellous human bone in patients undergoing endoprosthetic hip surgery at Uppsala University Hospital, Uppsala, Sweden. The specimens were cut into small pieces of 1–2 mm in diameter, thoroughly rinsed with PBS and cultured in 75 cm² tissue culture dishes containing α-MEM supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (PEST), amphotericin-B (0.5 mg/mL), L-glutamine (2 mmol/L), ascorbic acid (50 mmol/L) and 10% FCS. After 4–5 weeks, the culture dishes were confluent with cells that had migrated from the trabecular bone. The cells were detached with trypsin-EDTA, pelleted and thereafter reconstituted in 10 mL of α-MEM and counted in a hemocytometer (Bürker chamber).

T cells

Blood was collected from healthy volunteers. Peripheral blood leucocytes (PBL) were purified by Ficoll-Paque PLUS in accordance with the manufacturer's instructions (Amersham Biosciences, G.E. Healthcare). Culturing PBL was done overnight in RPMI with 10% human serum and with the addition of 1.0 ng/mL of IL-7 stimulated RANKL expression. Expression of RANKL on T cells was determined by flow cytometry, as described below.

Co-culture prostate cancer cell lines and human osteoblast-like cells

Primary cultures of human osteoblast-like cells were cultured as described above. Well inserts with semi-permeable membranes were used. PC-3, DU-145 and LNCaP cell lines were seeded in semi-permeable well inserts. The medium used was α -MEM with 10% FCS. After 96 h, the inserts were removed and the cells detached with trypsin-EDTA and FACS was performed.

RT-PCR (Paper I and II)

RT-PCR. Total ribonucleic acid from the prostate cancer cell lines was isolated according to the method described by Chomczynski and Sacchi(126). Briefly, confluent prostate cancer cells in 75 cm² 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 H₂O. 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 housekeeping gene GAPDH. For PCR amplification standard reagents and Taq-Gold polymerase were used together with custom-made primers.

Measurement of osteoprotegerin secretion (Paper I)

To measure the expression we used an enzyme-linked immunoabsorbent assay (ELISA) method(127). The expression was measured under normal conditions *in vitro* and also when stimulated by different cytokines, such as TNF- α and IL-1 β . 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% NaN₃ and 5% sucrose in PBS overnight at 4°C. The standard rhOPG or samples were added and incubated for 2 h at 37°C. Biotinylated goat anti-human OPG detecting anti-

body was supplied followed by another 2 h of incubation. Streptavidin horseradish peroxidase was then added to the plates for 30 min before development using 1 mM tetramethylbenzidine in 0.1 M potassium citrate (pH 4.25) containing H₂O₂. The reaction was terminated with 0.9 M H₂SO₄ 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.

Flow cytometry (Paper II)

To measure the expression of cell-surface bound RANKL on prostate cancer cell lines we used FACS with a primary RANKL antibody and a secondary FITC antibody. We measured how many in the cell population express RANKL as well as the degree of expression. The following antibodies were used for flow cytometry stainings: Fluorescein (FITC)-conjugated affinity purified donkey anti-mouse IgG (Jackson Immuno Research, Lab. Inc., West Grove, PA, USA), FITC-conjugated anti-human CD4 (BD), phycoerythrin-conjugated anti-human CD3 (BD) and FITC-conjugated Rabbit anti-mouse IgG (DAKO A/S, Denmark). As isotype control for RANKL expression, an unconjugated anti-mouse IgG2B antibody was used. Cells were detached by a brief trypsin-EDTA treatment and suspended in FACS buffer (2% FCS, 0.02% NaN₃ in PBS). Cell suspensions were incubated with primary antibody for 45 min in the dark. Cells were washed in FACS buffer by centrifugation for 10 min at 1100 rpm, resuspended and thereafter incubated with secondary fluorescent conjugate for another 30 min followed by washing as above. Cells were resuspended in FACS buffer and subjected to analysis. Cells, 30,000 of each sample, were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA) using Cellquest software.

In vivo studies

The Swedish MrOS Cohort (Paper III and IV)

The MrOS (The Osteoporotic Fractures in Men) study is a multicenter prospective fracture epidemiology investigation involving elderly men from Hong Kong, Sweden and the US (128). The Swedish part consists of 3,014 men aged 70 years or older. Approximately 1,000 participants were recruited at each of three academic medical centers: Sahlgrenska Academy in Göteborg, Malmö General Hospital and Uppsala University Hospital. The participants were randomly selected from the population registries and invited to participate by mail. Forty-five percent of the subjects who were asked to participate attended the study. To be eligible for the study, the subject had to

be able to walk without aids, they were not allowed to have bilateral hip prosthesis and be between 70 and 80 years of age. BMD was measured at total body, lumbar spine (L1-L4), femoral neck, femoral trochanter and total hip. At the clinic visit, participants completed questionnaires regarding medical history, current medication and lifestyle characteristics. Blood (n=2,961) and serum samples (n=2,908) were collected and stored at -20°C and -70°C, respectively, until processing and analysis. Informed consent was obtained for all subjects and the study was approved by the local ethics committees.

Incident fractures, all verified by radiographs, were registered according to the International Classification of Diseases (ICD) classification system. Osteoporotic fractures were considered as fractures of an vertebrae, distal forearm, proximal humerus, pelvis or femur. During the 5-year follow-up, 548 incident fractures were recorded.

Prostate cancer prevalence and incidence during a 3-year follow-up was determined by searching the Swedish Cancer Register. Also data on staging according to the TNM Classification of Malignant Tumors (TNM) were available, for some of the individuals, at the time of diagnosis. The TNM staging system for all solid tumors uses the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases to classify the progression of cancer.

Bone Densitometry (Paper III)

BMD (g/cm²) was estimated by DXA using either a Lunar Prodigy DXA scanner (GE Lunar Corp., Madison, WI, USA) (*Uppsala and Malmö*) or a Hologic QDR 4500 / A-Delphi densitometer (QDR 4500 W, Hologic, Inc., Waltham, MA, USA) (*Göteborg*). To be able to use DXA measurements obtained with equipment from two different manufacturers in the Swedish MrOS Study standardized BMD (sBMD) was calculated at the lumbar spine, femoral neck and total hip using previously reported algorithms (129, 130). The technical qualities of the equipment were continuously checked according to standard procedure and with phantom measurements. The long-term precision, expressed as the coefficient of variance, CV%, for the BMD measurements ranged from 0.5–3.0%, depending on application.

Genotyping the OPG haplotype (Paper III and IV)

DNA was isolated from whole blood extracted at baseline. In total, six variants in the 3'UTR (rs10955908, rs13439134 and rs4355801) and 5'UTR (rs4567065, rs6993813 and rs7842942) of the OPG gene previously associated with BMD (37, 39, 131) were selected and genotyped using the Sequenom MassARRAY® iPLEX Gold technology (Sequenom Inc., Newton, MA, USA) including single base primer extension and MALDI TOF Mass

Spectrometry. Allele frequencies for the six single nucleotide polymorphisms (SNPs) were calculated and found to be in Hardy-Weinberg (HW) equilibrium in the cohort.

Statistical analysis (Paper III and IV)

All statistical analyses were performed using the STATISTICA 10 software package (version 10 StatSoft, Tulsa, OK, USA). Descriptive BMD data were presented as mean \pm standard deviation (SD). Standardized BMD was analyzed versus presence of various polymorphic variations in the gene for OPG, using height, weight and age as covariates. The General linear model (GLM/ANCOVA) was used to analyze designs with any combination of categorical independent factors (e.g., genotype groups and study location) and continuous predictors (age, weight and height) with least square (LS) means. For categorical variables, such as fracture, the Chi-square test was used.

Results and discussion

Expression of osteoprotegerin in prostate cancer cell lines (Paper I)

To start our investigation to see whether the OPG/RANK/RANKL system could be involved in the communication between prostate cancer cells and bone cells we decided to use cultures of prostatic cancer cell lines to search for expression of OPG. Knowing that between the tumor cell and bone there is an interaction, or crosstalk, we also investigated the influence of the cytokines that are known to be part of the bone microenvironment, on the possible OPG expression.

Expression of OPG from prostate cancer cell lines (PC-3, DU-145, and LNCaP) was detected by real-time polymerase chain reaction (RT-PCR) (Figure 8) and confirmed by nucleotide sequencing.

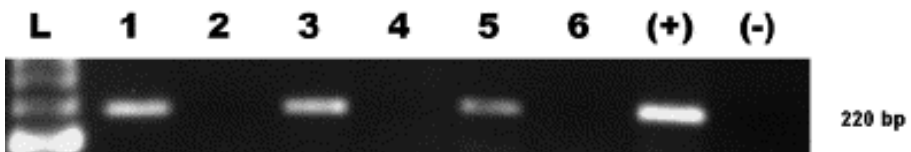


Figure 8 Constitutive expression of OPG mRNA in PC-3, DU-145 and LNCaP evaluated by real-time polymerase chain reaction (RT-PCR). L=ladder 123 bp/step. 1, PC-3; 2, PC-3 without addition of Superscript II (-Superscript II); 3, DU-145; 4, DU-145 - Superscript II; 5, LNCaP; 6, LNCaP - Superscript II. (+) Human osteoblasts used as positive control. (-) H₂O used as negative control.

Then the PC-3, LNCaP and DU-145 cells were treated with various doses of TNF- α (5–5000 pM), IL-1 β (5–5000 pM), TNF- β (1–1000 pM) and dexamethasone (0.1–100 nM) for 48 h. The cell count in the wells after treatment with cytokines did not show any corresponding increase when there was increase in the OPG production. The OPG levels in the culture media of prostate cancer cell lines were investigated using an OPG ELISA method (Figure 9).

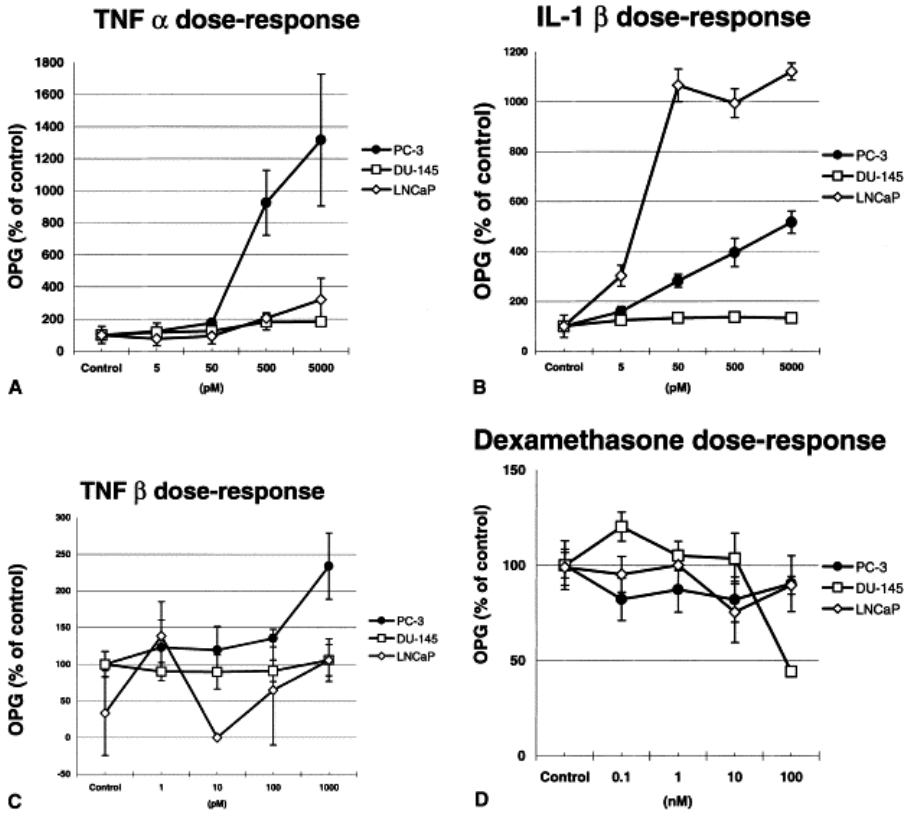


Figure 9. Effect of cytokines on OPG secretion in prostate cancer cell lines. PC-3, DU-145, and LNCaP cells were treated for 48 h with different doses of TNF- α (5–5000 pM), IL-1 β (5–5000 pM), TNF- β (1–1000 pM), and dexamethasone (0.1–100 nM).

The PC-3 cell line increased the OPG production by 1316% \pm 411 SD with TNF- α (5000 pM), 516% \pm 44 SD with IL-1 β (5000 pM) and 234% \pm 45 SD with TNF- β (1000 pM). The DU-145 cell line increased OPG production by 182% \pm 31 SD with TNF- α (5000 pM) and 132% \pm 3.8 SD with IL-1 β (5000 pM), but treatment with TNF- β (1000 pM) 106% \pm 21 SD showed no effect. The LNCaP cell line increased the OPG production by 319% \pm 133 SD with TNF- α (5000 pM) and 1121% \pm 43 SD with IL-1 β (5000 pM), but treatment with TNF- β (1000 pM) 106% \pm 29 SD showed no effect. Treatment with dexamethasone (0.1–100 nM), which is known to influence OPG secretion in other cell systems, slightly lowered levels in the PC-3 and LNCaP medium and halved the OPG levels compared with control in the DU-145 medium. As a control, human immortalized prostate cell lines were used. In these cell cultures OPG was detected in the media, but only very slight effects on the levels could be seen in the treatment with TNF- α / β , IL-1 β or dexamethasone (Figure 3, Paper I).

In conclusion OPG was secreted from prostate cancer cells *in vitro* and IL-1 and TNFs further stimulated this secretion. We suggest that OPG originating from tumor cells is involved in the pathogenesis of the lesions seen in bone metastasis from prostate cancer. This increase in expression of OPG can also be beneficial to the survival of the cancer cells through the effect of blocking the apoptotic effect of TRAIL.

Expression of RANKL in prostate cancer cell lines and the influence of human osteoblasts on its expression (Paper II)

The expression of OPG and the fact that cytokines in the bone microenvironment have a stimulating effect on the expression can have a part in explaining the osteoblastic appearance of the prostate cancer metastasis. Yet, we also know that the prostate cancer metastases have a lytic component. This could be because it needs room to grow and in that case it becomes a vicious cycle where the tumor cell is stimulated by the factors that are released by its lytical properties. If the OPG/RANK/RANKL system would be involved in this process, we then would look for RANKL expression by which it would be able to propagate the activity of osteoclasts via RANK. Again, to see whether this happens in the microenvironment of the metastases/bone interaction we wanted to determine the expression of RANKL on the surface of the prostate cancer cell lines as opposed to the soluble variant of RANKL that also exists. Further, we wanted to determine whether there is any crosstalk between the cancer cells and bone.

As shown by RT-PCR, expression of mRNA for RANKL was demonstrated in all three prostate cancer cell lines investigated (DU-145, PC-3 and LNCaP) (Figure 1, Paper II). The expected RANKL product was cloned and, by DNA sequencing, found to be identical to the published human RANKL sequence.

To develop a system to show RANKL expression on the cell surface we used T cells that are known to express RANKL(132) and also since there is a documented method to stimulate this expression, that is by exposing the cells to interleukin 7 (IL-7)(133), we wanted to test our method as to whether it could detect this expression. The expression of RANKL on the surface of human CD3⁺ T cells was demonstrated as a positive control (Figure 2, Paper II) to validate the antibody. When compared with the IgG isotype control 33% of CD3⁺ T cells expressed RANKL with a mean fluorescence intensity (MFI) of 282. When the cells were stimulated overnight with IL-7 the expression of RANKL increased 1.4 times (MFI 400) (Figure 2, Paper II). Thus, the RANKL antibody used demonstrated the expected pattern of

RANKL expression on T cells and increased its expression after a known stimulus.

FACS was then performed on the prostate cancer cell lines. Using an isotype control did not reveal any unspecific staining of the cell lines. The cell surface expression levels of RANKL measured as MFI were from 600 to 800 and thus similar to the levels as was demonstrated on T cells. Interestingly, the LNCaP cells demonstrated one intermediate RANKL-expressing population and one high RANKL-expressing population. RANKL was only demonstrated on a subpopulation of prostate cancer cells, but at high distinct levels. It was expressed on 2% of PC-3 cells, 1.5% of DU-145 cells and 4–5% of LNCaP cells.

Interaction between the prostate cancer cells and the osteoblast was investigated by measuring the RANKL expression after co-culture with hOB cells. Co-culture of PC-3, DU-145 and LNCaP cell lines with hOB cells demonstrate an increase in the number of cells expressing RANKL. When co-cultured for 96 h with hOB cells the RANKL-expressing cells increased in PC-3 2.5-fold, in DU-145 2-fold and in LNCaP 4-fold (Figure 11), suggesting a paracrine interaction between osteoblasts and prostate cancer cells. Subsequent experiments with cytokines known to be secreted from osteoblasts, such as IL-1, TNF- α , and IL-6 in doses about 0.5–1.0 nmol (data not shown) did not affect the expression levels of RANKL on cell surfaces of the prostate cancer cell lines. Thus, the identity of this paracrine factor is at present not known.

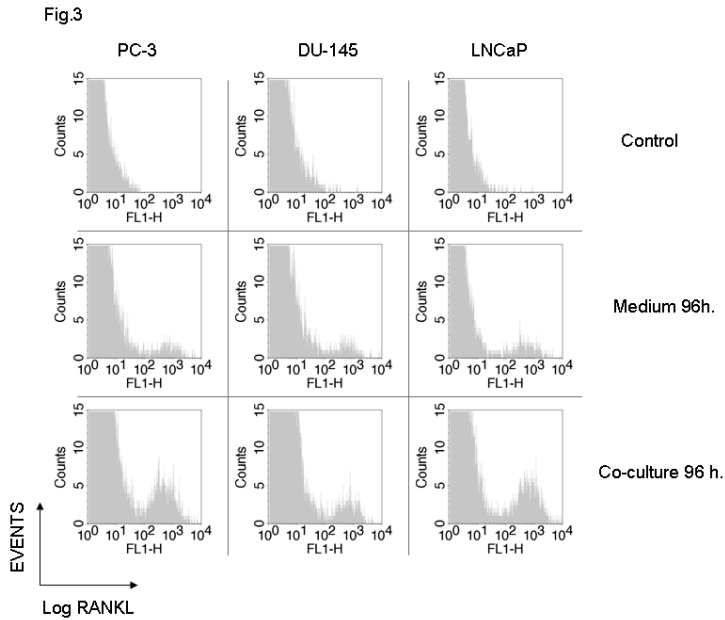


Figure10. RANKL expression of prostate cancer cell lines and co-culture with human osteoblasts. **Top panel:** PC-3, LNCaP and DU-145 cell lines stained with an isotype control. Cell lines were cultured alone (**mid panel**) or co-cultured with human osteoblasts (**bottom panel**) for 96 h in wells with a semi-permeable membrane. Thereafter cells were detached with trypsin-EDTA and analyzed for RANKL expression by FACS.

The expression of RANKL and the effect of the co-culture suggest a signaling mechanism between bone cells and prostate cancer cells that might increase bone resorption and thereby promote bone metastases.

Genetic polymorphisms of OPG have an effect on bone mineral density and fragility fractures (Paper III)

In Paper I and Paper II we presented evidence for OPG and RANKL expression in prostate cancer cell lines. The findings that OPG and RANKL formation can be regulated by various cytokines and by cell communication with osteoblasts might indicate that they play a role in the interaction between prostate cancer cells and bone cells. It is possible that OPG and RANKL are part of the pathophysiology behind the alteration in bone metabolism that is due to prostate cancer cell invasion to bone marrow and possibly involved in the mechanisms underlying prostate cancers ability to metastasize to bone. This view of course is speculative and it was not likely that

the *in vitro* systems could add further information on this matter. Rather, there was the need for either animal studies or *in vivo* investigations in clinical cohorts. We chose the latter in this thesis and aimed at investigating whether the OPG/RANK/RANKL system was involved in prostate cancer incidence or staging in bone metastases. In this respect we used data from the MrOS Sweden cohort (described in Materials and Methods). This study is a large multicenter randomly selected cohort of elderly men. DNA has been collected that gives the possibility to analyze genetic variations in genes involved in bone biology. Furthermore, through cross-examination with the Swedish Cancer Register, data on incidence and staging of prostate cancer can be collected. In the second part of this thesis we therefore first investigated the impact of known genetic polymorphic variations in the gene for OPG versus bone mineral density and fracture rate. This was done to confirm that variations in this gene, as has been suggested by previous reports have biological effects also in male populations because of the gender-specificity as discussed in the section on the genetics of bone biology. Thereafter, these variations could be investigated against prostate cancer incidence and staging.

If a significant correlation here could be established, these data would suggest that the OPG /RANK/RANKL system would not only be present and regulated in prostate cancer cells but also that it could play a role in the pathogenetic mechanisms involved in prostate cancer-induced bone metastases. Thus, the OPG/RANK/RANKL system would be a possible target to interfere with in order to reduce the bone-derived morbidity in prostate cancer.

The baseline characteristics were registered (Table 1, Paper III). In total, six variants in the 3'UTR (rs10955908, rs13439134 and rs4355801) and 5'UTR (rs4567065, rs6993813 and rs7842942) of the OPG gene previously associated with BMD (37, 39, 131) were selected and genotyped. Allele frequencies were calculated and found to be in HW equilibrium in the cohort (Figure 11).

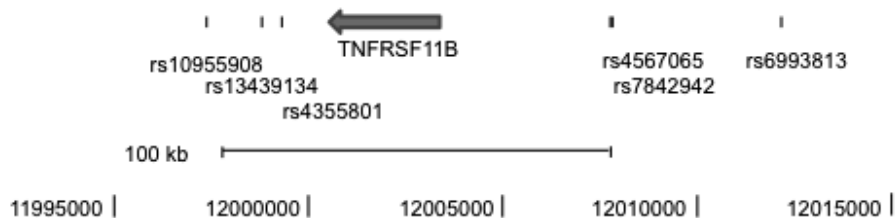


Figure 11 Osteoprotegerin (*TNFRSF11B*) gene SNPs analyzed denoted by their respective rs numbers. The gene including UTRs, exons and introns corresponds to the horizontal arrow and the SNPs are short vertical lines to the left and to the right of the gene. 5'UTR is to the right, and 3'UTR to the left. Bottom scale denotes position in bp on chromosome 8, genome build 36 (Data from UCSC genome browser).

BMD of the lumbar spine and femoral neck was measured using DXA. The results indicated strongly significant BMD differences associated with the different OPG SNPs. These differences were also present at both the right hip and spine in all six genetic variations. Furthermore, the associations were in the same direction at both the hip and the lumbar spine (Figure 12).

TNFRSF11B/OPG	rs#	Genotype	sBMD lumbar spine				sBMD right hip			
			N=	sBMD	Std.Dev.	overall p	N=	sBMD	Std.Dev.	overall p
rs10955908	AA	610	1157	195	0,0008	596	944	140	0,0008	
	CA	1431	1147	208		1390	939	145		
	CC	814	1120	195		792	920	145		
rs13439134	CC	616	1156	195	0,0014	601	944	139	0,0009	
	CG	1435	1147	208		1394	939	145		
	GG	810	1121	195		788	920	145		
rs4355801	CC	617	1156	195	0,0014	602	944	139	0,0010	
	CT	1434	1146	209		1393	939	145		
	TT	810	1121	195		788	921	145		
rs4567065	GG	553	1160	194	0,0014	541	946	138	0,0018	
	GT	1397	1146	210		1354	938	147		
	TT	910	1123	192		887	922	142		
rs6993813	AA	647	1156	197	0,0080	632	945	142	0,0130	
	AG	1456	1144	211		1413	936	147		
	GG	759	1123	187		739	924	139		
rs7842942	TT	553	1160	194	0,0020	541	946	138	0,0024	
	CT	1399	1146	210		1356	938	147		
	CC	910	1123	193		887	923	143		

Figure 12 Standardized BMD (g/cm^2), in the lumbar spine vertebra L1-4 and right hip. BMD was measured using dual-energy X-ray absorptiometry (DXA). The values have been weighted for age, height, weight and medical center. To allow pooling of DXA measurements standardized BMD values were used. Overall p-values are univariate tests of significance by ANCOVA.

Incident fractures, all verified by radiographs, were registered according to the ICD classification system. During the 5-year follow-up, 548 incident fractures were recorded (Table 3, Paper III). Fragility fractures were considered as fractures of a vertebrae, distal forearm, proximal humerus, pelvis or femur.

When calculating the association with fragility fractures an association with the SNP rs6993813 ($p=0.03$) was found when using the chi-square test. (Figure 13)

TNFRSF11B (OPG)		All fractures			Chi square	Fragility fractures			Chi-square
		Fracture	No fracture	Total		Fracture	No fracture	Total	
rs10955908	AA	68	549	617	$p= 0.1313$	45	572	617	$p= 0.1223$
	CA	196	1247	1443		133	1310	1443	
	CC	105	713	818		$p= 0.9881$	77	741	
rs13439134	CC	70	553	623	$p= 0.1704$	47	576	623	$p= 0.1866$
	CG	198	1249	1447		134	1313	1447	
	GG	103	711	814		$p= 0.8323$	75	739	
rs4355801	CC	70	554	624	$p=0.1739$	47	577	624	$p= 0.1822$
	CT	198	1248	1446		134	1312	1446	
	TT	102	712	814		$p=0.7636$	75	739	
rs4567065	GG	64	495	559	$p=0.2643$	41	518	559	$p= 0.1526$
	GT	179	1231	1410		123	1287	1410	
	TT	128	786	914		$p= 0.2147$	92	822	
rs6993813	AA	71	581	652	$p= 0.0876$	44	608	652	$p= 0.0301$
	AG	196	1274	1470		139	1331	1470	
	GG	104	659	763		$p= 0.4583$	73	690	
rs7842942	TT	64	495	559	$p= 0.2672$	41	518	559	$p= 0.1542$
	CT	179	1233	1412		123	1289	1412	
	CC	128	786	914		$p= 0.2110$	92	822	

Figure 13 The different alleles and their association with all fractures and to fragility fractures in the study group. Fragility fractures were considered as fractures of the vertebrae, distal forearm, proximal humerus, pelvis or femur. The p-value is the chi-square of the association of each homozygote versus the two other alleles.

These data showed that variations in the gene for OPG were strongly associated with bone density and further showed a trend towards increased susceptibility to fragility fractures in men.

Polymorphic variations in the gene for OPG do not predict prostate cancer incidence (Paper IV)

Because it could be confirmed that the genetic variations in the OPG gene had a biological impact we believe it is correct to test these variations against prostate cancer incidence and staging. The first step here was to investigate the incidence of prostate cancer because of the known ability of OPG to block the apoptotic effect of TRAIL. If there would be effects on incidence then that would have to be taken into consideration when it comes to the second step in the investigation, which is to evaluate staging, glandular involvement and metastases. Eventually, if a significant correlation here could

be established, this would be data suggesting not only that the OPG/RANK/RANKL system would be present, but also that it plays a role in the pathogenetic mechanisms involved in prostate cancer induced bone metastases. Thus, it would be a possible target to interfere with to reduce the bone derived-morbidity in prostate cancer.

In this study, we used the MrOS cohort that was characterized in the previous study. Data on prevalent prostate cancer at inclusion and on incident prostate cancers up until 3 years after inclusion were collected from the Swedish National Cancer Register.

Of the 3,011 elderly men included in the trial, 262 had a been diagnosed with prostate cancer at inclusion. During the 3-year follow-up, there were 168 incident prostate cancer diagnoses recorded. Thus, we were able to compare 430 cases with 2,581 controls within MrOS Sweden. We could not find any difference regarding age, height, weight or BMI between the cases and controls (Table 1, Paper IV)

The OPG polymorphisms that were used were rs10955908, rs13439134, rs4355801, rs4567065, rs6993813, and rs7842942. These were the SNPs we found to be (Study III) associated with bone mineral density in the MrOS cohort and therefore believed to carry a signal for altered OPG activity *in vivo*. However, when comparing the frequencies of these six SNPs in subjects with prevalent or incident prostate cancer, as compared with the rest of the cohort, there were no associations found (Table 2, Paper IV). These data therefore do not support the hypothesis that altered OPG activity might affect prostate cancer incidence.

A further analysis was performed where the scores for tumor invasiveness, lymph node involvement and metastases (TNM staging system) at diagnosis was evaluated against the six polymorphic variations. In this analysis there was a tendency towards association ($p=0,08-0,09$) for the various SNPs that did not reach statistical significance (Table 3 a, b, c, Paper IV). To illustrate this, Figure 14 shows invasiveness evaluated against the six SNPs.

rs number	Genotype	GLM procedure LS means				N=	P vs ref (min)	Overall P
		T Mean	SEM	CI-95%	CI+95%			
rs10955908	AA	4,47	0,14	4,19	4,75	58		0,25
rs10955908	CA	4,66	0,09	4,48	4,83	150	0,18	0,25
rs10955908	CC	4,78	0,12	4,54	5,02	81	0,09	0,25
rs13439134	CC	4,46	0,14	4,18	4,74	59		0,24
rs13439134	CG	4,66	0,09	4,49	4,84	149	0,15	0,24
rs13439134	GG	4,78	0,12	4,54	5,01	81	0,08	0,24
rs4355801	CC	4,46	0,14	4,18	4,74	59		0,24
rs4355801	CT	4,66	0,09	4,49	4,84	149	0,15	0,24
rs4355801	TT	4,78	0,12	4,54	5,01	81	0,08	0,24
rs4567065	GG	4,46	0,15	4,17	4,74	57		0,31
rs4567065	GT	4,72	0,09	4,54	4,90	142	0,08	0,31
rs4567065	TT	4,67	0,12	4,44	4,90	90	0,24	0,31
rs6993813	AA	4,60	0,14	4,32	4,87	61		0,82
rs6993813	AG	4,69	0,09	4,52	4,86	156	0,51	0,82
rs6993813	GG	4,62	0,13	4,37	4,88	72	0,91	0,82
rs7842942	CC	4,67	0,12	4,44	4,90	90	0,24	0,31
rs7842942	CT	4,72	0,09	4,54	4,90	142	0,08	0,31
rs7842942	TT	4,46	0,15	4,17	4,74	57		0,31

Figure 14 Tumor invasiveness evaluated against the six polymorphic variations.

To summarize we could not show any correlation between the OPG SNPs and the frequency of prostate cancer. Our view is that it is intriguing now to find out whether there might be an involvement of the OPG/RANK/RANKL system in prostate cancers extra prostatic and metastatic disease.

Summary of results

- Prostate cancer cell lines produce and secrete OPG (Paper I)
- The expression of OPG in prostate cancer cells is under the influence of cytokines that are abundant in the microenvironment of remodeling bone (Paper I)
- Prostatic cancer cell lines also express RANKL on the cell surface and therefore have the ability to influence other cells that express the receptor RANK, i.e. osteoclasts (Paper II)
- The expression of RANKL on the surface of prostate cancer cells is affected by the presence of osteoblast-like cells (Paper II)
- OPG polymorphisms have an effect on bone mineral density in elderly men and thus can serve as a tool to investigate the influence of the OPG system in prostate cancer development *in vivo* (Paper III)
- OPG polymorphisms are not associated with the incidence of prostate cancer (Paper IV)

General discussion and future perspectives

Prostate cancer is the second most common malignancy in men and the cause of severe morbidity and mortality in the society (46). One of the specific aspects with this malignancy is the pattern by which it metastasizes to bone. This is an almost obligatory step in the disease and the interplay between bone cells and tumor cells not only causes the skeletal lesions but might also be one of the more important steps in tumor growth and progression. It is frustrating that although the clinical pattern of preferential growth in bone has long been recognized, the mechanisms involved in this process are not known. It is assumed that knowledge of the most important cytokines or growth factors in this interplay between the skeletal cells and tumor cells will eventually lead to new possibilities to therapeutic intervention in order to decrease not only the morbidity related to skeletal events but possibly also tumor growth.

In this thesis we present *in vitro* data demonstrating that prostate cancer cell lines can produce and secrete OPG, as well as express RANKL on their cell surfaces. We further demonstrated that these are expressions that can be regulated by cytokines known to be present in the bone remodeling and also, most important, by secreted factors coming from bone cells. All this implies that the OPG/RANK/RANKL system might be central to the growth of prostate cancer in bone. The fact that the relative expression of OPG and RANKL could lead to either osteosclerosis or osteolysis is in accord with the clinical pattern of bone metastases in prostate cancer. There is also the possibility that by interacting with the normal bone remodeling process prostate cancer cells could receive growth factors, either from resorbed bone matrix or possibly from a crosstalk with the bone cells. These findings are therefore intriguing and future *in vitro* studies will reveal the exact components in this crosstalk.

In an attempt to investigate the clinical importance of this novel putative cell-to-cell communication in prostate cancer, we investigated a clinical cohort of elderly men (the MrOS Sweden). This cohort is well suited to study prostate cancer *in vivo*. Baseline data, including genetics, from more than 3,000 elderly men can be compared with data on prostate cancer prevalence and incidence. With such a cohort it is possible to search for associations such as between genetic variations in the OPG/RANK/RANKL system and

prostate cancer development. In these initial studies we chose variations in the gene for OPG and in order to verify that the SNPs were of biological significance we started by investigating the effects on BMD and fracture incidence in this cohort. It is previously known from other cohorts, mostly consisting of postmenopausal women that such an association might exist. We could here confirm that such a relation exists also in men, but that the actual effects caused on BMD were of the magnitude that differences in fracture incidence were only on the verge of significance. Nevertheless the study demonstrated that the genetic variations analyzed in the OPG gene are associated with biological differences and therefore could be used as a tool to investigate the possible influence on prostate cancer. This line of investigation will of course be a long and cumbersome process in which not only tumor incidence but also aspects of growth, tumor metastasizing pattern and skeletal morbidity will be analyzed. As an initial investigation in this field we analyzed the possible association between OPG variants and tumor incidence in this cohort of elderly men. However, no such relation could be demonstrated, suggesting that this system is not related to de novo tumor genesis. We could see though that there was a tendency towards more severe disease with some genetic variants but these tendencies were not significant. Accordingly, focus of future studies *in vivo* should be on progression of the skeletal metastases. With the data from this thesis as a starting point for future studies, we propose that also variations in the RANK and RANKL should be investigated. Furthermore, we suggest that the most intriguing hypothesis to put forward is not that the development of the primary tumor is affected by this system, but rather that the metastasizing pattern to bone might be influenced.

To summarize this research, we have presented data showing that the OPG/RANK/RANKL system might be relevant for prostate cancer growth in bone, as well as for the skeletal related morbidity in this disease. Future *in vitro* and *in vivo* studies will demonstrate the relative importance of this crosstalk, and whether pharmacological interference with the system might be used as a therapeutic tool aiming to decrease skeletal morbidity and possibly also prolong survival in prostate cancer.

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