

Monocyte Chemotactic Protein-1 Mediates Prostate Cancer–Induced Bone Resorption

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Abstract

Prostate cancer preferentially metastasizes to bone, resulting in high mortality. Strategies to inhibit prostate cancer metastasis include targeting both tumor-induced osteoblastic lesions and underlying osteoclastic activities. We and others have previously shown that blocking receptor activator of nuclear factor- κ B ligand (RANKL) partially blocks tumor establishment and progression in bone in murine models. However, levels of RANKL in the cell lines used in these studies were very low, suggesting that soluble factors other than RANKL may mediate the cancer-induced osteoclast activity. To identify these factors, a human cytokine antibody array was used to measure cytokine expression in conditioned medium collected from primary prostate epithelial cells (PrEC), prostate cancer LNCaP and its derivative C4-2B, and PC3 cells. All prostate cancer cells produced high amounts of monocyte chemotactic protein-1 (MCP-1) compared with PrEC cells. Furthermore, levels of interleukin (IL)-6, IL-8, GRO α , ENA-78, and CXCL-16 were higher in PC3 than LNCaP. These results were confirmed by ELISA. Finally, human bone marrow mononuclear cells (HBMC) were cultured with PC3 conditioned medium. Although both recombinant human MCP-1 and IL-8 directly stimulated HBMC differentiation into osteoclast-like cells, IL-8, but not MCP-1, induced bone resorption on dentin slices with 21 days of culture in the absence of RANKL. However, the conditioned medium–induced bone resorption was inhibited by MCP-1 neutralizing antibody and was further synergistically inhibited with IL-8 antibody, indicating that MCP-1, in addition to IL-8, mediates tumor-induced osteoclastogenesis and bone resorption. MCP-1 may promote preosteoclast cell fusion, forming multinucleated tartrate-resistant acid phosphatase–positive osteoclast-like cells. This study may provide novel therapeutic targets for treatment of prostate cancer skeletal metastasis. [Cancer Res 2007;67(8):3646–53]

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related death of men in the United States (1). The most common site of metastasis is the bone, with 70% to 80% of prostate cancer patients developing skeleton metastases (2).

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doi:10.1158/0008-5472.CAN-06-1210

It is estimated that more than 350,000 patients a year in the United States die with bone metastases (3). Prostate cancer skeletal metastases are most often radiographically characterized as osteoblastic lesions as opposed to osteolytic lesions with decreased bone mineral density. However, it is clear from histologic evidence that prostate cancer metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions (4, 5). Osteoblastic metastases clearly form on trabecular bone at sites of previous osteoclastic resorption and that such resorption may be required for subsequent osteoblastic bone formation (5). The mechanisms through which prostate cancer cells promote bone resorption and subsequent woven bone formation remain poorly understood.

Osteoclast activities are important to the development of bone metastasis in several solid cancer types, including prostate cancer, breast cancer, and lung cancer (3, 6). Because of the facts that increased osteoclastic activity is associated with tumor growth in bone microenvironment, antiresorptive therapies, such as administration of bisphosphonates or parathyroid hormone-related protein neutralizing antibody, have been used in cancer patients or in animal models to block the tumor development in bone (7–13). Bisphosphonates effectively reduce the metastasis-related pain and skeletal complications (14–16), and the decreased bone turnover rate may predict better outcomes in patients with bone metastasis (17, 18). In prostate cancer metastasis animal models, we and others have previously shown that blocking receptor activator of nuclear factor- κ B ligand (RANKL) partially prevents prostate cancer tumor establishment and progression in the bone in murine models (19–22). However, the levels of RANKL from the cells used in these studies were very low as tested in this study. Together with the clinical observations that there is no statistically significant relationship between serum or bone marrow soluble RANKL (sRANKL) levels and osteolytic disease of multiple myeloma (23) and bisphosphonate treatment does not affect serum levels of RANKL in hypercalcemic cancer patients (24), it is plausible to hypothesize that soluble factors other than RANKL may mediate the tumor-induced osteoclast activity.

Chemokines, a family of low molecular weight cytokines that play key roles in immune response and in development of several cell types, are classified mainly into CC and CXC subfamilies according to the location of the first two cysteine residues. Various types of cancer cells, including prostate cancer cells (25–28), express chemokines and chemokine receptors (29–32). In addition, osteoblasts, osteoclasts, and bone marrow endothelial cells in the bone microenvironment have been shown to produce chemokines, such as monocyte chemotactic protein-1 (MCP-1; refs. 28, 33–36). Importantly, these chemokines may have significant effects on tumorigenicity and angiogenesis and regulate tumor cell migration and metastasis (26, 29, 37).

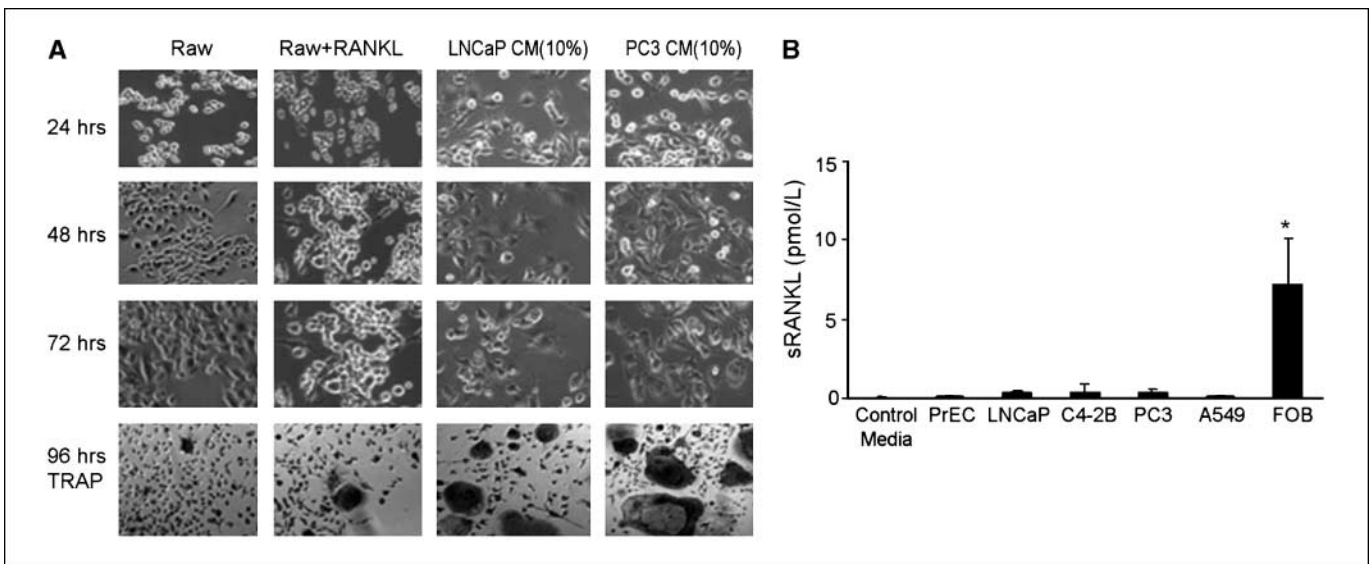
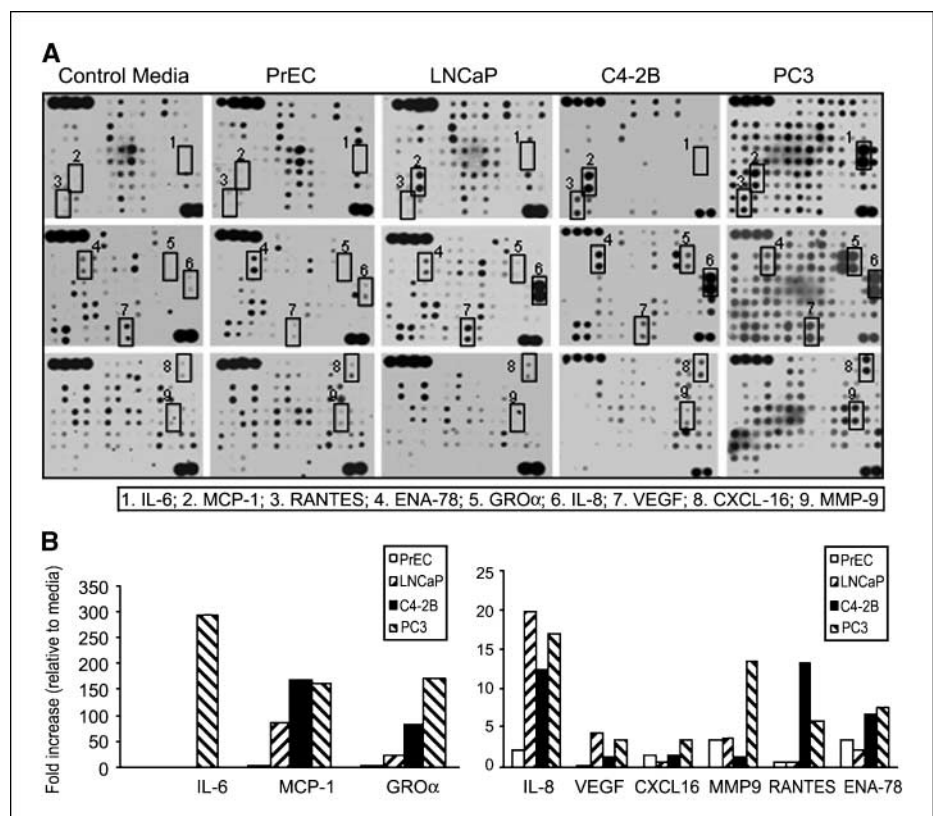


Figure 1. Prostate cancer conditioned medium induced osteoclastogenesis, and RANKL in the conditioned medium was at minimum levels. *A*, single-cell suspensions (3×10^4 cells per well) of RAW 264.7 cells were plated in a 96-well plate in DMEM plus 10% FBS. Cells were grown for 12 h, and then the medium was changed to DMEM plus 0.5% FBS. Conditioned medium from PrEC, LNCaP, C4-2B, PC3, and lung cancer A549 cells was harvested (as described in Materials and Methods) and added to a final concentration of 10% (v/v). Osteoclasts were identified as TRAP-positive multinucleated (more than three nuclei) cells. Representative micrographs of cultures for 24, 48, and 72 h. At 96 h, the cells were stained for TRAP. *B*, levels of sRANKL in control medium, PrEC, LNCaP, C4-2B, PC3, A549, and FOB cells were measured by ELISA. Columns, mean of triplicates; bars, SD. *, $P < 0.005$, compared with control medium.

MCP-1 is a member of the CC chemokine superfamily that plays a critical role in the recruitment and activation of leukocytes during acute inflammation. It is also expressed at the site of tooth eruption, rheumatoid arthritic bone degradation, and bacterially induced bone loss (38). In patients with breast and

ovarian cancers, increased MCP-1 serum levels were correlated with advanced tumor stage (39, 40). It has been further shown that MCP-1 acts as a potent chemotactic factor for prostate cancer and myeloma cells (28, 36, 41–43). Recently, it was shown that MCP-1 was induced by RANKL in osteoclast precursor cells (44)

Figure 2. Prostate cancer conditioned medium was assayed using a human cytokine antibody array. *A*, images of human cytokine antibody array that were assayed with conditioned medium obtained from control medium, PrEC, LNCaP, C4-2B, and PC3 cells. Details of the procedure are described in Materials and Methods. The signals of IL-6, MCP-1, RANTES, ENA-78, GRO α , IL-8, VEGF, CXCL-16, and MMP-9 were labeled with rectangle boxes. *B*, autoradiographs of the arrays were scanned to determine the density of the protein array position. Data as mean value of fold increase relative to control medium. The value from scans was adjusted based on the intensity of positive control spots on each membrane.



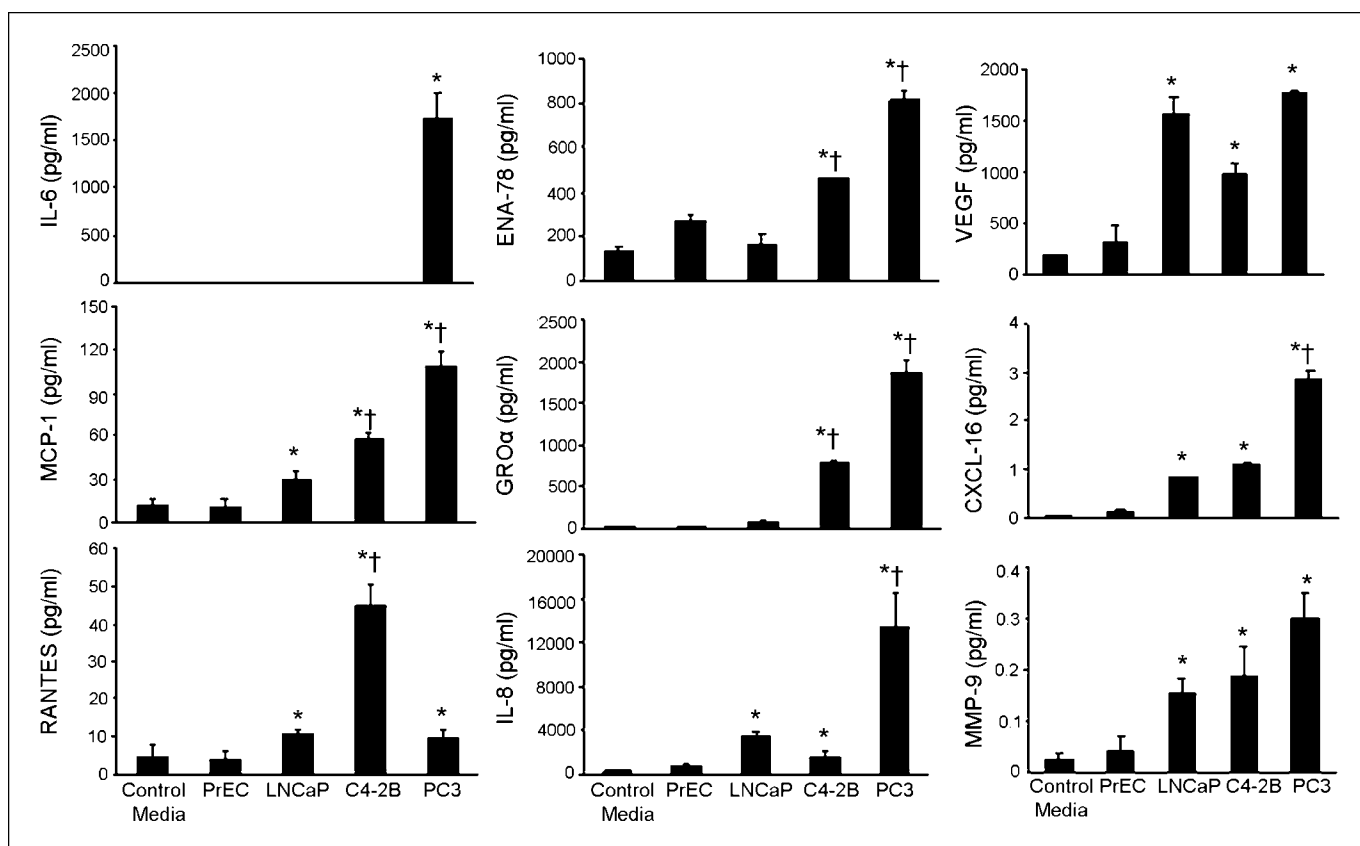


Figure 3. Prostate cancer cells produced IL-6, MCP-1, RANTES, ENA-78, GRO α , IL-8, VEGF, CXCL-16, and MMP-9. Conditioned media collected from control medium and PrEC, LNCaP, C4-2B, and PC3 cell cultures were measured by ELISA. Columns, mean of triplicates; bars, SD. Data are representative of two separate experiments. *, $P < 0.001$, compared with control medium; †, $P < 0.001$, compared with LNCaP.

and MCP-1-treated human peripheral blood mononuclear cells formed tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclast-like cells, but those cells were unable to resorb bone, indicating that MCP-1 may promote osteoclast fusion (45). However, the role of MCP-1 in tumor-induced bone resorption has not been studied.

In this study, we used a human cytokine antibody array (174 cytokines and chemokines included) to identify the factors produced by prostate cancer cells that mediate tumor-induced osteolytic activities. We evaluated the role of MCP-1 in prostate cancer-induced bone resorption *in vitro* by osteoclast formation assay and bone resorption pit assay.

Materials and Methods

Reagents. Recombinant human (rh) and murine macrophage colony-stimulating factor (M-CSF), RANKL, interleukin (IL)-8, MCP-1, IL-8 antibody, MCP-1 antibody, RANKL antibody, and isotype control antibodies were purchased from R&D Systems (Minneapolis, MN). All chemical reagents were purchased from Sigma (St. Louis, MO).

Cell culture. Human prostate cancer cell lines LNCaP and PC3 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI 1640 (Invitrogen Co., Carlsbad, CA). C4-2B cells (UroCor, Oklahoma City, OK), which are LNCaP sublines, were grown in T medium [80% DMEM, 20% Ham's F12 medium (Invitrogen), 5 μ g/mL insulin, 13.6 pg/mL triiodothyronine, 5 μ g/mL transferrin, 0.25 μ g/mL biotin, and 25 μ g/mL adenine]. Primary prostate epithelial cells (PrEC) were purchased from Cambrex Bioscience (Walkersville, MD) and grown in the

specific culture medium provided by the Cambrex Bioscience. RAW 264.7 cells, murine osteoclast precursors obtained from ATCC, were cultured in DMEM (Invitrogen). SV40 large T antigen transfected and immortalized human fetal osteoblast (FOB) cells (ATCC) were cultured in medium with 50% DMEM and 50% Ham's F12 at 34°C. The human lung adenocarcinoma cell line A549 was obtained from ATCC and cultured with DMEM. All the media were supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Conditioned medium. PrEC, LNCaP, C4-2B, PC3, and A549 cells at 2×10^6 were grown in 100-mm tissue culture dishes overnight in cell culture medium and washed twice with PBS, and the medium was changed to 1% FBS in RPMI 1640. After 48 h, the conditioned medium was collected. To normalize for differences in cell density because of proliferation during the culture period, cells from each plate were collected and total DNA content/plate was determined (spectrophotometric absorbance, 260 nm). Conditioned medium was then normalized for DNA content between samples by adding RPMI 1640.

Cytokine antibody array. RayBio Human Cytokine Antibody Array C Series 2000 kit was purchased from RayBiotech (Norcross, GA). This array was consisted of 174 different antibodies spotted in duplicate onto three membranes. Experiments were done as recommended by the manufacturer. Briefly, array membranes were incubated for 30 min in blocking buffer and then incubated for 2 h with 1.5 mL of the conditioned medium collected from PrEC, LNCaP, C4-2B, PC3, or medium of RPMI 1640 with 1% FBS as control (control medium). The membranes were washed, and a diluted cocktail of biotinylated antibodies was added for 90 min. Membranes were then washed again and the sandwiched antigens were detected by incubation for 2 h with a peroxidase-labeled streptavidin solution diluted to 1:1,000. Proteins were detected finally by enhanced chemiluminescence, and signals were captured on X-ray films. Array images were acquired at a

resolution of 300 ppi on a Microtek scanner and quantified with ImageQuant TL software (Amersham Biosciences, Inc., Piscataway, NJ). The fold changes among samples were analyzed by using RayBio Antibody Array Analysis Tool provided by the company. For each spot, the net density gray level was determined by subtracting the background gray level from the total raw density gray levels. The data after background subtraction were normalized according to positive control densities.

ELISA. High-sensitive sRANKL enzyme immunoassay kit was purchased from Biomedica Gruppe (Divischgasse, Austria). Quantikine Human IL-6, MCP-1, RANTES, ENA-78, GRO α , IL-8, vascular endothelial growth factor (VEGF), CXCL-16, and matrix metalloproteinase-9 (MMP-9) ELISA kits were purchased from R&D Systems. ELISAs were done according to the manufacturer's instructions.

Human bone marrow mononuclear cell culture. Human bone marrow mononuclear cell (HBMC) cultures were done as described previously (46). The studies were approved by the Institutional Review Board of the University of Pittsburgh. Briefly, the mononuclear cell fraction from healthy donors was obtained by density gradient centrifugation over Ficoll-Hypaque (Sigma), and the mononuclear cells were incubated in α -MEM with 20% FBS at 5×10^6 /mL overnight at 37°C in 100-mm tissue culture plates to separate nonadherent and adherent cells. The nonadherent cells were collected and used in cultures as the source for osteoclast formation assay and bone resorption assay as described below.

Osteoclast formation from HBMC and RAW 264.7 cells. Osteoclast formation assay was done by culturing 1×10^5 per well nonadherent HBMC or 3×10^4 per well RAW 264.7 cells either in 96-well plates in 0.1 mL of α -MEM with 20% horse serum for 3 weeks or in DMEM with 10% FBS for 4 days, respectively. For HBMC culture, half of the media with the same treatment refreshment were changed twice weekly. The cells were all incubated with rhM-CSF (10 ng/mL) and various treatment of rhMCP-1 (10 ng/mL), rhIL-8 (10 ng/mL), rhRANKL (50 ng/mL), or 10% conditioned medium from prostate cancer cells with or without MCP-1 neutralizing antibody at 1 μ g/mL or IL-8 neutralizing antibody at 200 ng/mL or RANKL neutralizing antibody at 1 μ g/mL. After 21 days of culture, the cells were fixed with 2% formaldehyde. Human osteoclast-like cells were identified by monoclonal antibody 23c6 (widely accepted antibody that specifically identifies human multinucleated cells expressing integrin $\alpha_v\beta_3$) using a Vectastain avidin-biotin complex/alkaline phosphatase kit (Vector Laboratories, Inc., Burlingame, CA). The 23c6-positive multinucleated cells were scored using an inverted microscope. RAW 264.7 cells were treated for 4 days with 10% conditioned medium from prostate cancer cells. The cells were then fixed and stained with K-ASSAY TRAP staining kit (Kamiya Biomedical Co., Seattle, WA) after 4 days of culture. The positive staining cells that contained three or more nuclei under an inverted microscope were scored as osteoclast-like cells. All osteoclast formation was scored from three independent experiments.

Bone resorption assays. To do bone resorption pit assay, HBMCs (1×10^5 per well) were seeded on the dentin slices in 96-well plate and treated as above. After 3 weeks of culture, osteoclasts on dentin slices were confirmed by TRAP staining and bone resorption lacunae were stained with hematoxylin. The mean area of resorption was determined microscopically with SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Statistical analysis. Statistical analysis was done using StatView software (Abacus Concepts, Berkeley, CA). ANOVA was used for initial analyses followed by Fisher's protected least significant difference for post hoc analyses. Student's *t* test was used for comparisons between two groups. Differences with a *P* < 0.05 were determined as statistically significant.

Results

Prostate cancer conditioned medium induces *in vitro* osteoclastogenesis through both RANKL-dependent and RANKL-independent pathways. We previously reported that prostate cancer conditioned medium induced osteoclast formation in RANKL-dependent manner (19). In this study, to further

determine whether prostate cancer cells also induce osteoclastogenesis through RANKL-independent pathway, we cultured murine osteoclast precursor cells RAW 264.7 with conditioned medium collected from LNCaP or PC3 cells. Within 24 h of culturing, RAW cells started to differentiate into multinuclear cells, and at 96 h, both LNCaP conditioned medium and PC3 conditioned medium induced TRAP-positive multinuclear cell formation (Fig. 1A). In contrast, at least 96 h were needed for osteoclast formation when the cells were treated with sRANKL at 50 ng/mL concentration. Furthermore, the levels of sRANKL in the conditioned medium were measured by ELISA. All prostate cancer cells, as well as lung cancer A549 used as a positive control, produced minimum amount of sRANKL at picomolar level compared with FOB cells (Fig. 1B). A549 is a known line that produces RANKL and induces a mixed osteolytic and osteoblastic lesion in bone (47). These results suggest that factors other than RANKL may mediate the tumor-induced osteoclastogenesis.

Prostate cancer cells produce high amounts of MCP-1 and IL-8. To identify factors that present in the conditioned medium that induce osteoclastogenesis, a human cytokine antibody array was used to analyze cytokine production in conditioned medium collected from PrEC, LNCaP, C4-2B, and PC3 cells. All prostate cancer cells produced high amount of MCP-1 and IL-8 compared with control medium (Fig. 2A and B). Levels of MCP-1 and IL-8 were greater in C4-2B and PC3 cells compared with PrEC cells. In addition, levels of IL-6 were greater in PC3 cells compared with LNCaP and C4-2B. Levels of GRO α were greater in LNCaP, C4-2B, and PC3 cells compared with PrEC cells. Levels of VEGF, CXCL-16, and MMP-9 were greater in PC3 cells compared with PrEC cells.

Confirmation of cytokine array data by ELISA. To validate the findings in cytokine array experiment, we measured the levels of IL-6, MCP-1, RANTES, ENA-78, GRO α , IL-8, VEGF, CXCL-16, and MMP-9 in the conditioned medium used for the cytokine array (Fig. 3). Consistent with the array observation, MCP-1 levels in LNCaP, C4-2B, and PC3 cells were significantly greater than PrEC cells. MCP-1, ENA-78, and GRO α levels were significantly greater in C4-2B and PC3 cells compared with LNCaP cells. RANTES levels were significantly greater in C4-2B cells compared with LNCaP cells. Levels of IL-8, VEGF, CXCL-16, and MMP-9 in LNCaP, C4-2B, and PC3 cells were significantly greater compared with PrEC cells, whereas PC3 produced the highest amounts.

Neutralizing antibodies for MCP-1 inhibited the prostate cancer conditioned medium-induced osteoclast formation. To determine the role of MCP-1, in addition to RANKL and IL-8, in the prostate cancer conditioned medium-induced osteoclastogenesis, the HBMCs were cultured with PC3 conditioned medium (10%) in the presence of neutralizing antibodies for MCP-1, IL-8, RANKL, or combination of the two or three antibodies. PC3 conditioned medium as well as LNCaP conditioned medium induced multinucleated osteoclast-like cell formation, and this induction was diminished significantly by each individual antibody, whereas the adding of three antibodies blocked >80% of osteoclast formation (Fig. 4A and B). As expected, the rhMCP-1 or rhIL-8 or rhRANKL induced osteoclast formation *in vitro*, and the combination of the three recombinant molecules showed the strongest induction. Importantly, anti-MCP-1 antibody efficiently blocked the osteoclast fusion compared with the anti-IL-8 or anti-RANKL-treated cells. These results indicate that MCP-1 may play a key role in tumor-induced osteoclast fusion process. Because the HBMCs

might contain marrow stroma or osteoblasts, these experiments did not differentiate if the inhibition of osteoclast formation in this system was due to a direct or indirect effect. Thus, RAW 264.7 cells, the osteoclast precursors, were further determined in the same way but in the absence of supporting stroma/osteoblasts cells (Fig. 4C and D). Exogenous MCP-1 itself stimulated osteoclast fusion in this *in vitro* system. Furthermore, PC3 conditioned medium induced osteoclast fusion and osteoclast formation that was inhibited by each individual antibody. These data provided strong evidence that MCP-1 promotes preosteoclast fusion. Of interest, within 7 days of culture, PC3 conditioned medium induced multinucleated osteoclast-like cell formation from HBMCs, whereas the sRANKL (50 ng/mL) required minimum 14 days (data not shown).

Neutralizing antibodies for MCP-1 and IL-8 synergistically diminished prostate cancer conditioned medium-induced bone resorption. To further evaluate the role of MCP-1, in addition to RANKL and IL-8, in the prostate cancer conditioned medium-induced bone resorption, HBMCs were seeded on the dentin slices and bone resorption pit assays were done. After 3 weeks of culture, the area of resorption was determined (Fig. 5A and B). PC3 conditioned medium induced the greatest bone resorption (45%), whereas LNCaP conditioned medium (15%) and C4-2B conditioned medium (19%) had less effect. As a control experiment, both rhIL-8 and rhRANKL, but not rhMCP-1, induced bone resorption. However, PC3 conditioned medium-induced bone resorption was inhibited by MCP-1 neutralizing antibody by 53% at

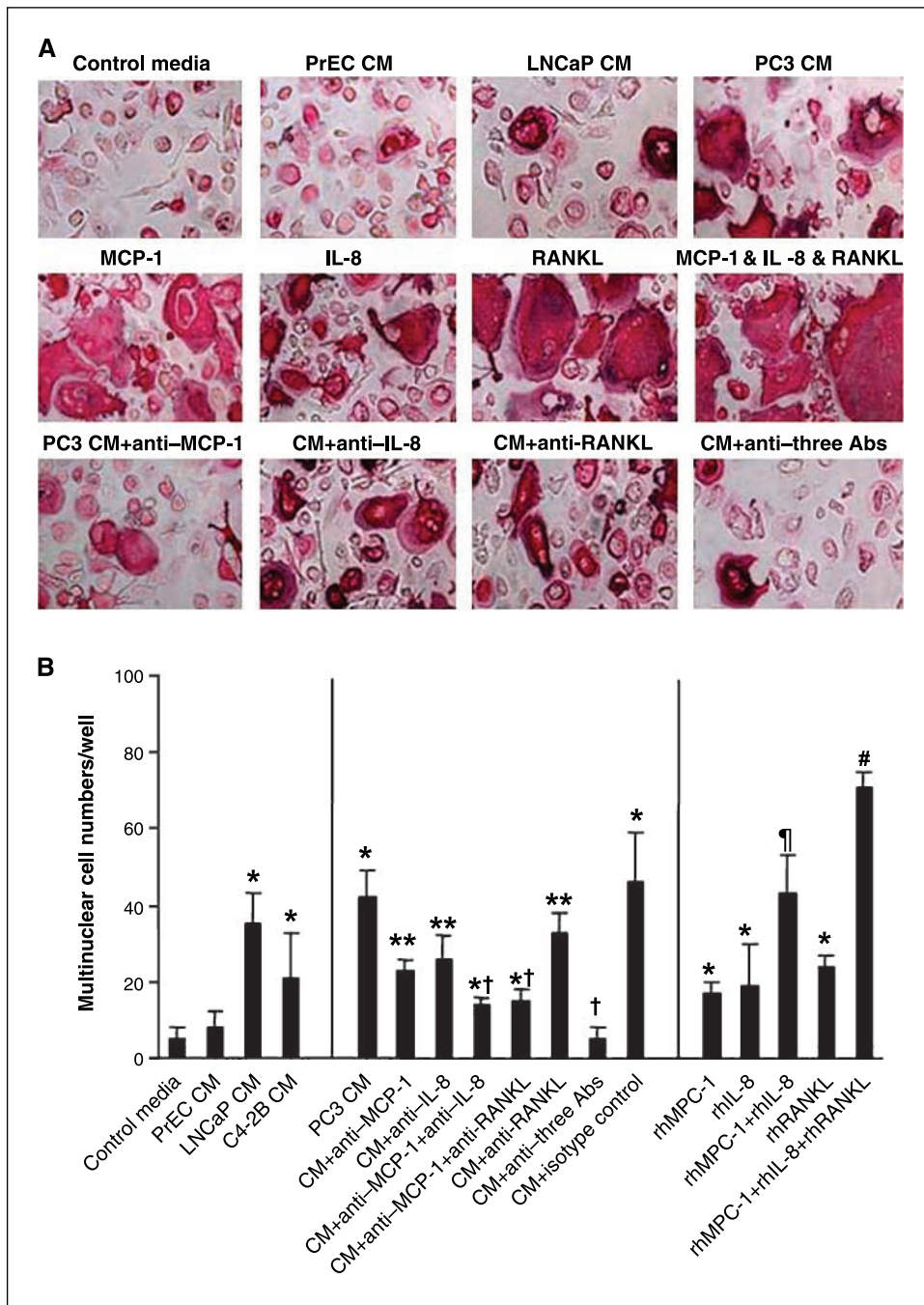


Figure 4. Neutralizing antibodies for MCP-1 diminished the prostate cancer conditioned medium (CM)-induced osteoclast formation. Nonadherent HBMC was cultured at 1×10^5 per well in 96-well plates for 3 wks. HBMC cultures were all incubated with rhM-CSF (10 ng/mL) and indicated treatment of rhMCP-1 (10 ng/mL), rhIL-8 (10 ng/mL), rhRANKL (50 ng/mL), or 10% conditioned medium from PC3 cells with or without MCP-1 neutralizing antibody at 1 μ g/mL or IL-8 neutralizing antibody at 200 ng/mL or RANKL neutralizing antibody at 1 μ g/mL. After 3 wks, the human osteoclast-like cells were identified by monoclonal antibody 23c6. The positive staining cells that contained three or more nuclei were scored as osteoclast-like multinucleated cells. *A*, representative micrographs of HBMC cultures stained for monoclonal antibody 23c6. *B*, a number of osteoclast-like multinucleated cells per well were quantified. Samples were evaluated in quadruplicate. Columns, mean; bars, SD. Data were analyzed using one-way ANOVA. *, $P < 0.001$, compared with control medium-treated group; **, $P < 0.01$, compared with PC3 conditioned medium-treated group; †, $P < 0.001$, compared with PC3 conditioned medium-treated group; ‡, $P < 0.005$, compared with rhMCP-1-treated group; #, $P < 0.0001$, compared with rhMCP-1-treated group.

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concentration of 1 $\mu\text{g}/\text{mL}$ and was further synergistically inhibited with either IL-8 antibody or RANKL antibody. This inhibition of bone resorption by MCP-1 antibody was in a dose-dependent manner (data not shown). IL-8 neutralizing antibody at concentration of 200 ng/mL inhibited 48% of PC3 conditioned medium-induced bone resorption, whereas RANKL neutralizing antibody at concentration of 1 $\mu\text{g}/\text{mL}$ inhibited only 16% of PC3 conditioned medium-induced bone resorption. The combination of the above three antibodies blocked 91% of the PC3 conditioned medium-induced bone resorption. These results indicate that MCP-1, in addition to IL-8 and RANKL, mediates tumor-induced osteoclastogenesis and bone resorption.

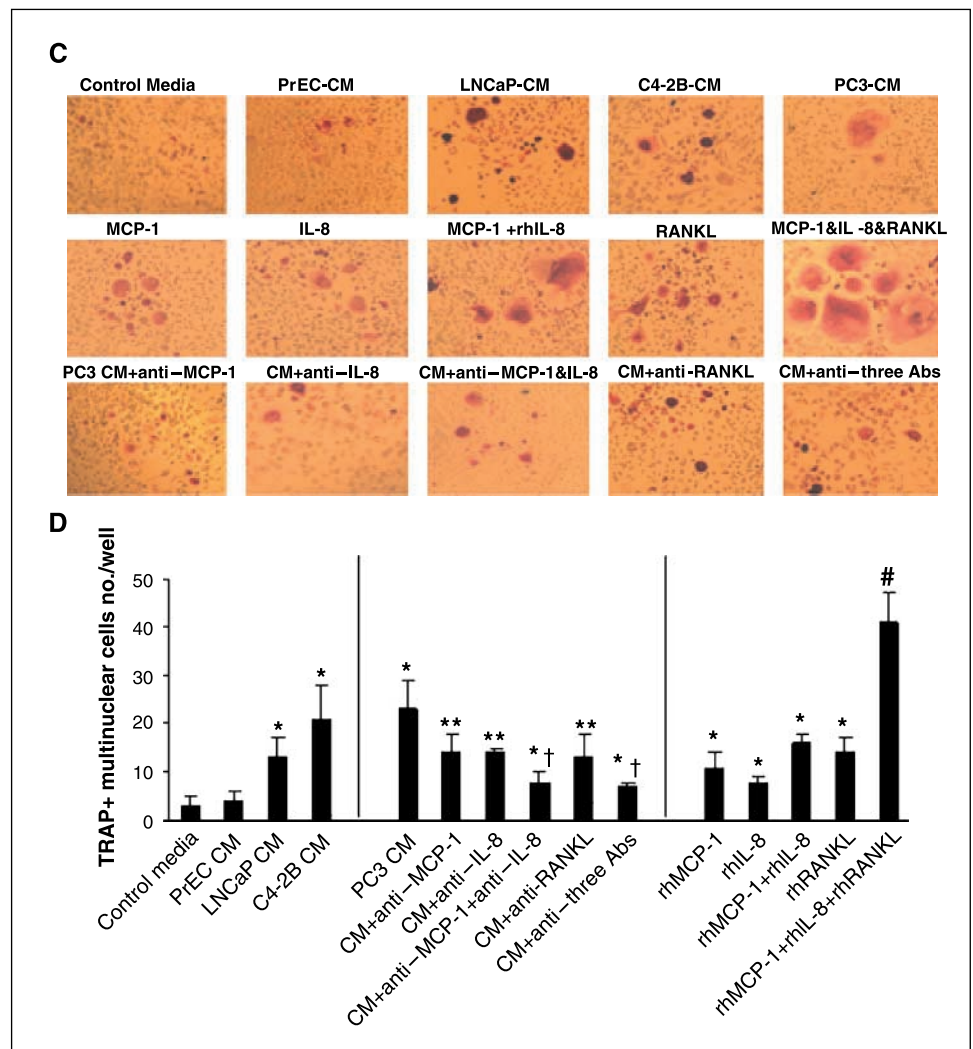
Discussion

By using a human cytokine array approach, we identified MCP-1 from conditioned medium collected from prostate cancer LNCaP and its derivative C4-2B and PC3 cells that mediate prostate cancer-induced osteoclastogenesis and bone resorption. Together with previous report that prostate cancer induces osteoclastogenesis through RANKL-dependent pathway (19), we further showed, in this study, that prostate cancer also induces

osteoclastogenesis through RANKL-independent pathway. To our knowledge, this is the first report that showed that MCP-1 plays key role in tumor-induced osteoclast differentiation and fusion.

Various types of cancer cell, including prostate cancer cells, express chemokines (25–27, 30) and chemokine receptors (29, 30). In addition to inducing inflammatory cell infiltration into the tumor, local chemokines may also mediate angiogenesis, serve as growth factors, and regulate tumor cell migration or metastasis. We found that prostate cancer cells produced significant amount of MCP-1, in addition to other cytokines and chemokines, such as IL-8, and more aggressive cells (PC3) secreted greater MCP-1 compared with less aggressive tumor cell lines (LNCaP). Consistent with the recent findings that MCP-1 induces osteoclast fusion (45), we observed that, although MCP-1 alone cannot induce bone resorption *in vitro*, both prostate cancer cell-induced osteoclastogenesis and bone resorption were inhibited by neutralizing antibody for MCP-1, and this effect was synergistic with neutralizing antibody for either IL-8 or RANKL. In the current study, we paid specific attention to the use of 20% horse serum in human bone marrow osteoclast formation and bone resorption assays and 10% FBS in murine osteoclast formation assay for RAW

Figure 4 Continued. C, representative micrographs of RAW cell cultures stained for TRAP. D, a number of osteoclast-like multinucleated cells per well were quantified. *, $P < 0.001$, compared with control medium-treated group; **, $P < 0.01$, compared with PC3 conditioned medium-treated group; †, $P < 0.001$, compared with PC3 conditioned medium-treated group; #, $P < 0.001$, compared with rhMCP-1-treated group.



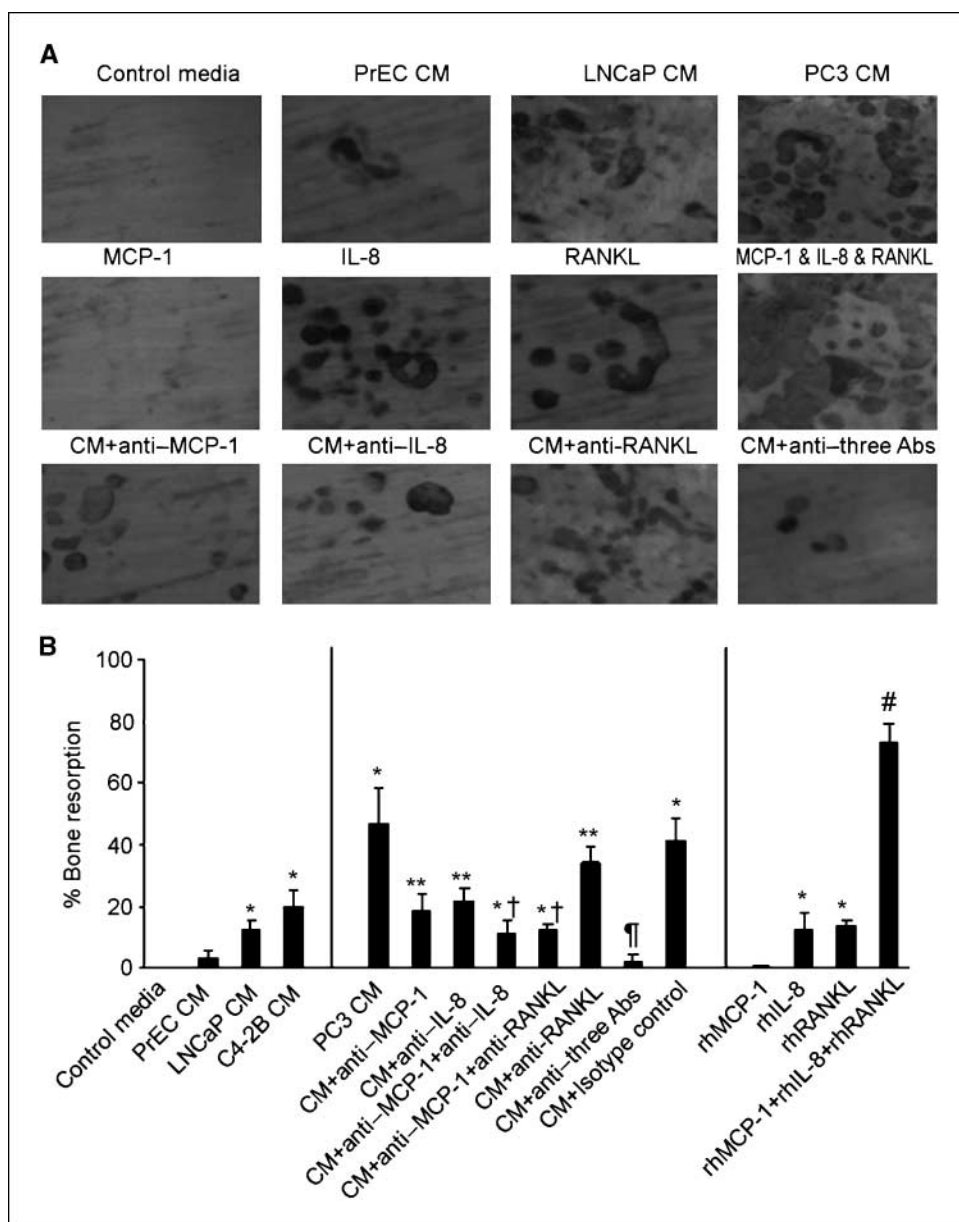


Figure 5. Neutralizing antibodies for MCP-1 and IL-8 synergistically diminished the prostate cancer conditioned medium-induced bone resorption. HBMCs at 1×10^5 per well were seeded on the dentin slices in 96-well plate and treated as in Fig. 4. After 3 wks, the dentin slices were stained with TRAP staining kit and then the cells on the dentin slices were then removed by rubbing the slices, and area of resorption was determined microscopically with SPOT software. **A**, representative images of resorption pits on dentin slices. **B**, samples were evaluated in triplicates. Columns, mean; bars, SD. *, $P < 0.001$, compared with control medium-treated group; **, $P < 0.005$, compared with PC3 conditioned medium-treated group; †, $P < 0.01$, compared with PC3 conditioned medium-treated group plus anti-MCP-1-treated group; ‡, $P < 0.001$, compared with PC3 conditioned medium-treated group; #, $P < 0.001$, compared with either rhIL-8-treated or rhRANKL-treated groups.

264.7 cells because there is a possibility that adding serum may confound the results. Accordingly, critical experiments of using control medium (RPMI 1640 plus 1% FBS) as a basal control and incorporating the neutralizing antibodies for MCP-1, IL-8, and RANKL into these functional assays were included, although we still might not exclude a possibility of existing other functional factors, other than MCP-1, IL-8, and RANKL, in this culture condition.

In this study, we further observed that IL-8 mediated prostate cancer-induced osteoclast activity and bone resorption and this induction is independent of RANKL. In accordance with this finding, a recent report in breast cancer bone metastasis animal model showed that IL-8 promotes the tumor-induced osteolysis (48). Another study showed that IL-8 promotes androgen-independent growth and migration of LNCaP cells (49), suggesting that IL-8 may facilitate transition of prostate cancer to an androgen-independent state. Therefore, our results provided more evidence of the functional significance of IL-8 in the multiple steps

of the metastatic cascade, specifically in prostate cancer-induced bone lesions.

The majority of chemokines are secreted as small soluble molecules. However, CXC chemokine ligand CXCL-16 is expressed on the cell surface as membrane-bound molecule. The membrane-bound form of CXCL-16 can be cleaved by a disintegrin and metalloproteinase-10, and the cleaved soluble form of CXCL-16 induces migration of activated T cells. It has been reported that CXCL-16 is expressed not only by dendritic cells, macrophages, B cells, T cells, smooth muscle cells, and umbilical endothelial cells but also by colorectal adenocarcinomas (50). In this study, CXCL-16 was shown to be produced by prostate cancer cells. Interestingly, the level of CXCL-16 was greater in PC3 cells compared with LNCaP cells. The possible role of CXCL-16 and its receptor CXCR6 in pathobiology of different tumors, including prostate cancer, certainly needs to be further studied.

In summary, we have found that MCP-1 is produced by prostate cancer cells. It is a critical mediator for prostate

cancer-induced osteoclastogenesis and bone resorption. Together with other cytokines and chemokines, such as IL-8 and RANKL, MCP-1 may form a network that mediates the increased bone destruction associated with the tumor growth. This study may provide a novel therapeutic strategy to target chemokine, such as MCP-1, for treatment of prostate cancer skeletal metastasis.

Acknowledgments

Received 4/3/2006; revised 1/22/2007; accepted 2/7/2007.

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We thank Drs. Deborah L. Galson and Noriyoshi Kurihara for their helpful discussions and Judy L. Anderson for her technical assistance with collecting human bone marrow cells.

References

- Greenlee RT, Hill-Harmon MB, Murray T, et al. Cancer statistics, 2001. *CA Cancer J Clin* 2001;51:15-36.
- Shah RB, Mehra R, Chinnaiyan AM, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* 2004;64:9209-16.
- Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004;350:1655-64.
- Roudier MP, True LD, Higano CS, et al. Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. *Hum Pathol* 2003;34:646-53.
- Carlin BI, Andriole GL. The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. *Cancer* 2000;88:2989-94.
- Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002;2:584-93.
- Asahi H, Mizokami A, Maeda Y, et al. Bisphosphonate therapy for hormone refractory prostate cancer with bone metastasis. *J Urol* 2003;169:281-2.
- Heidenreich A, Hofmann R, Engelmann UH. The use of bisphosphonate for the palliative treatment of painful bone metastasis due to hormone refractory prostate cancer. *J Urol* 2001;165:136-40.
- Guise TA, Yin JJ, Thomas RJ, et al. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted *in vitro* and enhances breast cancer metastasis to bone *in vivo*. *Bone* 2002;30:670-6.
- Corey E, Brown LG, Quinn JE, et al. Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. *Clin Cancer Res* 2003;9:295-306.
- Miwa S, Mizokami A, Keller ET, et al. The bisphosphonate YM529 inhibits osteolytic and osteoblastic changes and CXCR-4-induced invasion in prostate cancer. *Cancer Res* 2005;65:8818-25.
- Green JR, Clezardin P. Mechanisms of bisphosphonate effects on osteoclasts, tumor cell growth, and metastasis. *Am J Clin Oncol* 2002;25:S3-9.
- Yoneda T, Michigami T, Yi B, et al. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. *Cancer* 2000;88:2979-88.
- Saad F, Gleason DM, Murray R, et al. Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. *J Natl Cancer Inst* 2004;96:879-82.
- Lipton A, Small E, Saad F, et al. The new bisphosphonate, Zometa (zoledronic acid), decreases skeletal complications in both osteolytic and osteoblastic lesions: a comparison to pamidronate. *Cancer Invest* 2002;20 Suppl 2:45-54.
- Saad F, Gleason DM, Murray R, et al. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. *J Natl Cancer Inst* 2002;94:1458-68.
- Brown JE, Cook RJ, Major P, et al. Bone turnover markers as predictors of skeletal complications in prostate cancer, lung cancer, and other solid tumors. *J Natl Cancer Inst* 2005;97:59-69.
- Coleman RE, Major P, Lipton A, et al. Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. *J Clin Oncol* 2005;23:4925-35.
- Zhang J, Dai J, Qi Y, et al. Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. *J Clin Invest* 2001;107:1235-44.
- Zhang J, Dai J, Yao Z, et al. Soluble receptor activator of nuclear factor κ B Fc diminishes prostate cancer progression in bone. *Cancer Res* 2003;63:7883-90.
- Corey E, Brown LG, Kiefer JA, et al. Osteoprotegerin in prostate cancer bone metastasis. *Cancer Res* 2005;65:1710-8.
- Yonou H, Kanomata N, Goya M, et al. Osteoprotegerin/osteoclastogenesis inhibitory factor decreases human prostate cancer burden in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice. *Cancer Res* 2003;63:2096-102.
- Kraj M, Owczarska K, Sokolowska U, et al. Correlation of osteoprotegerin and sRANKL concentrations in serum and bone marrow of multiple myeloma patients. *Arch Immunol Ther Exp (Warsz)* 2005;53:454-64.
- Zojer N, Brenner K, Beke D, et al. Bisphosphonate treatment does not affect serum levels of osteoprotegerin and RANKL in hypercalcemic cancer patients. *Anticancer Res* 2005;25:3607-12.
- Moore BB, Arenberg DA, Stoy K, et al. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol* 1999;154:1503-12.
- Taichman RS, Cooper C, Keller ET, et al. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* 2002;62:1832-7.
- Reiland J, Furcht LT, McCarthy JB. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. *Prostate* 1999;41:78-88.
- Lu Y, Cai Z, Galson DL, et al. Monocyte chemoattractant protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion. *Prostate* 2006;66:1311-8.
- Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-7.
- Vaday GG, Peehl DM, Kadam PA, et al. Expression of CCL5 (RANTES) and CCR5 in prostate cancer. *Prostate* 2006;66:124-34.
- Inoue K, Slaton JW, Eve BY, et al. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res* 2000;6:2104-19.
- Lu Y, Cai Z, Xiao G, et al. CCR2 expression correlates with prostate cancer progression. *J Cell Biochem. In press* 2007.
- Lisignoli G, Toneguzzi S, Grassi F, et al. Different chemokines are expressed in human arthritic bone biopsies: IFN- γ and IL-6 differentially modulate IL-8, MCP-1 and rantes production by arthritic osteoblasts. *Cytokine* 2002;20:231-8.
- Grassi F, Piacentini A, Cristino S, et al. Human osteoclasts express different CXC chemokines depending on cell culture substrate: molecular and immunocytochemical evidence of high levels of CXCL10 and CXCL12. *Histochem Cell Biol* 2003;120:391-400.
- Yu X, Huang Y, Collin-Osdoby P, et al. CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *J Bone Miner Res* 2004;19:2065-77.
- Loberg RD, Day LL, Harwood J, et al. CCL2 is a potent regulator of prostate cancer cell migration and proliferation. *Neoplasia* 2006;8:578-86.
- Manes S, Mira E, Colomer R, et al. CCR5 expression influences the progression of human breast cancer in a p53-dependent manner. *J Exp Med* 2003;198:1381-9.
- Wise GE, Frazier-Bowers S, D'Souza RN. Cellular, molecular, and genetic determinants of tooth eruption. *Crit Rev Oral Biol Med* 2002;13:323-34.
- Lebrecht A, Hefler L, Tempfer C, et al. Serum cytokine concentrations in patients with cervical cancer: interleukin-4, interferon- γ , and monocyte chemoattractant protein-1. *Gynecol Oncol* 2001;83:170-1.
- Hefler L, Tempfer C, Heinze G, et al. Monocyte chemoattractant protein-1 serum levels in ovarian cancer patients. *Br J Cancer* 1999;81:855-9.
- Vanderkerken K, Vande Broek I, Eizirik DL, et al. Monocyte chemoattractant protein-1 (MCP-1), secreted by bone marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. *Clin Exp Metastasis* 2002;19:87-90.
- Vande Broek I, Asosingh K, Vanderkerken K, et al. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemoattractant proteins MCP-1, -2 and -3. *Br J Cancer* 2003;88:855-62.
- Johrer K, Janke K, Krugmann J, et al. Transendothelial migration of myeloma cells is increased by tumor necrosis factor (TNF)- α via TNF receptor 2 and autocrine up-regulation of MCP-1. *Clin Cancer Res* 2004;10:1901-10.
- Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor- κ B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. *J Biol Chem* 2005;280:16163-9.
- Kim MS, Day CJ, Selinger CI, et al. MCP-1-induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NF- κ B ligand for bone resorption. *J Biol Chem* 2006;281:1274-85.
- Kurihara N, Reddy SV, Menaa C, et al. Osteoclasts expressing the measles virus nucleocapsid gene display a paucic phenotype. *J Clin Invest* 2000;105:607-14.
- Feeley BT, Liu NQ, Conduah AH, et al. Mixed metastatic lung cancer lesions in bone are inhibited by noggin overexpression and rank:Fc administration. *J Bone Miner Res* 2006;21:1571-80.
- Bendre MS, Margulies AG, Walser B, et al. Tumor-derived interleukin-8 stimulates osteolysis independent of the receptor activator of nuclear factor- κ B ligand pathway. *Cancer Res* 2005;65:11001-9.
- Lee LF, Louie MC, Desai SJ, et al. Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* 2004;23:2197-205.
- Wagsater D, Hugander A, Dimberg J. Expression of CXCL16 in human rectal cancer. *Int J Mol Med* 2004;14:65-9.