

Prostate Cancer Promotes CD11b Positive Cells to Differentiate Into Osteoclasts

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ABSTRACT

Bone is the preferred site of prostate cancer metastasis, contributing to the morbidity and mortality of this disease. A key step in the successful establishment of prostate cancer bone metastases is activation of osteoclasts with subsequent bone resorption causing the release of several growth factors from the bone matrix. CD11b⁺ cells in bone marrow are enriched for osteoclast precursors. Conditioned media from prostate cancer PC-3 cells induces CD11b⁺ cells from human peripheral blood to differentiate into functional osteoclasts with subsequent bone resorption. Analysis of PC-3 conditioned media revealed high amounts of IL-6 and IL-8. CD11b⁺ cells were cultured with M-CSF and RANKL, IL-6, IL-8, and CCL2, alone or in combination. All of these conditions induced osteoclast fusion, but cells cultured with M-CSF, IL-6, IL-8, and CCL2 were capable of limited bone resorption. Co-incubation with IL-6 and IL-8 and the RANK inhibitor, RANK-Fc, failed to inhibit osteoclast fusion and bone resorption, suggesting a potential RANKL-independent mechanism of functional osteoclast formation. This study demonstrates that functional osteoclasts can be derived from CD11b⁺ cells derived from human PBMCs. Prostate cancer cells secrete factors, including IL-6 and IL-8, that play an important role in osteoclast fusion by a RANKL-independent mechanism. *J. Cell. Biochem.* 106: 563–569, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: OSTEOCLAST; CD11B; PROSTATE CANCER; RANKL; IL-6; IL-8; CCL2

Prostate cancer is the most commonly diagnosed malignancy in US men [Walczak and Carducci, 2007]. Advanced prostate cancer is first treated through chemical or surgical castration because a large percentage of the cancer cells are androgen dependent. The large majority of patients, however, relapse within a few years of treatment because of the emergence of a castration resistant clone of cancer cells. Castration independent prostate cancer is an incurable disease with little effective therapy and there is a great need for novel therapeutic strategies that target the molecular basis of castration-independent, chemo-resistant prostate cancer [Isaacs, 2005]. Previous studies have demonstrated that 83–90% of men had evidence of bone metastasis when they died of prostate cancer [Loberg et al., 2005; Keller et al., 2007; Kingsley et al., 2007]. Bone is the preferred metastasis site of advanced prostate cancer, and in many patients, the only clinically evident site of metastasis at the time of death [Taichman et al., 2007]. There is a great need of novel

therapeutic strategies that target the molecular basis of advanced prostate cancer–bone interactions. A key step in the successful establishment of prostate cancer bone metastases is osteoclast formation and bone resorption followed by the release of several growth factors from the bone matrix that promotes tumor growth [Taichman et al., 2007]. The mechanisms by which prostate cancer cells promote osteoclast formation and bone resorption remain unclear.

Previous reports suggested that RANKL, Interleukin-6 (IL-6), Interleukin-8 (IL-8), and C-C chemokine ligand 2 (MCP-1, CCL2) mediate osteoclast formation from human bone marrow mononuclear cells (HBMCs) and human peripheral blood mononuclear cells [Kudo et al., 2003; Lu et al., 2007]. Furthermore, prostate cancer cells have been demonstrated to secrete these factors that promote osteoclastogenesis of HBMCs [Lu et al., 2007]. The purpose of this study was to further delineate the cell-types and mechanisms involved in prostate cancer induced osteoclastogenesis.

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Monocyte-macrophages are known to be the precursors of osteoclasts [Fujikawa et al., 1996; Quinn et al., 1998]. The cell surface antigen CD11/CD18 is known to be a heterodimeric structure, consisting of a common β subunit (CD18) and a distinct α subunit (CD11a, CD11b, CD11c). CD11b/CD18 is also known as Mac-1/C R3, and $\alpha_M\beta_2$ -integrin and is expressed in human monocytes, macrophages, neutrophils, and eosinophils [Murdoch et al., 2008]. Hayashi et al. [2008] demonstrated that CD11/CD18 is required for nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation.

RANKL induces osteoclast formation and activation in the presence of macrophage colony stimulating factor (M-CSF) through NFATc-1 and MMP9 activation [Takayanagi, 2005; Boyce and Xing, 2008; Del Fattore et al., 2008]. Furthermore, IL-6 and IL-8 are known to be highly expressed in prostate cancer metastatic sites [Lu et al., 2007]. CCL2 is a member of the CC beta chemokine family and is classically known for activating chemotaxis for monocyte/macrophages and other inflammatory cells via its receptor CCR2. The precise mechanism underlying the role of CCL2 on osteoclast formation remains to be explored.

In this study, human recombinant chemokines were used to identify which factors were important in osteoclast formation and bone resorption from human peripheral mononuclear CD11b+ cells.

MATERIALS AND METHODS

REAGENTS

Recombinant human M-CSF, IL-6, IL-8 and neutralizing antibodies for IL-6 and IL-8 and RANKFc were purchased from R&D systems (Minneapolis, MN). RANKL and rhMCP-1 were purchased from PEPROTECH, Inc. (Rocky Hill, NJ) and APOLLO (Alexandria, Sydney, Australia), respectively.

CELL CULTURE

Human prostate cancer PC-3 cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) under a humidified atmosphere of 5% CO₂ at 37°C. For preparing conditioned media to induce osteoclasts, PC-3 cells were cultured in 150 mm² tissue culture flasks to 90% confluency. Cells were then washed with PBS and cultured with 1% serum media for 48 h. The conditioned media was normalized for cell number (1.0×10^6 cells/ml). The conditioned media was centrifuged and supernatant was collected and stored in -20°C until use.

CYTOKINE ARRAY

Cytokine arrays were purchased from R&D systems and used per manufacturer's instructions. Briefly, after blocking, membranes were incubated overnight with 1 ml of the conditioned medium collected from PC-3 cells. Membranes were then washed and immunoreactive spots were detected with chemiluminescence.

ELISA ASSAYS

ELISA analysis for IL-6, IL-8, and CCL2 (R&D systems) were performed following the manufacturer's instructions. ELISA analysis kit for RANKL was purchased from Biomedica Medizinprodukte GmbH & Co., KG (Austria).

QUANTITATIVE RT-PCR

RNA was extracted according to manufacturer's instructions by using RNAeasy Micro Kit (Qiagen, Inc., USA). Briefly, cells were lysed directly in the wells and homogenized by passing through a needle several times. RNA was precipitated with 70% ethanol and applied to RNAeasy MiniElute columns. Columns were washed with buffers and 80% ethanol. Total RNA was eluted from the columns with RNAase-free distilled water. One microgram of total RNA of each sample was reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ). Primers (NFATc-1, MMP9) were purchased from Applied Biosystems. The housekeeping transcript, GAPDH, was used as a control for standardization. PCR was carried out with TaqMan Universal PCR Master Mix (Applied Biosystems).

OSTEOCLAST FORMATION ASSAYS

Human peripheral mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation from healthy male volunteers. Cells of the monocyte fraction (CD11b+) were isolated from the PBMCs by MACS CD11b Micro Beads (Miltenyi Biotec) as recommended by the manufacturer. CD11b cells (5×10^5) were seeded on coverslips (Nalge Nunc International, Rochester, NY) in MEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and M-CSF (25 ng/ml) in 24 wells. Twenty-four hours after seeding, media was changed to fresh media and incubated in the presence of M-CSF (25 ng/ml), RANKL (30 ng/ml), IL-6 (5 ng/ml), IL-8 (30 ng/ml), RANKFc (1 μ g/ml) or combination of PC-3 conditioned medium and MEM (1:1) and anti-IL-6 (0.6 μ g/ml) and anti-IL-8 antibody (1.8 μ g/ml). Cells were cultured for 14 days and then fixed and stained with a TRAP staining kit (Sigma). Coverslips were also fixed in methanol and stained immunocytochemically using an antibody against vitronectin receptor (Abcam, Cambridge, MA). TRAP+ cells that resembled RANKL-induced multinuclear cells were considered to be osteoclasts and counted under the microscope. Stained cell images were obtained using a BX51 microscope (Olympus, Japan).

FLOW CYTOMETRY

CD11b+ cells were stained with anti-human CD11b-FITC (PharMingen) antibodies and their matching isotype controls according to the manufacturer's protocols. The cells were incubated with antibodies for 30 min at 4°C and washed with PBS. The samples were analyzed by using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Bedford, MA).

BONE RESORPTION ASSAYS

CD11b+ cells (5×10^5) were seeded on the resorbable artificial bone film-coated disks (Becton Dickinson) and incubated as described above. After 3 weeks of culture, cells were removed by sodium 5% hypochlorite solution and bone resorption area images were obtained by a BX51 microscope. The mean area of resorption from five randomly selected fields was analyzed with Adobe Photoshop software (Adobe).

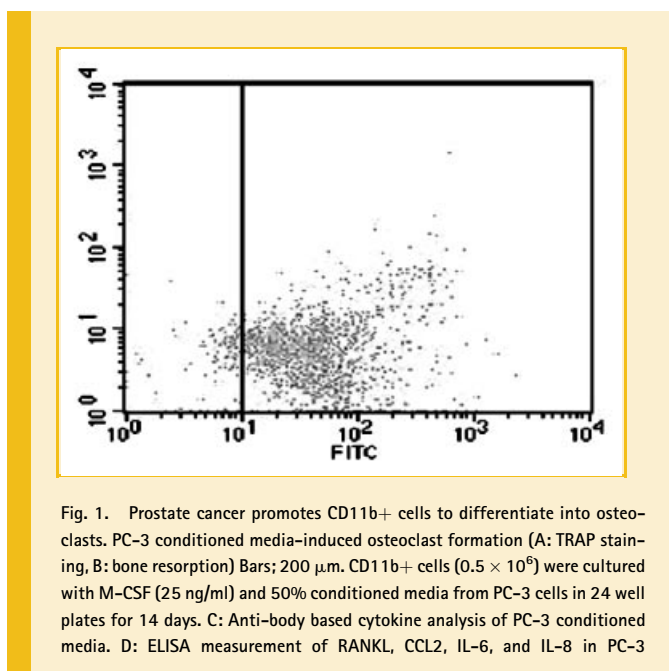
RESULTS

IDENTIFICATION OF CYTOKINES AND RANKL SECRETION BY PROSTATE CANCER, PC-3 CELLS

The fact that conditioned medium of several prostate cancer cell lines induces osteoclast from HBMCs or the general PBMC population is well established. Prior to the osteoclast formation experiment, we investigated the population of cells isolated by CD11b beads. More than 90% of isolated cells were CD11b positive (Fig. 1). Osteoclasts were induced from the CD11b+ fraction of PBMCs by PC-3 conditioned media (Fig. 2A). These osteoclasts were considered to be functional as evidenced by bone resorption *in vitro* (Fig. 2B). Identification of factors from prostate cancer cells is essential to explain the mechanism of prostate cancer induced bone resorption. A limited cytokine antibody array demonstrated that PC-3 cells secrete high amounts of GM-CSF, GRO, IL-6, IL-8, IGF-BP2, TGF- β 2, TIMP-1, and TIMP-2 but very little CCL2 (Fig. 2C). Of these factors, IL-6, IL-8, and CCL2 are reported to be important for osteoclast formation. To quantify these factors in PC-3 conditioned medium, the amount of IL-6, IL-8, CCL2, and RANKL by ELISA was measured. IL-6 and IL-8 were highly secreted in PC-3 conditioned medium; however, the level of RANKL (<5 pg/ml) and CCL2 (undetectable) were low (Fig. 2D).

IL-6 AND IL-8 PROMOTE CD11b POSITIVE CELLS TO OSTEOCLAST LIKE CELL BUT NOT BONE RESORPTION

At day 14 after incubation in the presence of each factors plus M-CSF, osteoclast fusion was detected by TRAP staining and vitronectin. As shown in Figure 3A–D; IL-6, IL-8 alone and in combination induced TRAP-positive multinuclear cells from CD11b+ cells. The number of IL-6 and IL-8 induced osteoclast-like cells were similar and approximately half of the number of those seen in M-CSF + RANKL stimulated conditions or in the IL-6 + IL-



8 + M-CSF stimulated conditions. Co-incubation with M-CSF, IL-6, IL-8, and RANKFc, a biologic inhibitor of RANKL, failed in inhibiting osteoclast fusion and bone resorption. This result implies that IL-6 and IL-8 have a potential RANKL independent mechanism of osteoclast fusion. M-CSF and CCL2 induced CD11b+ derived osteoclast fusion; however, the number of nuclei in CCL2 induced multinuclear cells was fewer compared to RANKL, IL-6, and IL-8 induced multinuclear cells (Fig. 3A–D).

To confirm the ability of CD11b+ derived osteoclast-like cells were functional and capable of bone resorption, CD11b+ cells were cultured on artificial bone discs. As expected, the soluble RANKL induced strongest bone resorption (Fig. 3C–E). CD11b+ cells cultured with other factors (IL-6, IL-8, CCL2, and their combination), demonstrated limited or no resorption as compared to RANKL stimulation (Fig. 3C–E). Interestingly, osteoclast-like cells that were induced by co-incubation with IL-6 and IL-8 made significantly less pit formation, regardless of the same amount of TRAP positive multinuclear cells compared with RANKL stimulation, perhaps suggesting that CD11b+ require key factors like soluble RANKL for of bone resorption activity after formation of TRAP positive multinuclear, osteoclast-like cells (Fig. 3C,D).

PC-3 CONDITIONED MEDIA PROMOTES CD11b POSITIVE CELLS TO DIFFERENTIATE TO OSTEOCLASTS

PC-3 conditioned medium has been shown to promote HBMCs to osteoclasts [Lu et al., 2007]. CD11b+ cells from mice are known precursors of osteoclasts [Li et al., 2004]. To investigate the effect of PC-3 conditioned media on osteoclast formation, human CD11b+ cells were cultured with conditioned media. PC-3 conditioned media promoted CD11b+ cells to fuse to form TRAP positive multinuclear cells and the cells had the capability of bone resorption (Fig. 4). The results from the cytokine array and osteoclast formation assay indicated that RANKL, IL-6, and IL-8 may play important roles in CD11b+ osteoclast fusion and bone resorption. To determine the role of RANKL, IL-6, and IL-8 in PC-3 conditioned media induced CD11b+ osteoclast formation, CD11b+ cells were cultured with conditioned medium in the presence of RANK-Fc and neutralizing antibodies for IL-6 and IL-8. RANKFc and the combination of anti-IL-6 and anti-IL-8 failed to inhibit conditioned media induced osteoclast formation (Fig. 4). To verify that prostate cancer cells induced osteoclast differentiated from CD11b+ cells, bone resorption area was observed (Fig. 4A). The resorption was qualitatively different than the resorption demonstrated by stimulation with M-CSF + RANKL (Fig. 3A). RANKFc had no effect on conditioned media induced osteoclast resorption while combination treatment of anti-IL-6 and anti-IL-8 antibodies slightly reduced PC-3 conditioned media induced bone resorption. These results imply that IL-6 and IL-8 may have a role in CD11b+ osteoclastogenesis.

EXPRESSION OF NFATC-1 AND MMP-9 BY EACH SIMULATION

NFATc-1 and MMP-9 are known to be activated during osteoclast fusion and function [Takayanagi, 2005; Kim et al., 2006; Boyce and Xing, 2008; Del Fattore et al., 2008]. Osteoclast-related gene expression after 21 days incubation of CD11b+ cells with each factor was examined by quantitative RT-PCR. Both NFAT-c-1 and MMP-9 expression were increased by RANKL compared to M-CSF

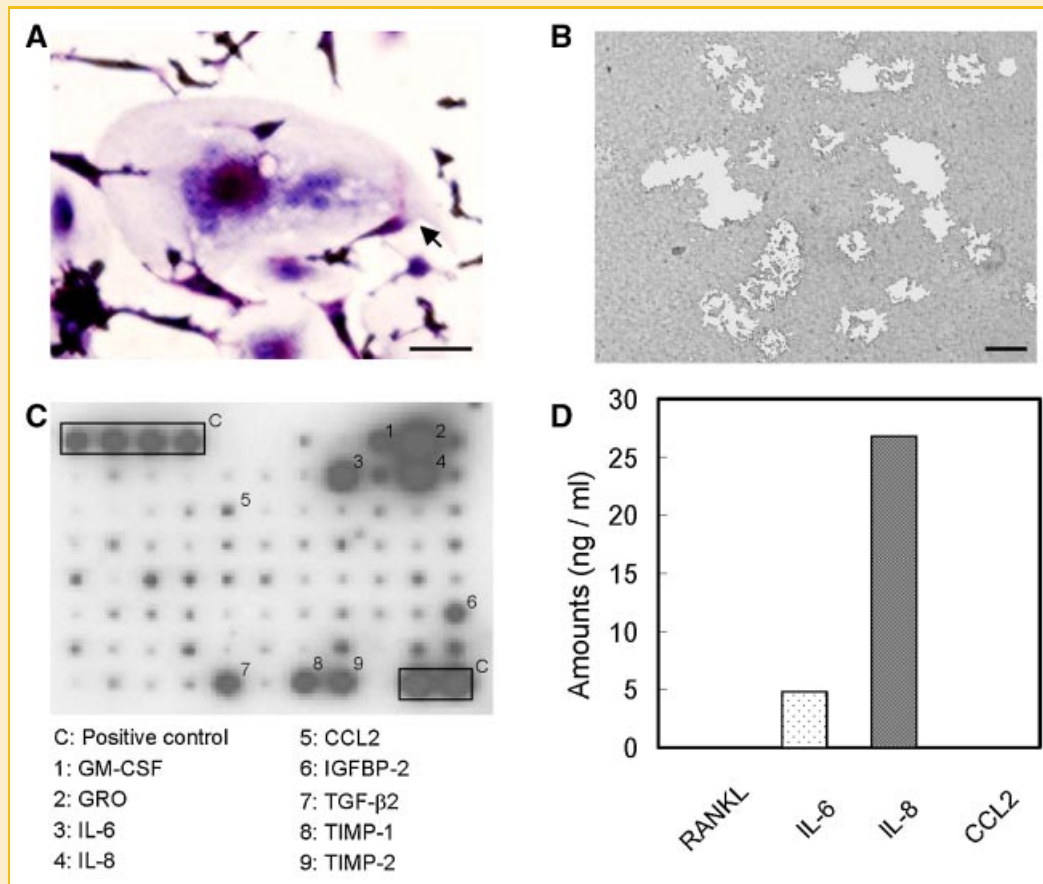


Fig. 2. Cell population analysis of isolated cells from PBMCs. Eighty to 95% of isolated cells using CD11b beads were CD11b positive. Isolated cells were incubated anti-human CD11b antibody that was conjugated with FITC and analyzed by flow cytometry.

alone (Fig. 5). Expression levels induced by other factors did not show significant difference. Interestingly, expression levels of NFATc-1 decreased to 63% as compared to control (MCSF) conditioned media.

DISCUSSION

The results of the present study demonstrated that functional osteoclasts can be induced to be differentiated from human peripheral blood derived CD11b+ cells. Recently, it is reported that reduction of activity of CD11b by siRNA and neutralizing antibody inhibited CD11b+ mononuclear cells osteoclastogenesis by M-CSF and RANKL [Hayashi et al., 2008]. Hence, CD11b+ is thought to be a marker of osteoclast precursors. Detailed studies of prostate cancer induced CD11b positive cell-osteoclastogenesis have not been reported. Results demonstrated that RANKL, IL-6, and IL-8 promote osteoclast fusion from CD11b+ cells. RANKL has been previously demonstrated to promote osteoclastogenesis through the up-regulation of NFATc-1 in HMBCs and PBMCs [Takayanagi, 2005; Lu et al., 2007; Boyce and Xing, 2008; Del Fattore et al., 2008]. This

study extends these results and demonstrated that RANKL plays an important role in both osteoclast fusion and bone resorption of human CD11b+ cells with up-regulation of NFATc-1.

IL-6 and IL-8 are known as key factors in HBM and PBMC osteoclastogenesis [Lu et al., 2007]. Kudo et al. [2003] had reported that IL-6 induced osteoclast formation by a RANKL independent mechanism. In this study, IL-6, IL-8, and CCL2 did not induce RANKL expression from CD11b+ cells (data not shown). RANKL expression is not induced by IL-6 in human bone marrow and osteoblasts [Hofbauer et al., 1999; Han et al., 2001]. The findings demonstrate that IL-6 effects osteoclast fusion but not bone resorption, suggesting other factor(s) are necessary for bone resorption. IL-8 is also multifunctional cytokine correlating with tumor growth, chemotactic activities and osteoclastogenesis. Bendre et al. [2005] had reported IL-8 secreted by tumor cells induce osteolysis independent of the RANKL pathway. Osteoclasts without bone resorption activity has been reported [Kim et al., 2006]. TRAP-positive multinuclear cells induced by IL-6 and IL-8 with negative activity for bone resorption may represent a precursor stage of functional osteoclasts. The results of no induced RANKL expression by IL-6 and IL-8 as well as co-culture with IL-6 and IL-8 and RANKFc may indicate that osteoclast fusion occurs by a RANKL-

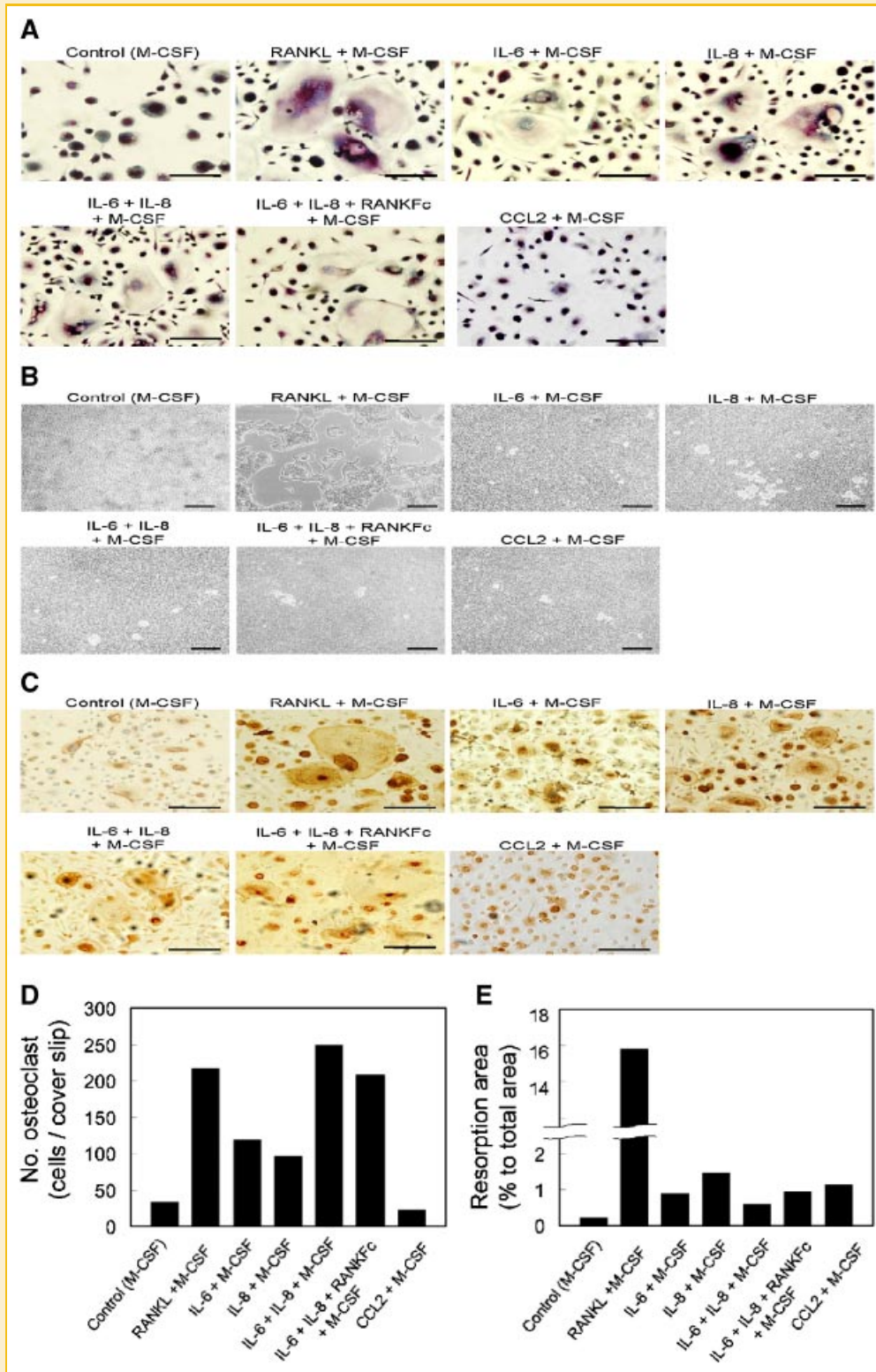


Fig. 3. Representative micrographs of TRAP positive (A) and vitronectin (B) multinuclear cells that are induced by each stimulator. CD11b⁺ cells (0.5×10^6) were all cultured with M-CSF (25 ng/ml) and IL-6 (5 ng/ml), IL-8 (30 ng/ml), RANKFc (1 μ g/ml) and CCL2 (100 ng/ml) or 50% conditioned media from PC-3 cells in 24 well plates for 14 days. C: Representative micrographs of artificial bone resorption by osteoclasts. Twenty-one days after incubation with each stimulator, cells were removed and bone resorption area was visualized. Bars; 100 μ m. Graphical representation of number of osteoclast-like multinuclear TRAP positive cells (C) and bone resorption area (D). After TRAP staining multi nucleated and expanded cells per slide were counted. The mean area of bone resorption from five randomly selected fields was quantified.

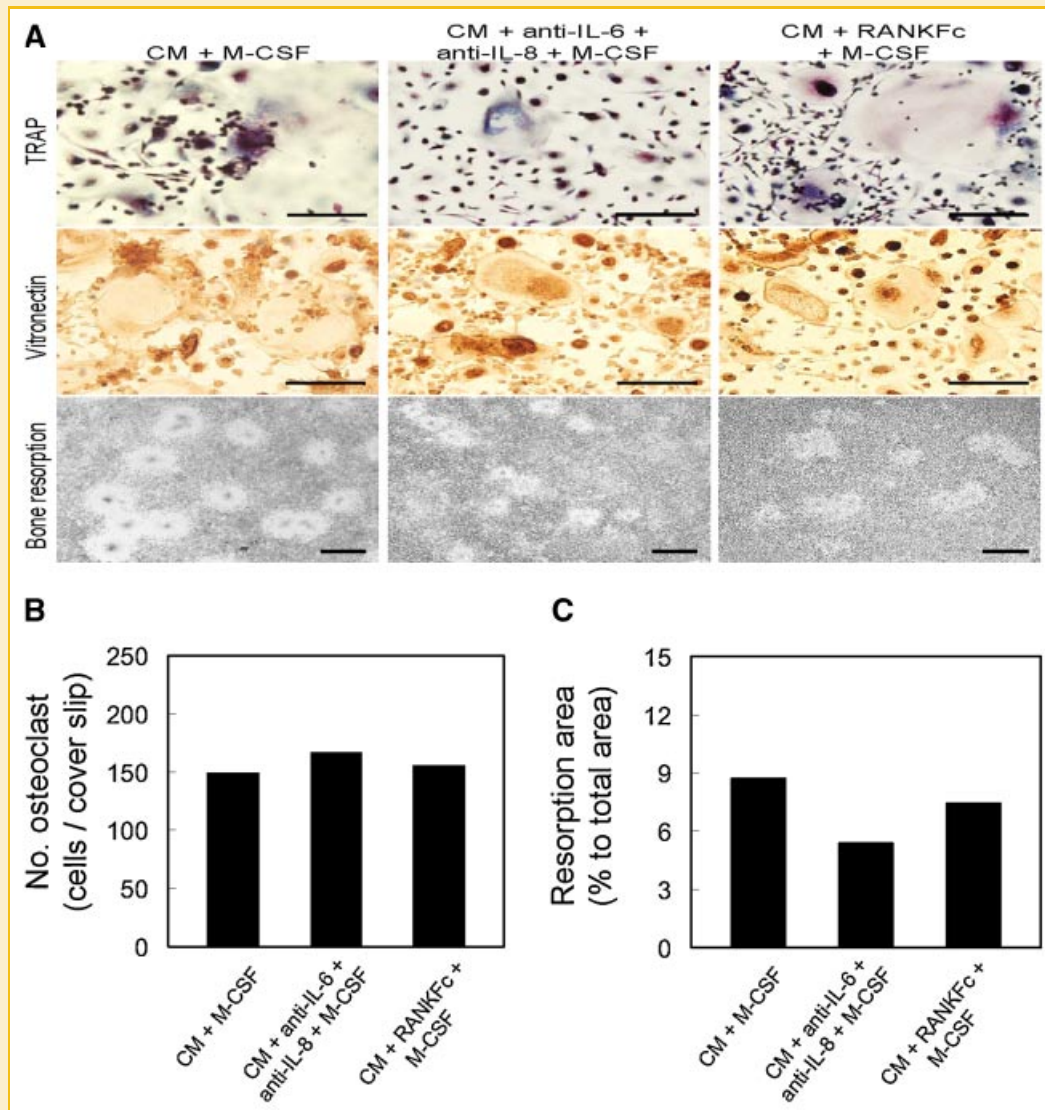


Fig. 4. A: Representative micrographs of TRAP positive (upper panels), vitronectin positive (middle panels) multinuclear cells, and bone resorption (lower panel). CD11b+ cells (0.5×10^6) were all cultured with M-CSF (25 ng/ml) and neutralizing antibodies for IL-6 and IL-8 and RANKFc with 50% conditioned media from PC-3 cells. Graphical representation of number of osteoclast-like multinuclear TRAP positive cells (B) and bone resorption area (C).

independent mechanism in CD11b+ cells. Direct osteoclast differentiation by IL-6 through NFATc-1 activation has been previously reported [Del Fattore et al., 2008]. Up-regulation of NFATc-1 and bone resorption by IL-6 were not observed in this study. This may be due to the different concentrations of IL-6 used in the different studies.

CCL2 is a member of the CC beta chemokine family and is classically known for activating chemotaxis for monocyte/macrophages and other inflammatory cells. CCL2 has been demonstrated to promote osteoclast fusion and function; however, the exact cell type that is induced to differentiate into functional osteoclasts from PBMCs remains unclear [Kim et al., 2005, 2006; Li et al., 2007; Lu

et al., 2007]. These results suggest CCL2 had limited effect on both osteoclast fusion and bone resorption of CD11b+ cells in vitro.

The studies conducted here confirm that conditioned medium from prostate cancer induces osteoclast formation [Lu et al., 2007]. The results demonstrate that osteoclast formation occurs via a RANKL independent mechanism. Prostate cancer induced osteoclastogenesis, however, also appears to be independent of IL-6 and IL-8. Further experiments are needed to address the role of other factors produced by prostate cancer cells that effect osteoclast formation. For example, it has been demonstrated that PTHrP produced by prostate cancer cells can facilitate osteoclastogenesis and osteoblastogenesis [Li et al., 2007; Liao et al., 2008]. This study

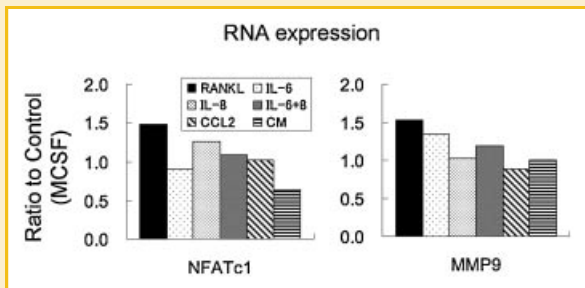


Fig. 5. Graphical representation of molecular characterization of osteoclastogenesis. Twenty-one days after incubation, mRNA was extracted and amplified. cDNA was used for quantitative real time PCR using primers of NFATc1 and MMP9.

demonstrates that functional osteoclasts can be derived from CD11b+ cells derived from human PBMCs. Prostate cancer cells secrete factors, including IL-6 and IL-8, that play an important role in osteoclast fusion by a RANKL-independent mechanism.

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