Tumor-Induced Pressure in the Bone Microenvironment Causes Osteocytes to Promote the Growth of Prostate Cancer Bone Metastases

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Abstract

Cross-talk between tumor cells and their microenvironment is critical for malignant progression. Cross-talk mediators, including soluble factors and direct cell contact, have been identified, but roles for the interaction of physical forces between tumor cells and the bone microenvironment have not been described. Here, we report preclinical evidence that tumor-generated pressure acts to modify the bone microenvironment to promote the growth of prostate cancer bone metastases. Tumors growing in mouse tibiae increased intraosseous pressure. Application of pressure to osteocytes, the main mechanotransducing cells in bone, induced prostate cancer growth and invasion. Mechanistic investigations revealed that this process was mediated in part by upregulation of CCL5 and matrix metalloproteinases in osteocytes. Our results defined the critical contribution of physical forces to tumor cell growth in the tumor microenvironment, and they identified osteocytes as a critical mediator in the bone metastatic niche. Cancer Res; 75(11): 1–8. ©2015 AACR.

Introduction

Prostate cancer is the second most common tumor of men in the United States with bone being the primary location of metastases. There are few therapeutic options for patients with bone metastases. Bone is a relatively inelastic tissue, with tumor growth restricted by mineralized extracellular matrix (ECM). Furthermore, prostate cancer bone metastases are typically osteoblastic, promoting poorly organized bone formation. The lack of potential space present in bone, and continual expansion of tumor, creates a paradox in which bone must be degraded or physical pressure within the tumor will increase resultant of continuous cellular division. Therefore, we hypothesized that tumor growth in bone would increase pressure within the bone microenvironment.

Bone is comprised of osteoblasts, cells responsible for bone production, osteoclasts, cells responsible for bone resorption, and osteocytes, the cells that coordinate the responses of osteoblasts and osteoclasts. Osteocytes are terminally differentiated osteoblast lineage cells embedded in mineralized bone (1). The primary role of osteocytes is maintaining bone homeostasis by translating physical forces into biochemical signals, a process called mechanotransduction (2). Alteration of physical forces in bone modulates osteocytes biochemical responses regulating bone remodeling. Similarly, tumor growth in bone should induce physical forces that effect osteocytes. The role of osteocytes has yet to be established in tumor biology even though osteocytes are the most abundant cell in bone (2). Accordingly, the goal of this study was to determine whether tumor growth in bone induces physical forces and determine whether these forces educate osteocytes to promote tumor progression.

Materials and Methods

Cell lines

The MLO-Y4 murine osteocytes cell line (courtesy of Dr. Lynda Bonewald; University of Missouri, Kansas City, MO) was maintained as previously described (3). DU145, LNCaP, and PC3 (human prostate cancer; BPH-1 (benign prostatic hyperplasia-1); ACE1 (canine prostate cancer; courtesy of Dr. Thomas Rosol, The Ohio State University, Columbus, OH); and H441 and A549 (human lung carcinoma) cell lines were maintained in RPMI with 10% FBS and 1% penicillin/streptomycin. MDA-MB-231 and MCF7 were cultured in DMEM-based media. All cells were serially passaged by trypsinization and maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Animals

All animal studies were performed in an American Association for Laboratory Animal Care-approved facility, with approval of the University Committee on Use and Care of Animals. Male SCID mice 8 to 10 weeks of age were used for all experiments. Four mice per group were used for all in vivo experiments.

In vivo pressure measurement in tumor-bearing mice

Mice were anesthetized with isoflurane and 1 × 10⁶ tumor cells were injected in the intramedullary cavity of the tibia. Sham-treated mice were injected with an equivalent volume of PBS. 24 hours after tumor challenge, implantation of a wireless pressure transmitter was performed similar to previous description (4). Briefly, a DSI PhysioTel PA-C10 pressure transmitter (Data

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Sciences International) was implanted in the abdomen and catheter inserted into the intramedullary cavity of the tibia where tumor was previously implanted (Fig. 1A).

Real-time measurements of intramedullary pressure (ImP) were collected and analyzed using Dataquest A.R.T. System software (DSI international). Analysis of measurements was initiated 7 days after catheter implant to allow for inflammation to subside. Measurements were averaged for each mouse and reported. Radiographs were obtained weekly using a Faxitron MX-20 at ×4 magnification.

**Conditioned media**

Conditioned medium (CM) was prepared by incubating near confluent MLO-Y4 cells on collagen-coated plates for 24 hours with RPMI media containing 0.1% FBS and 1 × penicillin/streptomycin. Supernatant was removed and centrifuged at 500 × g for 5 minutes. MLO-Y4 cells were counted and CM was normalized by adjusting to a final concentration of 2 × 10⁷ cells per 30 mL. BCA assay was used to measure CM protein concentration.

**Application of hydrostatic pressure in vitro**

Application of hydrostatic pressure to cells in vitro was accomplished as previously described (5–7) using modifications. Briefly, cells were seeded into Opticell (Thermo Fisher Scientific) cassettes and allowed to adhere overnight. Pressure was created by connecting the Opticell to an IV bag filled with cell culture media using arterial line tubing. Opticells were clamped between perforated stainless steel sheets secured by binder clips. Hydrostatic pressure was modulated by adjusting the height of the IV bag. Pressure was determined using the following formula: $P = \rho \cdot h$; where $P$, hydrostatic pressure; $\rho$, fluid density; and $h$, the height of the IV bag in relation to the Opticell.

Viability was assessed by pressurizing cells for 24 hours and counting live cells by Trypan blue staining. CM was isolated from MLO-Y4 cells by pressurizing cells for 24 hours in RPMI containing 0.1% FBS at 0, 20, and 40 mmHg. CM was prepared as described above. BCA assay was used to quantify protein concentration of cells; no significant ($P > 0.05$) differences were observed after normalization.

**Viability**

Prostate cancer cells were plated in 96-well plates at a density of 3,000 cells per well and incubated overnight. Media were aspirated and replaced with CM or control medium (RPMI containing...
Migration and invasion

Screening of compounds identified in the reagents section was performed utilizing Culturex Migration and low BME Invasion assays (Trevigen). Validation of these results were performed using commercially available Boyden chamber assays with (invasion) and without (migration) Matrigel according to the manufacturer’s directions (Corning). Prostate cancer cells (ACE1 = 1 × 10⁵; DU145 = 5 × 10⁵; LNCaP = 5 × 10⁵; and PC3 = 1 × 10⁴) were plated in the top well in 0.1% FBS RPMI. In the bottom well, control media or CM was added as a chemoattractant. Blocking or neutralizing compounds were incubated with CM or control media for 1 hour at room temperature before plating. To recapitulate the protein concentration of CCL5 and MMP2 found in 40 mmHg CM, recombinant respective protein was added to 0 mmHg before plating as a chemoattractant. Cell number from five random ×400 fields were counted, averaged for each membrane, and reported as the mean ± SEM.

Cytokine array

Identification of MLO-Y4 secreted mediators was achieved using a commercially available mouse cytokine array (R&D Systems) according to the manufacturer’s instructions. Protein concentration was normalized before performing the assay. Protein density of each spot was measured using Photoshop CS3 extended (Adobe Systems Inc.). Spots were averaged, background subtracted, and average values reported for each cytokine.

Reagents

The following reagents were used in screening effector molecules identified in the cytokine array: Rat anti-mouse CCL5 (R&D Systems), rat IgG2a isotype control (R&D Systems), recombinant mouse CCL5 (R&D Systems), recombinant MMP2 (R&D Systems), CCR2 antagonist (Calbiochem), rat anti-mouse CXCL10 (R&D Systems), cFMS Receptor III inhibitor (Calbiochem), and Batimastat (Calbiochem).

Real-time qPCR

RNA was extracted using TRIzol (Invitrogen), purified by the RNeasy Mini-Kit (Qiagen), and reverse transcribed using the SuperScript III Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR was performed in triplicate using SYBR Green qPCR Master Mix (Qiagen) in a 10 μL reaction volume on a Roche LightCycler 480 (Roche). Primers were purchased from SABiosciences (Qiagen). Measurements from triplicate Cₚ values were normalized to GAPDH, averaged, and reported.

ELISA

Commercially available ELISA for murine CCL5/RANTES (R&D Systems) and MMP2 (R&D Systems) were used according to the manufacturer’s instructions.

Zymography

Zymography was performed using CM from osteocytes and prostate cancer cell lines normalized by total protein as measured by BCA assay. Ten percent gelatin zymogram gels (Life Technologies) were used according to the manufacturer’s directions and counterstained using SimplyBlue SafeStain (Life Technologies). Washed gels were scanned and densitometry performed using ImageJ (8).

Statistical analysis

All experiments were repeated independently two to three times. Statistical analysis was performed using Prism 5 (GraphPad Software). In vivo pressure experiments were analyzed using two-way repeated measures ANOVA with a Bonferroni posttest. Multiple groups were compared using one-way ANOVA with Bonferroni posttest. Data comprising only two groups were analyzed by a t test. Zymography and neutralization or pressure CM with anti-CCL5 antibody were compared using two-way ANOVA with the Bonferroni posttest. For all statistical analyses, a cutoff of P < 0.05 was used to assess statistical significance.

Results

Tumor growth in bone increases ImP

The primary site of prostate cancer metastasis is bone, in which tumor growth may induce pressure due to the lack of expansible space due to mineralized ECM. Therefore, we sought to determine whether prostate cancer growing in bone significantly altered ImP. Prostate cancer cells were injected into the tibiae of SCID mice, which were subsequently cannulated with a wireless pressure transmitter (Fig. 1A) allowing for real-time monitoring of ImP. DU145 and ACE1 tumor growth significantly increased ImP, peaking approximately 15 to 16 days posttumor challenge (Fig. 1B and D). Approximately 21 days posttumor injection, ImP declined; albeit was still increased compared with baseline. Radiographic loss of cortical bone was observed at the same time period of declining ImP (Fig. 1C and E). This observation suggests that cortical bone loss may mitigate tumor-induced pressure. Challenge with the highly osteolytic prostate cancer cell line PC3 did not induce a significant increase in ImP (Supplementary Fig. S1). From these data, it was determined that mice have a basal ImP of 19.12 ± 8.18 mmHg; similar to previous reports in mice without tumors (4). An average peak ImP of 38.51 ± 8.63 mmHg was observed in ACE1 and DU145 across all experiments. Investigation of the role of tumor-generated pressures on prostate cancer using the values identified from the in vivo studies was then investigated in vitro.

Effects of increased ImP on osteocytes and prostate cancer

To investigate the impact of increased pressure on osteocytes and prostate cancer viability in vitro, we applied hydrostatic pressure, based upon our in vivo findings, to cultures of osteocytes and prostate cancer cells. Pressure increased MLO-Y4 osteocyte viability (Fig. 2A). Even though osteocytes are not thought to proliferate in vivo, this observation speaks to the ability of osteocytes to survive stressful conditions (1, 2). In contrast, pressure either had no effect (DU145) or decreased prostate cancer (LNCaP and PC3) cell viability (Fig. 2A).

Osteocytes coordinate bone remodeling by releasing biochemical mediators. Thus, CM was collected from osteocytes and found to induce prostate cancer viability (Supplementary Fig. S2). Osteocyte CM also increased cell viability of breast and lung cancer cell lines, suggesting that the effect is not unique to prostate cancer (Supplementary Fig. S3). Osteocyte CM induced prostate cancer migration and invasion when used as a chemoattractant.
To determine whether increased pressure altered the protumorigenic phenotype conferred by osteocytes, CM was collected from MLO-Y4 cells pressurized at 0 (normal culture conditions), 20 (physiologic baseline ImP), and 40 mmHg (peak pressure observed). CM from pressurized cells dose dependently increased prostate cancer viability, migration, and invasion (Fig. 2B). These data provide evidence that tumor-induced pressure promotes secretion of factors associated with prostate cancer survival and motility in response to physical forces induced by tumor growth.

Identification and neutralization of CM-derived CCL5

To identify candidate mediators of prostate cancer protumorigenic activity, the cytokine profile of 0 mmHg osteocyte CM was compared with CM produced at 40 mmHg pressure. Increased pressure induced numerous chemokines and cytokines (Fig. 3A; detailed in Supplementary Table S1). Several of the factors with altered expression were screened for function on prostate cancer using neutralizing antibodies and receptor antagonists to inhibit prostate cancer viability, migration, and invasion. Inhibition of CCL2, MCSF, and CXCL10 was found to have no effect (data not shown). CCL5-neutralizing antibody inhibited osteocyte CM-induced prostate cancer migration and invasion. Therefore, CCL5 mRNA was measured by qPCR (Fig. 3B), and ELISA to measure CCL5 protein concentration in CM (Fig. 3C and Supplementary Fig. S4A). Pressure induced a dose-dependent increase in CCL5 production from MLO-Y4 cells.

CCL5 is a chemoattractant for T cells that has been implicated in promoting prostate cancer migration and viability (9, 10). To further characterize the tumor-promoting role of CCL5, prostate cancer cells were incubated with recombinant CCL5 (rCCL5). Although rCCL5 promoted DU145 migration and invasion, it promoted only migration, and not invasion of LNCaP and PC3 cells (Fig. 3D). Because these cells lines were tested under similar conditions.
Figure 3. Identification of CCL5/RANTES as an osteocyte-secreted mediator of enhanced prostate cancer invasion. A, CM prepared from MLO-Y4 cells pressurized at 0 and 40 mmHg for 24 hours were compared using a cytokine screening antibody array and subjected to densitometry. Results are shown as change in optical density of the cells subjected to 40 mmHg relative to the 0 mmHg. B, real-time PCR of CCL5 from pressurized MLO-Y4 cells was performed and normalized to GAPDH. C, CCL5 secreted from osteocytes, normalized by protein concentration, increases as the level of hydrostatic pressure increases as measured by ELISA. D, recombinant murine CCL5 (rCCL5; 10 ng/mL) was used as a chemoattractant for prostate cancer migration and invasion. Neutralization of rCCL5 was accomplished by incubating with anti-CCL5-neutralizing antibody (1 mg/mL) but not with isotype control antibody (1 mg/mL). rCCL5 neutralization inhibited migration across all cell lines. However, invasion of LNCaP and PC3 was not altered by the neutralization of rCCL5. E, CM derived from MLO-Y4 cells pressurized at 0, 20, and 40 mmHg was incubated with CCL5-neutralizing antibody before being used as a chemoattractant in migration and invasion assays. Neutralization of CM-derived CCL5 led to significant inhibition of prostate cancer migration and invasion despite increasing pressure. Secretion of CCL5 by MLO-Y4 was induced by tumor-generated pressure, leading to subsequent increases in tumor cell invasiveness. One-way ANOVA with Bonferroni posttest (C and D) or two-way ANOVA (E) was performed; bars, significant differences where $P < 0.05$. 

Pressure Promotes Prostate Bone Metastases

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conditions it is likely there may be cell specificity to rCCL5; however, it is possible that even with the replicate experiments, there was insufficient statistical power to detect the invasion in LNCaP and PC3 cells. Neutralization of rCCL5 using an anti-CCL5 antibody negated the promigratory effects conferred by rCCL5 (Fig. 3D). The observation that invasion was not affected by increased CCL5 alone suggests that osteocyte-produced CCL5 is insufficient to promote invasion by itself.

To investigate whether CCL5 in the context of osteocyte CM promotes prostate cancer migration and invasion, we evaluated neutralization of osteocyte-derived CCL5. CCL5 neutralization inhibited migration in nonpressurized CM (Supplementary Fig. S4B). Furthermore, neutralization of CM derived from pressurized osteocytes inhibited migration and invasion at all pressures examined (Fig. 3E). However, inhibition of CCL5 did not completely block the pressure-induced migration and invasion, indicating that other factors contribute to this activity. To determine whether CCL5 was sufficient to promote the pressure-induced migration and invasion, we recapitulated the concentration of CCL5 found in 40 mmHg CM (3 ng/mL) by adding rCCL5 to 0 mmHg CM. The addition of CCL5 promoted migration and invasion to levels similar to 40 mmHg CM (Supplementary Fig. S4C). These invasion results contrast with the studies using rCCL5 alone (Fig. 3D), suggesting that other factors produced by osteocytes contribute to promoting invasion of prostate cancer in addition to CCL5.

Neutralization of CM-derived CCL5 and MMP mitigates prostate cancer proinvasive phenotype

Matrix metalloproteinases (MMP) are known to be expressed by osteocytes to modulate ECM during bone-resorptive processes (11). To determine whether osteocytes modulate prostate cancer invasion through MMPs, CM from MLO-Y4 and prostate cancer cell lines was isolated and compared using zymography. MLO-Y4 CM produced up to 67× more MMP2 than prostate cancer cell lines (Supplementary Fig. S5A). MMP9 expression was elevated to a lesser degree. Pressurization of MLO-Y4 increased secretion of MMP2 and MMP9 as measured by zymography (BPH-1 was used as a positive control; Fig. 4A). Quantification of latent versus active MMPs showed increased MMP2 activity associated with increased pressure (Fig. 4A and Supplementary Fig. S5B). The effect of pressure on MMP2 and MMP9 mRNA expression in osteocytes was evaluated demonstrating pressure induced a dose-dependent increase of both MMP2 and MMP9 mRNA (Supplementary Fig. S5C). MMP2 quantitation by ELISA showed increased protein expression associated with pressure (Fig. 4A). To determine whether pressure promotes invasion through MMP production by osteocytes, we added a broad spectrum MMP inhibitor, batimastat to CM. Batimastat inhibited invasion but not migration of prostate cancer cells in both nonpressurized CM (Supplementary Fig. SS5D) and 40 mmHg CM (Fig. 4B) cultures, indicating that in addition to basal expression of MMPs by osteocytes, pressure-induced MMP expression by osteocytes increases invasion. Furthermore, addition of recombinant MMP2 (rMMP2) to 0 mmHg CM, to recapitulate the concentration of MMP2 found in 40 mmHg CM, promoted invasion to levels similar to 40 mmHg CM alone (Supplementary Fig. S5E), indicating the ability of MMPs to contribute to the pressure-induced invasion. Our finding that pressure induced CCL5 (Fig. 3B and C) along with previous reports, indicating that CCL5 promotes MMP expression led us to explore whether CCL5 promotes MMP expression in osteocytes (13, 14). Accordingly, rCCL5 was added to MLO-Y4 and found to increase MMP2 and MMP9 mRNA expression (Supplementary Fig. S6), suggesting that pressure could induce MMP2 and MMP9, in part, through CCL5.

Discussion

The consequences of tumor-generated physical forces on the tumor microenvironment, in general, and the bone microenvironment specifically, have yet to be elucidated. Our data provide evidence that tumor growth–induced pressure promotes a pro-proliferative and proinvasive response from osteocytes. It achieves this, in part, through pressure-induced osteocytes production of CCL5 and MMPs (model in Fig. 4C). This is the first demonstration of tumor modulating the microenvironment through a physical force so that the microenvironment enhances cancer progression.

Herein, data are presented showing that tumors that produce radiocytically mixed lesions (i.e., ACE1 and DU145 cells) increase ImP. However, no elevation of ImP was observed from lytic PC3 cells, suggesting that osteolysis provided sufficient space for PC3 growth without inducing changes in ImP. Consistent with this possibility is the observation that breast cancer cells respond to matrix rigidity through upregulation of MMPs (15). It is plausible that matrix rigidity, through its resistance to conformational changes, would influence the magnitude of pressure generated by a growing tumor. Furthermore, in tumors that create mixed osteoblastic lesions, the decreased ability to resorb bone and subsequent inability to create space for tumor growth may result in the increased ImP.

The primary role of osteocytes is coordination of bone production and resorption in response to physical stimuli (1, 2). Our report is the first to demonstrate that osteocytes support cancer progression. Bone is a complex tissue, and even though osteocytes are the primary mechanotransducing cells, it is plausible that other cells may be affected by altered ImP. We observed numerous cytokines produced from osteocytes in response to hydrostatic pressure. CCL5 was observed to have a significant response on promoting tumor invasion and migration. CCL5/RANTES is a chemokine whose primary role has been defined as a chemoattractant for immune cells. Prostate cancer cells DU145, LNCaP, and PC3 have previously been shown to express multiple receptors for CCL5 (10). CCL5 has previously been shown to promote migration of numerous tumor types, including prostate, breast, lung, and osteosarcoma (9, 10, 16–18). Our results are consistent with CCL5’s known activity on invasion and migration; however, as indicated in Supplementary Table S1 and the observation that CCL5 only partially inhibited of migration and invasion (Fig. 3E), multiple other factors most likely also contribute to the pressure-induced migration and invasion.

In addition to CCL5, osteocyte MMP2 and MMP9 basal expression was higher than that of prostate cancer cell lines and further induced by pressure. Batimastat inhibited invasion of prostate cancer toward osteocyte-derived CM, indicating a role for MMPs in osteocyte-mediated invasion. Although MMPs are known to play a role in cancer invasion their expression from osteocytes as potentiating tumor invasion is a novel finding (19). Osteocytes have been observed to have some bone resorption properties, and MMP expression is likely a consequence of this function (11) and MMP2 is important in osteocyte canalicul network formation and maintenance (20). Osteocyte-derived MMPs provide
A mechanism for nutrient release and development of a vicious cycle previously associated with tumor growth in bone (21, 22). Similarly in prostate cancer, integrins, such as αvβ6, may interact with TGFβ, leading to MMP2 induction and osteolytic degradation (12). This is especially interesting in the context that MMP activation of TGFβ is important in osteoblast viability and osteocyte differentiation (23). Furthermore, our results showing that CCL5 induces MMPs in osteocytes supports previous findings where CCL5 promoted MMP production in tumor cells (13, 14) and provides a potential mechanism to account for pressure-induced MMP expression. In this setting, tumor promotion of bone degradation may elicit growth factor release from mineralized bone, further promoting tumor growth through initiation of a positive feedback loop. The response of osteocytes to tumor-generated pressure has great potential to promote the vicious cycle as osteocytes are the most abundant cell in bone and have a greater surface area than that of trabeculae (24).

**Conclusion**

The current report demonstrates that tumor growth promotes ImP and these physical forces promote osteocyte secretion of the tumor-promoting factors CCL5 and MMPs. Furthermore, osteocytes were identified as novel protumorigenic cellular mediators. Exploration of osteocytes and their response to physical forces is necessary to develop novel therapeutic targets for the inhibition of bone metastases.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J.L. Sotnik, E.T. Keller
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Sotnik, J. Dai, B. Campbell
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Sottnik, J. Dai, B. Campbell, E.T. Keller
Writing, review, and/or revision of the manuscript: J.L. Sottnik, J. Dai, E.T. Keller
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Sottnik
Study supervision: E.T. Keller

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