Parathyroid Hormone–Related Protein Drives a CD11b⁺Gr1⁺ Cell–Mediated Positive Feedback Loop to Support Prostate Cancer Growth

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Abstract

In the tumor microenvironment, CD11b⁺Gr1⁺ bone marrow–derived cells are a predominant source of protumorigenic factors such as matrix metalloproteinases (MMP), but how distal tumor cells regulate these cells in the bone marrow is unclear. Here we addressed the hypothesis that the parathyroid hormone–related protein (PTHrP) potentiates CD11b⁺Gr1⁺ cells in the bone marrow of prostate tumor hosts. In two xenograft models of prostate cancer, levels of tumor-derived PTHrP correlated with CD11b⁺Gr1⁺ cell recruitment and microvessel density in the tumor tissue, with evidence for mediation of CD11b⁺Gr1⁺ cell–derived MMP-9 but not tumor–derived VEGF-A. CD11b⁺Gr1⁺ cells isolated from mice with PTHrP-overexpressing tumors exhibited relatively increased proangiogenic potential, suggesting that prostate tumor–derived PTHrP potentiates this activity of CD11b⁺Gr1⁺ cells. Administration of neutralizing PTHrP monoclonal antibody reduced CD11b⁺Gr1⁺ cells and MMP-9 in the tumors. Mechanistic investigations in vivo revealed that PTHrP elevated Y418 phosphorylation levels in Src family kinases in CD11b⁺Gr1⁺ cells via osteoblast-derived interleukin-6 and VEGF-A, thereby upregulating MMP-9. Taken together, our results showed that prostate cancer–derived PTHrP acts in the bone marrow to potentiate CD11b⁺Gr1⁺ cells, which are recruited to tumor tissue where they contribute to tumor angiogenesis and growth. Cancer Res; 73(22): 6574–83. ©2013 AACR.

Introduction

The tumor microenvironment provides primary tumor cells to mix with multiple types of stroma such as endothelium, fibroblasts, and immune cells (1). Such heterogeneity of cell populations presents a major impediment for developing a cure for cancer. Increasing evidence suggests that stromal cells in the tumor microenvironment not only occupy a significant fraction of the tumor bulk, but also play critical roles in proliferation, invasion, and/or metastasis of tumor cells (2). In this regard, bone is an essential partner for tumor progression, because bone marrow serves as the supplying organ for numerous critical cells in the tumor microenvironment (3, 4). However, it is unclear how tumor cells co-opt the bone and/or bone marrow to facilitate a favorable tumor microenvironment.

Among the bone marrow–derived cells, CD11b⁺Gr1⁺ cells [commonly referred to as myeloid-derived suppressor cells (MDSC)] correlate with tumor progression (5). MDSCs were originally investigated for their roles in evasion of host immune surveillance, especially via suppression of T-cell–dependent antitumor immunity by production of arginase, reactive oxygen species, and inducible nitric oxide synthase (6). Subsequent studies demonstrated that MDSCs are increased in tumor-bearing mice and cancer patients, and infiltrate primary tumor tissue to promote angiogenesis by secreting matrix metalloproteinases (MMP), and also by direct incorporation into tumor endothelium (7, 8). More recently, MDSCs have been shown to play key roles in recovery after radiation therapy (9, 10) and antiangiogenic therapy (11).

In parallel, multiple mechanisms have been proposed to explain the increased recruitment of MDSCs in tumor tissue. Yang and colleagues demonstrated that CXC chemokine ligand (CXCL)-5/CXC receptor (CXCR)-2 and stromal-derived factor-1/CXCR-4 axes recruit circulating MDSCs to tumor tissue (12). More recently, expression of a single integrin (α4β1) promotes MDSC invasion into tumors via activation of phosphatidylinositol 3-kinase (PI3K; ref. 13). However, despite such clear evidence supporting the tumorigenic functions of MDSCs and also the potential mechanisms of recruitment to the tumor tissue, MDSCs are poorly understood about their regulation in the supplying organ (i.e., bone marrow) of the tumor host, and also their potential crosstalk with distant primary tumor cells.

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This study was designed to elucidate how CD11b⁺Gr1⁺ cells are regulated in the bone marrow of prostate tumor hosts, contributing to tumor growth and angiogenesis. Prostate cancer provides a unique perspective on this process because of its devastating mortality and morbidity associated with its preferential metastasis to the skeleton (14). Accordingly, prostate cancer cells secrete numerous bone-modulating cytokines, leading to osteoblastic/osteolytic reactions that facilitate growth factor and cytokine release from bone cells and matrix (15). In particular, parathyroid hormone–related protein (PTHrP) is expressed by prostate cancer cells, and stimulates osteoblasts in an endocrine manner to secrete factors such as receptor activator of NF-κB ligand (RANKL), interleukin (IL)-6, C-C chemokine ligand (CCL)-2, and VEGF-A. Thus, PTHrP is well known to regulate tumor growth via autocrine, paracrine, and intracrine manners (17–19, 26, 27), hence providing a unique perspective on this process because of the potential crosstalk between tumor and the bone marrow, the femoral bone marrow was flushed and dissociated, followed by antibody staining and flow cytometry (23). For analyses of phospho-Y418 Src family kinase (SFK), the bone marrow cells were fixed, permeabilized, stained, and analyzed according to the BD PhosFlow Cell Signaling protocols. All materials were from BD Biosciences.

**Flow cytometry**

For analyses of CD11b⁺Gr1⁺ cells in the tumor tissue, tumors were mechanically dissociated, followed by digestion in complete RPMI-1640 media supplemented with type I collagenase (5 mg/mL; Sigma-Aldrich). Viable cells were counted and resuspended in fluorescence-activated cell sorting buffer containing combinations of antibodies including FITC-conjugated anti-mouse CD11b, PE-conjugated anti-mouse Gr1, or isotype controls. For analyses or sorting CD11b⁺Gr1⁺ cells from the bone marrow, the femoral bone marrow was flushed and dissociated, followed by antibody staining and flow cytometry (23). For analyses of phospho-Y418 Src family kinase (SFK), the bone marrow cells were fixed, permeabilized, stained, and analyzed according to the BD PhosFlow Cell Signaling protocols. All materials were from BD Biosciences.

**Immunohistochemistry**

Tumors were surgically removed and bisected, a portion fixed in formalin and a portion snap-frozen. Murine endothelial cell–specific CD31/PECAM immunostaining (clone MEC13.3; BD Biosciences) was performed according to a previously described method (24). Rat anti-mouse CD11b (clone M1/70; BD Biosciences) and anti-mouse Ly-6G (clone RB6-8C5; eBioscience) were fluorescently labeled and used to detect CD11b⁺Gr1⁺ cells in the tumor tissue. Three to five randomly selected microscopic images per sample were obtained, and positively stained cells were counted using ImageJ software.

**Quantitative PCR**

mRNA samples were prepared from the bone marrow or tumor tissues using TRIzol reagent (Invitrogen), followed by reverse transcription-quantitative PCR (25). All quantitative PCR probes and reagents were from Applied Biosystems.

**Statistical analyses**

All *in vivo* data sets were tested for normality by Shapiro–Wilk test. Statistical analyses were performed by GraphPad Prism software. Student *t* test or Mann–Whitney *U* test were used to compare 2 groups and all statistical tests were 2-sided.

**Results**

**Reduction of PTHrP in PC-3 prostate tumors decreased CD11b⁺Gr1⁺ bone marrow cell recruitment and angiogenesis**

As a first approach to investigate the role of PTHrP in the potential crosstalk between tumor and the bone marrow, the *PTHLH* gene (encoding PTHrP) was targeted via lentiviral shRNA vectors in PC-3, human prostate cancer cells (19). Two clones expressing high and low levels of PTHrP (961.8 ± 12.8 vs. 457.8 ± 4.1 pg ml⁻¹ 1 × 10⁶ cells⁻¹ 48 h⁻¹; measured in the culture supernatant by immunoradiometric assays) were selected and designated PTHrP⁺ and PTHrP⁻, respectively. PTHrP is well known to regulate tumor growth via autocrine, intracrine, and paracrine manners (17–19, 26, 27), hence alterations in the host response (e.g., recruitment of host-derived cells) could simply be secondary to the differences in...
the tumor size, not in PTHrP expression levels. Therefore, PTHrPLo tumors were grown for a longer period until they reached a similar mean tumor volume as PTHrPHi tumors (38 days). B, PTHrPHi and PTHrPLo tumors were surgically dissected on the same day and photographed. Mean tumor volume between the 2 groups was not significantly different (P = 0.68, Student t-test). Scale bar, 1 cm. C, percentages of CD11b+Gr1+ double-positive cells in the tumor tissues were analyzed by flow cytometry. D, tumor tissues were sectioned for H&E and murine CD31/PECAM immunohistochemical staining. Original magnification, ×20. Scale bars, 50 μm. E and F, microscopic images were analyzed for tumor mean vessel density (MVD) or CD31+ vascular area with normalization to total nuclear area. G and H, host-derived (i.e., murine) Mmp9 and tumor-derived (i.e., human) VEGFA mRNA levels were measured by quantitative RT-PCR using species-specific probes (n = 9–10 per group). All P values are from Student t test. NS, not significant. Data in all graphs are mean ± SEM.

Ectopic PTHrP increased the recruitment of CD11b+Gr1+ cells in prostate tumor tissue

An additional prostate tumor model was utilized to establish the causal relationship between PTHrP and CD11b+Gr1+ cells. Ace-1 prostate cancer cells produce predominantly osteoblastic lesions in vivo, a phenotype that recapitulates human prostate cancer more realistically than the majority of currently available prostate cancer cell lines (17, 29). Ace-1 cells, expressing undetectable basal levels of PTHrP, were stably transfected with PTHrP overexpression (designated PTHrP OE) or empty control (designated pcDNA) vectors. In the same approach as the PC-3 tumor model (i.e., growth in differential periods), 2 groups of similarly sized tumors, PTHrP OE and pcDNA control, were produced. To directly examine the effects of systemic PTHrP on CD11b+Gr1+ cell recruitment, one group of mice carrying pcDNA control tumors was treated with recombinant PTHrP, while the other group of mice carrying PTHrP OE tumors was treated with recombinant PTHrP (Fig. 2A and Supplementary Fig. S1). Both PTHrP OE and recombinant PTHrP-treated groups had significantly increased CD11b+Gr1+ cells in the tumor tissue compared with pcDNA control tumors (Fig. 2B).
significantly increased percentages of CD11b+Gr1+ cells in the bone marrow (Fig. 2C), recombinant PTHrP treatment failed to show such an increase in the bone marrow. This may be explained by either the different modes of PTHrP administration (i.e., intermittent injection vs. continuous expression) or the reduced duration (7 days) of PTHrP treatment compared with tumor burden (21 days). Immunohistochemical analyses of tumor tissue showed that both PTHrP OE and recombinant PTHrP tumors had significantly increased evidence of angiogenesis (Fig. 2D and Supplementary Fig. S1B). In addition, host-derived Mmp9 expression was significantly increased in PTHrP OE tumor tissue (Fig. 2E), suggesting contribution of the CD11b+Gr1+ cell recruitment, at least in part, to angiogenesis. Collectively, data in Figs. 1 and 2 suggest that prostate cancer-derived PTHrP is a crucial regulator of CD11b+Gr1+ cells.

**CD11b+Gr1+ cells promoted tumor growth in vivo**

The protumorigenic functions of CD11b+Gr1+ cells are relatively well characterized using multiple tumor models (5, 7, 30, 31). To more rigorously examine the effects of CD11b+Gr1+ cells on tumor growth in the prostate tumor model, 2 fractions of bone marrow cells, that is CD11b+Gr1+ double positive or negative cells, were isolated and coimplanted with parental Ace-1 tumor cells in vivo (Fig. 3A). Increasing numbers of CD11b+Gr1+ cells mixed with tumor cells correspondingly increased the tumor size within 15 days (Fig. 3B and C). More importantly, Ace-1 tumor coimplanted with 0.5 × 10⁶ CD11b+Gr1+ cells grew significantly larger than tumors coimplanted with the same number of CD11b+Gr1+ cells, suggesting that altered tumor size in Figs. 1 and 2 were secondary to the altered recruitment of CD11b+Gr1+ cells in the tumor tissue.

**Tumor-derived PTHrP confers increased angiogenic potential to CD11b+Gr1+ cells**

To examine whether tumor-derived PTHrP regulates CD11b+Gr1+ cells within the bone marrow of tumor hosts, CD11b+Gr1+ bone marrow cells were isolated from 2 groups of mice bearing either PTHrP-overexpressing or pcDNA control tumors for 3 weeks, resulting in 2 fractions of CD11b+Gr1+ cells (i.e., PTHrP-activated vs. control). Parental Ace-1 tumor cells were mixed with the isolated CD11b+Gr1+ cells and xenografted into male athymic mice (Fig. 4A). Tumors coimplanted with PTHrP-activated CD11b+Gr1+ cells were significantly larger than the tumors with control CD11b+Gr1+ cells (Fig. 4B), potentially because increased MMP-9 and angiogenesis as determined by immunohistochemistry (Fig. 4C and D and Supplementary Fig. S2).

**PTHRP increased expression of phospho-[Y418] Src family kinases in CD11b+Gr1+ cells**

The molecular mechanism for the observed PTHrP-dependent CD11b+Gr1+ cell potentiation was subsequently investigated. Recently, Liang and colleagues demonstrated that dasatinib, an SFK inhibitor, suppressed prostate tumor growth as well as the numbers of CD11b+ myeloid cells in tumor tissues (32). Accordingly, the effects of PTHrP administration
on SFK in CD11b<sup>+</sup>Gr1<sup>+</sup> cells were investigated. A single administration of PTHrP (1–34) to male athymic mice significantly increased the activating phosphorylation of Tyr-418 residue of SFK in CD11b<sup>+</sup>Gr1<sup>+</sup> cells (Fig. 5A). As SFK activation requires intramolecular conformational changes and interaction with activated receptor kinases via the SH-2 domain, phosphorylation of [Y418] in the SH-2 domain indicates the status of full activation. However, because CD11b<sup>+</sup>Gr1<sup>+</sup> cells do not express receptors for PTHrP (as determined by quantitative reverse transcription (RT)-PCR for Pthr1; Supplementary Fig. S3), phosphorylation of [Y418] SFK was reasoned to be indirect through cytokines from osteoblasts, the predominant cells expressing the PTH/PTHrP receptor (PTH1R) in the bone marrow. Potential candidate cytokines from PTHrP-stimulated osteoblasts included IL-6, VEGF-A, C-C chemokine ligand (CCL)-2, and RANKL (16, 33–35). Therefore, CD11b<sup>+</sup>Gr1<sup>+</sup> cells were isolated from femoral bone marrow and treated with these osteoblastic cytokines. Although all 4 cytokines (IL-6, VEGF-A, CCL-2, and RANKL) have been shown to upregulate SFKs (36–38), only IL-6 and VEGF-A increased the expression of phospho-[Y418] SFK in MDSCs (Fig. 5B).

**Phospho-[Y418] SFK by osteoblastic VEGF-A and IL-6 increased MMP-9 expression in CD11b<sup>+</sup>Gr1<sup>+</sup> cells**

To further investigate the functional significance of phospho-[Y418] SFKs in MDSCs, several published markers of...
CD11b\(^{+}\)Gr1\(^{+}\) cell activation were examined in combination with PTHrP-dependent osteoblastic cytokines and an SFK selective inhibitor, PP2 (5, 13, 28). Only VEGF-A and IL-6 increased \textit{Mmp9} gene expression, whereas \textit{Cxcr2}, \textit{Cxcr4}, or \textit{Itgb1} expression remained unaffected in CD11b\(^{+}\)Gr1\(^{+}\) cells, and this increase was reversed by PP2 treatment (Fig. 6A–D).

Figure 5. PTHrP phosphorylated \([Y418]\) Src family kinases in CD11b\(^{+}\)Gr1\(^{+}\) cells. A, male athymic mice \((n = 3\) per group) were stimulated with a single administration of PTHrP \((1–34)\) or saline control, 8 hours before sacrifice and flow cytometric analyses of phospho-\([Y418]\) Src family expression levels in CD11b\(^{+}\)Gr1\(^{+}\) bone marrow cells. Representative histograms from the control group (shaded) and the PTHrP-stimulated group (open) were overlapped to show the intensity of phospho-\([Y418]\) Src expression. CD11b\(^{+}\)Gr1\(^{+}\) cells expressing high levels of phospho-\([Y418]\) Src family kinases (indicated by a bracket [M]) were quantified and plotted. Data are mean ± SEM. \(P < 0.01\), Student \(t\) test. B, CD11b/Gr1 double positive cells were sorted from the femoral bone marrow of male athymic mice, followed by treatment with saline, IL-6, VEGF-A, CCL-2, RANKL, or PTHrP \((100 \text{ ng/mL for } 0.5 \times 10^{6} \text{ cells})\) for 1 hour at 37°C \((n = 3\) per group). Representative histograms (open) were overlapped onto unstimulated controls (shaded) to show the intensity of staining.

Figure 6. Phosphorylation of Src family kinases by osteoblastic VEGF-A and IL-6 increased MMP-9 expression in CD11b\(^{+}\)Gr1\(^{+}\) cells. A–D, CD11b\(^{+}\)Gr1\(^{+}\) cells were sorted from the femoral bone marrow of male athymic mice via flow cytometry, followed by treatment with saline (control), VEGF-A, IL-6, PTHrP, or CCL-2 \((100 \text{ ng/mL for } 0.5 \times 10^{6} \text{ cells})\) in combination with PP2 \((100 \text{ nmol/L})\) or dimethyl sulfoxide \((\text{DMSO})\) control for 1 hour at 37°C \((n = 3\) per group). mRNA levels of \textit{Mmp9}, \textit{Cxcr4}, \textit{Cxcr2}, and \textit{Itgb1} were determined via quantitative RT-PCR. Data are mean ± SEM. \(P < 0.01\) compared with the DMSO control group. No other combination had statistical significance \((P > 0.05\), Student \(t\) test). E–G, conditioned media were collected from primary calvarial osteoblasts stimulated with saline (control) or PTHrP \((1–34)\). Femoral bone marrow CD11b\(^{+}\)Gr1\(^{+}\) cells were treated with the control or PTHrP-conditioned media in combination with neutralizing antibodies against VEGF-A and/or IL-6, followed by quantitative PCR (E) for \textit{Mmp9} gene expression \((n = 3\) per group) or zymography (F). Data are mean ± SEM. \(P < 0.05\); NS, not significant by Student \(t\) test. G, CD11b\(^{+}\)Gr1\(^{+}\) cells were treated with control or PTHrP-conditioned media in combination with PP2 or DMSO control, followed by zymography.
Furthermore, to confirm the requirement of osteoblasts in PTHrP-dependent potentiation of CD11b⁻Gr1⁺ cells, primary osteoblasts were established from murine calvaria and treated with PTHrP (1–34) or saline for 24 hours and conditioned media harvested (39). CD11b⁻Gr1⁺ cells were isolated from femoral bone marrow and stimulated with osteoblast-derived control- or PTHrP-conditioned media in combination with neutralizing antibodies against VEGF-A and/or IL-6. Consistent with the previous data, PTHrP-conditioned media from osteoblast cultures increased Mmp9 gene expression (Fig. 6E) and functional MMP-9 (Fig. 6F) in the MDSCs, and these effects were blocked by anti-VEGF-A and/or anti-IL-6 neutralizing antibodies. Furthermore, the effect of PTHrP-conditioned media on MMP-9 expression was suppressed by PP2 (Fig. 6G).

**Anti-PTHrP monoclonal antibody treatment decreased MDSC recruitment in PC-3 tumors**

Finally, to more rigorously determine the causal relationship between PTHrP and MDSC recruitment, mice bearing PTHrP⁺⁺ PC-3 tumors were treated with nonspecific control IgG or anti-human PTHrP monoclonal antibodies. Anti-PTHrP antibodies significantly suppressed tumor growth, but not to the level of PTHrP⁻⁻ tumors (Fig. 7A and B). As anti-PTHrP monoclonal antibodies potentially suppress tumor growth via inhibition of autocrine PTHrP effects on tumor cells (Supplementary Fig. S4), tumor tissues were analyzed for MDSC recruitment by immunofluorescence colocalization of CD11b⁻Gr1⁺ cells (Fig. 7C and D). Numbers of CD11b⁻Gr1⁺ cells were decreased in anti-PTHrP antibody-treated or PTHrP⁻⁻ tumor tissues, suggesting that reduced PTHrP is causal to decreased MDSCs found in tumor tissues. Serum calcium levels were correlated with PTHrP levels, indicating the functional activity of PTHrP (Fig. 7E). Quantitative RT-PCR analysis in tumor tissues revealed that shRNA-mediated PTHrP knockdown was stable in PC-3 tumor cells during in vivo tumor growth, and the correlation between PTHrP and Mmp9 gene expression (Fig. 7F and G).

Figure 7. Anti-human PTHrP monoclonal antibody decreased MDSC recruitment in PC-3 tumors. A, tumor growth curve of PC-3 PTHrP⁺⁺ tumors treated with control IgG or anti-PTHrP monoclonal antibodies (mAb) and PC-3 PTHrP⁻⁻ tumors (n = 10 per group). Both P values are from linear regression comparison with PC-3 PTHrP⁺⁺ IgG tumor group. Data are mean ± SEM. B, individual tumor weight was measured upon necropsy and plotted. Dots, individual measurements (mg); Horizontal lines, mean ± SEM (n = 10 per group). C, tumor tissues were sectioned and stained for CD11b (Alexa-Fluor 488), Gr1 (Alexa-Fluor 546), and DAPI. Original magnification, ×40. Scale bars, 50 μm. D, immunofluorescent images were merged and analyzed for CD11b⁻Gr1⁺ cell per microscopic field. Three positively stained nonnecrotic tumor areas were randomly selected for quantification (5 tumors/group). E, sera were collected upon necropsy, followed by calcium assay. F and G, PTHLH or Mmp9 mRNA levels in the pulverized tumor tissue were measured by quantitative RT-PCR (n = 10 per group). Data in all bar graphs are mean ± SEM. All P values, unless indicated otherwise, are from Student t test.
mobilize and/or expand MDSCs. Still, questions remain about whether and how PTHrP stimulates differentiation of MDSCs from bone marrow precursors. Nevertheless, this work provides a biological rationale for the clinical application of SFK inhibitors in targeting 2 compartments (i.e., tumor and the microenvironment) simultaneously, of which the mechanism requires further studies. The data in this study demonstrate that activation of SFKs is one of the key signal transduction mechanisms of MDSCs' angiogenic potential, in addition to two other factors, STAT3 and PI3K, that have previously been shown to be implicated in MDSC functions (13, 31). Indeed, SFKs mediate crucial regulatory functions in both tumor cells and stromal cells (e.g., endothelial cells and osteoclasts), suggesting that SFKs are promising therapeutic targets for the suppression of tumor as well as stromal compartments (24, 50).

In conclusion, this study provides evidence that prostate cancers positively regulate the bone marrow microenvironment via PTHrP, IL-6, VEGF-A, and SFKs, thereby increasing the angiogenic potential of CD11b+Gr1+ MDSCs, leading to increased tumor growth.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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