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14. ABSTRACT The dissemination of prostate cancer to the skeleton is the main cause of death from this tumor. Despite the effective palliative management of patients with advanced disease, curative measures are still lacking and metastatic prostate cancer is still an unmet medical need. The overarching goals of this study were to: 1) conclusively establish the role of PDGFR α in conferring bone metastatic potential to prostate cancer cells; 2) provide pre-clinical evidence that a fully human, monoclonal antibody against this receptor impairs metastatic progression in the skeleton of animal models; 3) identify specific genes and their encoded protein products underpinning the bone-metastatic behavior of prostate cancer cells. During these three years of funding we have fully addressed all goals of our study, published several papers in high profile journals, contributed to the pre-clinical phase of an antibody currently completing phase-II clinical trials and identified a three-gene set that define the bone-metastatic behavior of human prostate cancer cells in animal models and ex-vivo human samples.					
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INTRODUCTION

Skeletal metastases are responsible for substantial morbidity and represent the main cause of death in patients with advanced prostate adenocarcinoma^{1 2}. The treatment options currently available for this condition, mostly based on targeting osteoclasts and bone resorption, can alleviate pain and reduce the occurrence of fractures and nerve compression but fail to cure the patients^{3 4}. This strategy derives from the observation that bone metastatic tumors can recruit and activate osteoclasts at the site of lesion, inducing degradation of the bone matrix, release of trophic factors for the cancer cells that will recruit more osteoclasts, in a sort of “vicious cycle”^{5 6}. However, results from clinical trials with osteoclast-targeting compounds have not been encouraging^{7 8}, likely due to the fact that cancer cells disseminated to the skeleton benefit from trophic support that does not exclusively derive from osteoclasts’ intervention. In fact, we have previously reported that isolated cancer cells and small foci located in the bone marrow after their extravasation from the systemic circulation are either independent of, or much less reliant on spatial interactions with osteoclasts that are larger, more advanced lesions⁹.

The alpha-receptor for Platelet-Derived Growth Factor (PDGFR α) is a receptor tyrosine kinase that has been found associated with skeletal metastases by us and others^{10 9}.

More importantly, we were the first to report a direct correlation between PDGFR α expression and bone-metastatic potential of human prostate cancer cell lines in animal models^{11 12}.

The overarching goals of this three-year study were to:

- a) Conclusively establish the role of PDGFR α in conferring bone metastatic potential to prostate cancer cells;
- b) Provide pre-clinical evidence that antibodies against this receptor can impair metastatic progression in the skeleton;
- c) Identify specific molecular mediators that are regulated by PDGFR- α and underpinning the bone-metastatic behavior of prostate cancer cells.

BODY

As required, the research accomplishments for the three-year funding period are associated with each of the tasks outlined in the modified SOW, as follows:

Specific Aim 1: The role of PDGFR α in the survival of early micro-metastases in the bone marrow –

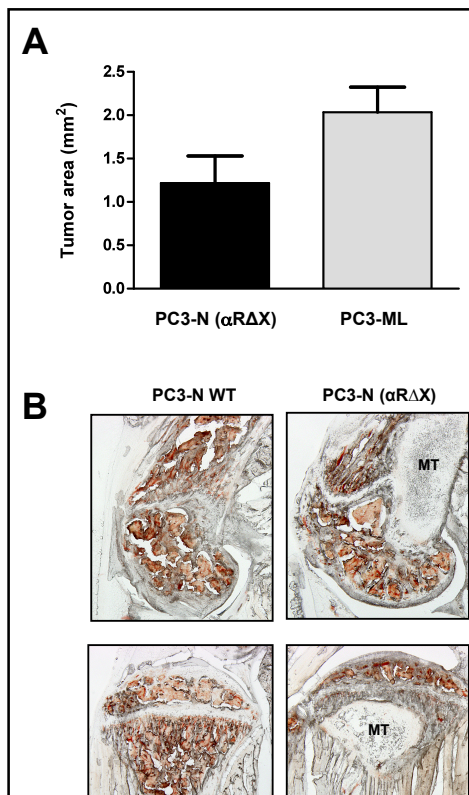
— We have conclusively established that human prostate cancer cell lines differ significantly in their ability to colonize the skeleton of animal models and progress into macroscopic metastatic lesions (For a full description and technical details of our pre-clinical model please refer to the paper we published in *Oncogene* 2009).

For instance, LNCaP, C4-2b, VCaP, DU-145 and LAPC-4 are all human cell lines that we found can home to the skeleton but consistently fail to produce bone lesions when inoculated in the systemic arterial circulation of mice via the left cardiac ventricle. Interestingly, all these cells also lack the expression of PDGFR- α .

In contrast, two sub populations of the PC3 cell line, named PC3-ML and PC3-N, can lodge and colonize the skeleton. However, PC3-ML cells produce large metastatic lesions in >90% of animals at four weeks post-inoculation, whereas PC3-N cells progress for only three weeks and only in 20% of animals. Interestingly, these different metastatic behaviors correlate with high and low expression of PDGFR α , respectively. The implication of this PDGFR α in the bone tropism of prostate cancer cells is confirmed by the fact that the exogenous over-expression of this receptor in PC3-N cells confers full metastatic potential to these cells and allows them to progress at the skeletal levels in a manner indistinguishable from that of PC3-ML cells.

We also found that cells that lack PDGFR α expression (see above) are nevertheless capable of homing to the skeleton via arterial circulation and survive for a very short period of time in the bone marrow microenvironment. These results further indicate that PDGFR α plays a paramount role in supporting the early survival of prostate cancer cells disseminated to the bone and provide conceptual support for the experiments included in SA2 of this study.

— We had previously shown that PDGFR α is transactivated by the acellular, soluble fraction of human bone marrow in a ligand-independent fashion. Therefore, we engineered PC3-N cells to stably express a truncated form of PDGFR α ($\alpha\Delta X$), which lacks the extracellular-binding domain. These cells were inoculated in our



animal model and showed an increase in bone-metastatic potential, which was indistinguishable from that of PC3-N cells overexpressing the full-length receptor and comparable to the highly-metastatic PC3-ML cells (**Figure 1**). These results conclusively demonstrate that PDGFR α is activated by soluble component of the bone marrow, which most likely recruit an additional receptor-signaling system. The concurrent inhibition of PDGFR α and this yet unidentified plasma membrane receptor should produce even better anti-metastatic effects than the blockade of PDGFR α alone. These results were included in a manuscript published in Cancer Research (2010) (appended).

Figure 1. (A) PC3-N cells expressing the truncated form of PDGFR α ($\alpha\Delta X$) and inoculated in mice produced bone-metastatic lesions comparable in size to that caused by the highly bone-metastatic PC3-ML cells; **(B)** mice showed large tumors in both tibiae (bottom) and femora (top) at 4-week post-inoculation of prostate cancer cells. (MT = metastasis). Further details are available in the published manuscript included in the appendix (Cancer Research, 2010).

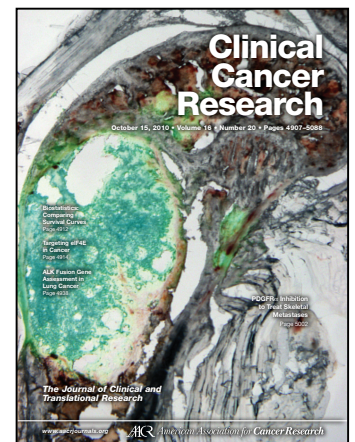
Specific Aim 2: The effects of antibody-mediated targeting of PDGFR α on skeletal metastasis -

— We showed that targeting PDGFR α with IMC-3G3, a humanized, monoclonal antibody, exerts both a prophylactic and curative effect on skeletal metastases from prostate cancer cells.

Our study also provided pre-clinical evidence that IMC-3G3, alone and in combination with the bisphosphonate Zoledronic Acid prolongs the overall survival of inoculated animals.

A paper including the results obtained from these experiments was published in Clinical Cancer Research (2010) (appended) and one of the images also selected for the cover page.

Furthermore, our study contributed in a determinant manner to persuade ImClone/Eli Lilly to pursue the testing of the IMC-3G3 antibody, renamed Olaratumab, in clinical trials. Phase-I trials showed no toxicity for this antibody, which is now on phase-II trials for several forms of solid tumors including advanced prostate adenocarcinoma. This trial will be concluded in the summer of 2013.



Specific Aim 3: Identification of molecular mediators responsible for bone-metastatic potential and regulated by PDGFR α .

— We conducted comparative gene-expression analyses of bone-metastatic prostate cells (PC3-ML), cells lacking metastatic ability (DU-145 and PC3-N), cells that were converted to a bone-metastatic phenotype upon exogenous PDGFR α expression (PC3-N-alpha) and cells that failed to show this conversion in phenotype (DU-145-alpha). This approach allowed us to identify seven genes that were related to bone-metastatic potential in our pre-clinical animal model. A further analysis was then conducted including two clonal sub-populations derived from PC3-ML cells, both capable of producing skeletal metastases in SCID mice. Through this

additional filtering, we finally identified three genes that were found to be directly up-regulated by PDGFR α expression and correlated with the acquisition of bone-metastatic potential by PCa cells. Interestingly, these three newly identified genes all encode for soluble, secreted proteins: the cytokine Interleukin-1 β (IL-1 β), the chemokine CXCL6 (GPC-2) and the protease inhibitor Elafin (PI3).

The clinical significance of these findings was corroborated by mining prostate cancer data sets publically available through the Oncomine repository, showing that IL-1 β , CXCL6, and Elafin are significantly up regulated in tumors as compared to normal prostate tissues (**Fig. 2a**). Furthermore, a meta-analysis indicated a strong association of both IL-1 β and CXCL6 with prostate cancer with Gleason scores (≥ 7) (**Fig. 2b**). In light of these observations, we screened human tissue arrays including 227 cases of prostate adenocarcinoma for IL-1 β protein expression and correlated signal intensities with the Gleason score attributed to each tissue specimen (**Fig. 2c,d**). This approach validated the results from the Oncomine analysis and conclusively demonstrates that prostate tumors with medium and high Gleason Scores, which have the highest propensity to metastasize express increased levels of IL-1 β as compared to tumors with Gleason Scores (<7) or normal tissues (**Fig. 2e**).

— Thus, we reasoned that IL-1 β could be a crucial player in the establishment of skeletal secondary lesions by prostate cancer. To test this hypothesis, we first used a pre-clinical animal model of metastasis and employed short-hairpin RNA (shRNA) to deplete IL-1 β in PC3-ML cells to expression levels lower than the levels observed in PC3-N cells (**Fig. 3a**). The resulting PC3-ML(sh-IL-1 β) cells were delivered in the systemic arterial circulation of mice euthanized four weeks later and showed significantly impaired metastatic abilities. The inspection of femora and tibiae of inoculated animals showed that PC3-ML and PC3-ML(sh-IL-1 β) cells produced bone metastases in a comparable number of animals (**Fig. 3b**). However, the lesions generated by PC3-ML(sh-IL-1 β) cells were 70% smaller than those observed in mice inoculated with PC3-ML cells expressing endogenous levels of IL-1 β . (**Fig. 3c,d**).

— To further define the role of IL-1 β in skeletal metastasis, we conducted complementary experiments in which this cytokine was exogenously over-expressed in prostate cancer cells with demonstrated inability to progress into macroscopic bone lesions. We first studied PC3-N cells, which routinely produce small lesions in only 20% of animals inspected at three weeks post-inoculation and then regress thereafter. After homing to the skeleton from the blood circulation, PC3-N(IL-1 β) cells were able to fully progress into tumors comparable in number and size to the lesions produced by PC3-N(R α) cells (**Fig. 4a-d**). More importantly, analogous results were obtained with DU-145 cells, which are widely reported to lack bone-tropism in mouse models. We have previously shown that this lack of metastatic behavior is caused by the inability of these cells to survive for

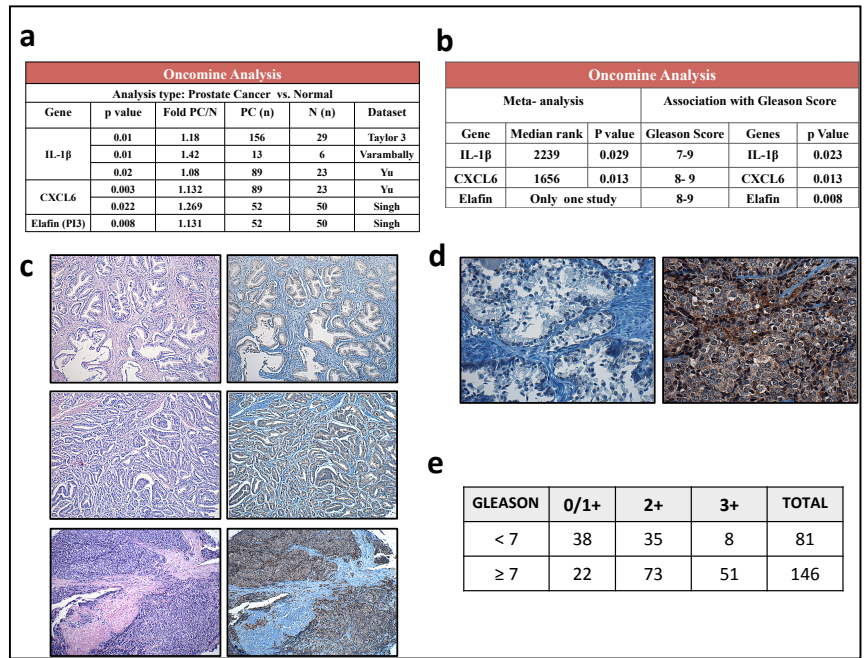


Figure 2. Up regulation of the genes for IL-1 β , CXCL6 and Elafin in prostate cancer and correlation of IL-1 β protein expression with Gleason scores. (a) The Oncomine database shows a consistent increase in the expression of these three genes in tumor as compared to normal prostate tissue and (b) a significant correlation between IL-1 β and CXCL6 expression in tumors with Gleason scores (7-9) and (8-9), respectively. The results from the only study available analyzing Elafin expression in tumor versus normal and Gleason scores (8-9), respectively, were statistically significant; (c) TMAs including 227 cases of primary prostate adenocarcinoma were stained for IL-1 β with signal intensities that were scored as weak (1, top), moderate (2, middle) and strong (3, bottom). ; (d) higher magnification of two representative tissue specimens that stained negative (left) and strongly positive (right) for IL-1 β , respectively. Hematoxylin-Eosin counterstaining was used; (e) contingency table showing that prostate tumors with Gleason scores (≥ 7) expressed higher levels of the IL-1 β protein as compared to tumors with lower Gleason scores (<7). Chi-square = 33.08 and $p < 0.0001$

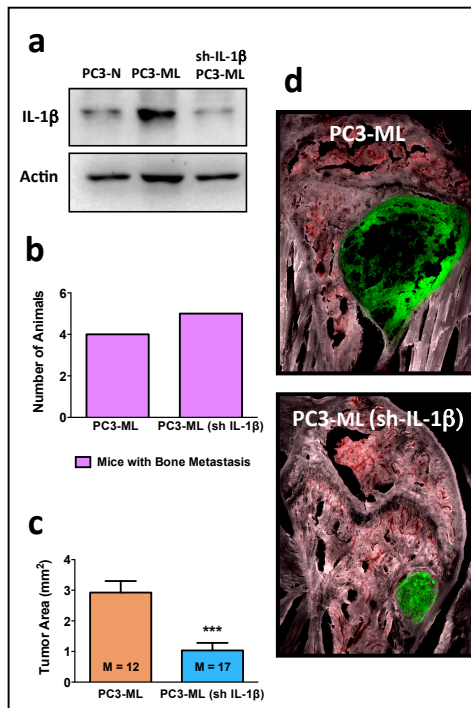


Figure 3. Effects of IL-1 β silencing on the metastatic potential of prostate cancer cells *in vivo*. (a) RNA interference reduced IL-1 β protein expression in highly metastatic PC3-ML cells; (b) four weeks after intracardiac inoculation of PC3-ML or PC3-ML(sh-IL-1 β) cells all mice developed bone metastatic tumors; (c-d) however, the lesions generated by PC3-ML (sh-IL-1 β) cells were significantly smaller in size. M = number of metastatic tumors. *** p=0.002

more than three days after homing to the bone marrow. Interestingly, DU-145 cells do not express IL-1 β ; however, upon over-expression of this cytokine (Fig. 5a) these cells generated skeletal metastases in 40% of mice examined at four weeks post-inoculation (Fig. 5b). Although these lesions were smaller in size than the skeletal tumors produced by either PC3-ML or PC3-N(IL-1 β) cells after the same time interval (Fig. 5c), this data provides compelling evidence that, in addition to potentiating the weak bone-tropism of PC3-N cells, IL-1 β can induce *de novo* metastatic behavior in prostate cancer cells. This newly identified pro-metastatic role of IL-1 β might be exerted through either autocrine stimulation or paracrine recruitment of cells other than osteoclasts. In the latter scenario IL-1 β would cross talk with cells of the bone stroma and induce them to reciprocate with an increased or *ex-novo* production of trophic factors supporting the survival and growth of DTCs. In both circumstances disrupting the functional

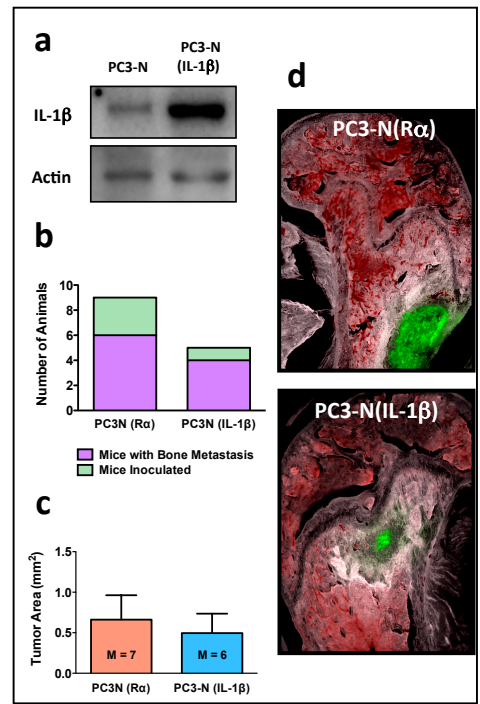


Figure 4. Effects of IL-1 β over-expression on the metastatic potential of prostate cancer cells *in vivo*. (a) IL-1 β over-expression in low metastatic PC3-N cells; (b) the resulting PC3-N(IL-1 β) cells were as effective as PC3-N(Rα) cells in generating skeletal lesions in mice sacrificed four weeks after intracardiac inoculation; (c,d,e) IL-1 β over-expression in low metastatic PC3-N cells; (f) the resulting PC3-N(IL-1 β) cells were as effective as PC3-N(Rα) cells in generating skeletal lesions in mice sacrificed four weeks after intracardiac inoculation; (g-h) bone lesions produced by PC3-N(IL-1 β) and PC3-N(Rα) cells were comparable in size. PC3-N cells transduced with an empty pLSXN vector were inoculated in the arterial circulation of five mice that were euthanized four weeks later and found free of skeletal tumors (not shown). M = number of metastatic tumors) bone lesions produced by PC3-N(IL-1 β) and PC3-N(Rα) cells were comparable in size. PC3-N cells transduced with an empty pLSXN vector were inoculated in the arterial circulation of five mice that were euthanized four weeks later and found free of skeletal tumors (not shown).

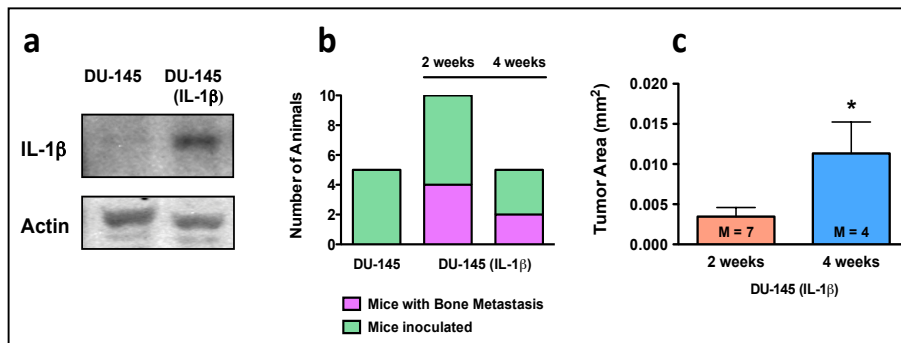


Figure 5. Exogenous expression of IL-1 β in non-metastatic DU-145 cells induces a bone-metastatic phenotype. (a) Expression of IL-1 β in DU-145 cells, which are non-metastatic; (b) the resulting DU-145(IL-1 β) cells generated bone lesions in 40 % of mice inoculated via the intracardiac route and sacrificed either 2 or 4 weeks post-inoculation; (c) the size of skeletal lesions increased in a time-dependent manner, thus suggesting metastatic progression. M = number of metastatic tumors. *p=0.037.

interactions between IL-1 β and its receptors, most likely IL-1R, would substantially attenuate the progression of prostate cancer at the skeletal level and possibly reduce the secondary involvement of soft tissue organs.

The results described above for SA3 were included in a manuscript currently under review for publication by Cancer Research.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of PDGFR α as a critical determinant for the bone-metastatic potential of prostate cancer cells.
- Provided conclusive evidence that the soluble component of the human bone marrow activates PDGFR α in a ligand-independent fashion (transactivation).
- Pre-clinical validation of a targeted therapy with a fully human, monoclonal antibody against PDGFR α , which was shown to exert both preventative and curative anti-metastatic effects in animal models.
- Identification of a three-gene set (IL-1 β , CXCL6 and Elafin) directly up-regulated by PDGFR α in prostate cancer cells and strongly correlated with bone-metastatic behavior in animal models.
- Functional validation of IL-1 β as a potential novel therapeutic target for the treatment of metastatic prostate cancer.

REPORTABLE OUTCOMES

Manuscripts.

1. Russell MR, Liu Q, Lei H, , Kazlauskas A, and Fatatis A. The alpha-receptor for platelet derived growth factor confers bone-metastatic potential to prostate cancer cells by ligand- and dimerization-independent mechanisms. **Cancer Research** 70, 4195-4203, (2010)
2. Russell MR, Liu Q and Fatatis A. Targeting the alpha receptor for Platelet-Derived Growth Factor as a primary or combination therapy in a preclinical model of prostate cancer skeletal metastasis. **Clinical Cancer Research** 16, 5002-5010, (2010).
3. Liu Q., Jernigan D., Zhanh, Y., and Fatatis A. Implication of platelet-derived growth factor receptor alpha in prostate cancer skeletal metastasis. **Chinese J. Cancer** 30, 612-619 (2011).

Abstracts

1. Annual Meeting of the American Association for Cancer Research. "Antagonism of Platelet-Derived Growth Factor Receptor alpha as potential therapeutic for prostate cancer bone metastases. Washington, DC (2010).
2. Innovative Minds in Prostate Cancer Today, CDMRP. "The alpha-receptor for Platelet-Derived Growth Factor is transactivated by bone marrow and its antibody-mediated targeting counteracts the establishment and progression of skeletal metastases". Orlando FL (2011).
3. Drexel University College of Medicine, Discovery Day. "A gene-expression signature promoting the bone-metastatic potential of prostate cancer cells". Philadelphia PA (2011).
4. Annual Meeting of the American Association for Cancer Research. "Molecular determinants for the bone-metastatic potential of prostate cancer cells". Chicago, IL (2012).
5. Drexel University College of Medicine, Discovery Day. "IL-1b promotes skeletal colonization and progression of metastatic prostate cancer cells.". Philadelphia PA (2012).

Presentations

- Kimmel Cancer Center – Thomas Jefferson University.
“Molecules and events implicated in skeletal metastasis from prostate cancer”.
Philadelphia, PA (Presented by Alessandro Fatatis) (2009).
- ImClone – Eli Lilly ‘PDGFR α in skeletal metastasis from prostate cancer”. New York City, NY. (Presented by Alessandro Fatatis) (2009).
- University of Florida. Shands Cancer Center
“Molecules and signaling events implicated in skeletal metastasis from prostate cancer”. Gainesville, FL.
(Presented by Alessandro Fatatis) (2010).
- University of Illinois at Chicago – Department of Biopharmaceutical Sciences. College of Pharmacy.
“Molecular and mechanisms for arrival and survival of cancer cells in skeletal metastasis”. Chicago, IL.
(Presented by Alessandro Fatatis) (2011).

Ph.D. awarded

- Mike Russell, degree awarded in April 2010
- Whitney Jamieson, degree awarded in April 2010
- Julia Fox D'Ambrosio, degree awarded in April 2010

Development of cell lines

- PC3-N cells stably expressing PDGFR α
- PC3-N cells stably expressing IL-1 β
- DU-145 stably expressing PDGFR α
- DU-145 stably expressing IL-1 β
- PC3-ML cells stably silenced for IL-1 β

Applications for funding

1R01CA175701-01

NCI

“A three-gene set promotes bone-metastatic prostate cancer”.

SRG meeting: October 22 and 23, 2012.

CONCLUSIONS

Complications from metastatic dissemination to the skeleton of cancer cells represent the main cause of death for prostate cancer patients. Currently, only palliative measures are available to patients with bone-disseminated prostate cancer and there is an unmet need for effective therapeutic strategies to antagonize the progression of prostate cancer cells in the skeleton.

We have identified at least one novel potential target to counteract the progression of prostate cancer cells at the skeletal level. This is particularly relevant as therapeutics that target either IL-1 β or IL-1R are currently available and prescribed for inflammatory conditions of non-neoplastic etiology but affecting the skeleton, such as rheumatoid arthritis. The evidence provided by our study should lead to the repositioning of these drugs and rapidly translate into novel strategies for the treatment of existing bone lesions and/or counteracting the tertiary seeding of lesions in the skeleton.

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APPENDICES

The α -Receptor for Platelet-Derived Growth Factor Confers Bone-Metastatic Potential to Prostate Cancer Cells by Ligand- and Dimerization-Independent Mechanisms

Mike R. Russell¹, Qingxin Liu¹, Hetian Lei³, Andrius Kazlauskas³, and Alessandro Fatatis^{1,2}

Abstract

Prostate adenocarcinoma is the second leading cause of cancer death among men, due primarily to the fact that the majority of prostate cancers will eventually spread to the skeleton. Metastatic dissemination requires a complex series of coordinated events that result in cells that escape from the primary tumor into the circulation and eventually colonize a distant organ. The ability of these cells to evolve into macroscopic metastases depends strongly on their compatibility with, and ability to utilize, this new microenvironment. We previously showed that bone-metastatic prostate cancer cells exposed to human bone marrow respond by activation of cell survival pathways, such as phosphoinositide 3-kinase/Akt, and that these events are mediated by the α -receptor for platelet-derived growth factor (PDGFR α). Our studies and others have shown that PDGFR α may be activated by mechanisms independent of PDGF ligand binding. Here, we provide conclusive evidence that soluble components of human bone marrow can activate PDGFR α through a mechanism that does not require the canonical binding of PDGF ligand(s) to the receptor. In particular, we found that dimerization of PDGFR α monomers is not induced by human bone marrow, but this does not prevent receptor phosphorylation and downstream signaling from occurring. To establish the relevance of this phenomenon *in vivo*, we used a PDGFR α mutant lacking the extracellular ligand-binding domain. Our studies show that this truncated PDGFR α is able to restore bone-metastatic potential of prostate cancer cells as effectively as the full-length form of the receptor. *Cancer Res*; 70(10); 4195–203. ©2010 AACR.

Introduction

Prostate adenocarcinoma is the second leading cause of cancer-related death among men, despite a largely successful treatment of the primary tumor following early detection (1). The main problem for therapy is that the majority of prostate cancers will eventually disseminate to the skeleton. This complication leads to a significant decline in quality of life and is currently untreatable, representing the main cause of death in patients with advanced disease (2).

Metastatic dissemination is a concerted multistep process in which cancer cells spread from the primary tumor into the vasculature, survive in the circulation, and reach distant organs (3). Cancer cells that spread to secondary sites must initially adhere to the luminal surface of endothelial cells and subsequently migrate in response to chemoattractant cues produced by the tissue microenvironment (4, 5). Further

progression into clinically significant metastases depends strongly on the ability of cancer cells to support their survival and proliferation in the parenchyma of the secondary organ. In fact, it is widely agreed that cancer cells that fail to adapt to a specific organ microenvironment will either perish or remain dormant, incapable of causing harm to the patient unless growth is resumed (6, 7).

Cancer cells that disseminate to the skeleton through the circulatory system encounter the bone marrow immediately following extravasation from the vascular sinusoids. Their propensity to grow into macroscopic secondary tumors is most likely dictated by favorable conditions offered by this tissue, such as compatible trophic factors (8, 9). Thus, interference with these symbiotic interactions has been proposed as a powerful means to counteract skeletal metastases (10).

Effective therapies against skeletal metastases might be achieved by identification and blockade of molecular targets that result in disruption of the host/tumor relationship. To this end, we exposed bone-metastatic prostate cancer cells to bone marrow aspirates from human donors, and found that they responded by activation of downstream cell survival pathways, such as phosphoinositide 3-kinase (PI3K)/Akt (11). We also determined that these events are mediated by the α -receptor for platelet-derived growth factor (PDGFR α), a receptor tyrosine kinase expressed at higher levels in bone-metastatic cells than in cells that lack bone tropism (12). In addition, we showed that targeting PDGFR α with a

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humanized monoclonal antibody dramatically prevents the growth of skeletal lesions in an animal model of disseminated prostate cancer (13). When PDGFR α was overexpressed in prostate cancer cells that lacked bone-metastatic potential, it restored their ability to survive during the first days following extravasation in the bone marrow. This allowed these cells to progress into secondary bone tumors that were comparable in number and size to those produced by metastatic cancer cells that endogenously express higher levels of the receptor (13). The emphasis we place on the role of PDGFR α in prostate cancer progression and dissemination is also based on studies by other groups, which reported detection of PDGFR α in human samples from both primitive prostate adenocarcinoma (14, 15) and bone secondary tumors (16). Whereas the contribution of PDGFR α in the promotion of skeletal metastases from prostate and breast cancers is progressively emerging (17, 18), studies focusing on the mechanisms of receptor activation and recruitment of downstream signaling pathways have provided some exciting results. In particular, recent evidence suggests that PDGFR α may be activated by novel mechanisms, alternative to the conventional binding of PDGF ligand(s) to the receptor. For instance, Lei and Kazlauskas have shown that generation of reactive oxygen species (ROS) is sufficient for Src kinase-mediated activation of PDGFR α (19). Additional evidence shows that PDGFR α associates with, and is activated in response to, infection by human cytomegalovirus (20). In agreement with these studies, we have found that activation of PDGFR α by human bone marrow could occur despite blockade of the extracellular ligand-binding domain (11).

Here, we provide conclusive evidence that the acellular fraction of human bone marrow can activate PDGFR α through a mechanism that does not require the canonical binding of PDGF ligand(s) to the receptor. In particular, we found that human bone marrow does not induce dimerization of PDGFR α monomers, but this does not prevent receptor phosphorylation and downstream signaling from occurring. To establish the relevance of this phenomenon *in vivo*, we used a PDGFR α mutant lacking the extracellular ligand-binding domain. Our studies show that this truncated PDGFR α is able to restore bone-metastatic potential of prostate cancer cells as effectively as the full-length form of the receptor.

Materials and Methods

Reagents. 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine (PP2) and *N*-acetyl-L-cysteine (NAC) were obtained from Calbiochem.

Cell lines and cell culture. PC3-ML and PC3-N cell lines were derived from the parental PC3 cell line [American Type Culture Collection (ATCC)] as previously described (21). DU-145 cells were purchased from ATCC. All cell lines were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 0.1% gentamicin (Invitrogen). Cells were engineered to stably express enhanced green fluorescent protein (EGFP) as described below.

Ectopic PDGFR α expression. Human full-length PDGFR α (NM_006206) and its truncated form ($\alpha\Delta X$) were expressed in the identical lentiviral vector (America Pharma Source) used to obtain stably fluorescent cells as previously described (13). The truncated PDGFR($\alpha\Delta X$) lacks amino acids 45 to 524, corresponding to the entire extracellular domain with the exception of the signal peptide (amino acids 1–44). Amino acid 45 in this truncated receptor corresponds to leucine 525 of the full-length form, which is the beginning of the transmembrane domain (22). Cells were transduced using a multiplicity of infection of 50 infectious units/cell. As the lentiviral vector contains an EGFP-IRES site, the cells expressing PDGFR α were isolated and purified by flow cytometry and sorting based on their fluorescence intensity.

Human bone marrow acquisition and processing. Bone marrow samples from healthy male donors (ages 18–45 years) were supplied by Lonza Biosciences (Poeitics Donor Program). Samples were shipped and maintained at 4°C throughout processing. Briefly, samples were centrifuged (1,500 rpm for 20 minutes) to separate the acellular and cellular phases. Supernatant containing the acellular phase was removed and filtered using 0.8- and 0.22- μ m filters in succession followed by storage at –80°C.

In vitro experimental protocols. Cells were starved of serum for 4 hours before being exposed to bone marrow or PDGF-AA (30 ng/mL). Fifty microliters of processed bone marrow were administered to cells in 1 mL of experimental medium for a final 1:20 dilution. PP2 was used at a concentration of 10 μ mol/L and IMC-3G3 was used at a concentration of 20 μ g/mL. Receptor cross-linking experiments were carried out on ice and/or at 4°C using the chemical cross-linker BS₃ (Pierce) at a concentration of 2 mmol/L. Briefly, cells were incubated with either ligand or bone marrow for the time periods indicated, followed by incubation with BS₃ for 30 minutes at 4°C. Cross-linking was then quenched by the addition of 20 mmol/L Tris for 5 minutes at room temperature.

SDS-PAGE and Western blotting. Cell lysates were obtained and SDS-PAGE and Western blot analysis performed as previously described (23) with few modifications. Membranes were blotted with antibodies targeting phospho-Akt (Ser-473, Cell Signaling Technology), PDGFR α (R&D Systems), and total Akt (Cell Signaling). Primary antibody binding was detected using a horseradish peroxidase-conjugated secondary antibody (Pierce). Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce) and detected with the Fluorochem 8900 imaging system and relative software (Alpha Innotech). Densitometry analysis was done using the UN-SCAN IT software (Silk Scientific). Samples were run on the same gels for effective comparison of intensity levels. Each experiment was repeated at least three times and provided similar results.

Detection of PDGFR α phosphorylation. Cells were washed twice with ice-cold PBS and lysed with immunoprecipitation buffer (50 mmol/L Tris, 150 mmol/L NaCl, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1% NP40) supplemented with protease and phosphatase inhibitors (Protease Inhibitor Cocktail Set III, Phosphatase Inhibitor

Cocktail Set II, Calbiochem). Cell lysates (750 μ g) were incubated with agarose-conjugated anti-PDGFR α primary antibody (Santa Cruz) for 1 hour at 4°C. Immunoprecipitation was carried out according to the manufacturer's protocol, with immunoprecipitated protein run on a 7.5% polyacrylamide gel. Western blotting was carried out as previously described using an antibody directed against phospho-tyrosine (Cell Signaling) or phospho-PDGFR α (Tyr-742, Cell Signaling). Equal loading was confirmed by stripping the membrane and blotting for total PDGFR α .

Animal model of metastasis. Five-week-old male immunocompromised mice (CB17-SCRF) were obtained from Taconic and housed in a germ-free barrier. At 6 weeks of age, mice were anesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine and successively inoculated in the left cardiac ventricle with cancer cells (5×10^4 in 100 μ L of serum-free DMEM/F12). All cancer cells used in our *in vivo* studies were stably transduced using a lentiviral vector (America Pharma Source) expressing EGFP, either with an exogenous gene insert (full-length PDGFR α or its α Δ X truncated form) or as an empty vector. PC3-ML and PC3-N cells expressing the empty vector showed metastatic abilities identical to their wild-type counterparts, as established in our previous studies (11, 13). As also previously described, the only soft-tissue organs we found to be harboring cancer cells at 4 weeks postinoculation were the adrenal glands. However, these tumors remained contained in size and never produced tumor burden in the inoculated animals (13).

Mice were sacrificed at specified time points following inoculation, and tissues prepared as described below. All experiments were conducted in accordance with the NIH guidelines for the humane use of animals. All protocols involving the use of animals were approved by the Drexel University College of Medicine Committee for the Use and Care of Animals.

Tissue preparation. Bones and soft-tissue organs were collected and fixed in 4% formaldehyde solution for 24 hours and then transferred into fresh formaldehyde for an additional 24 hours. Soft tissues were then placed either in 30% sucrose for cryoprotection or in 1% formaldehyde for long-term storage. Bones were decalcified in 0.5 mol/L EDTA for 7 days followed by incubation in 30% sucrose. Tissues were maintained at 4°C for all aforementioned steps and frozen in optimum cutting temperature medium (Electron Microscopy Sciences) by placement over dry ice-chilled 2-methylbutane (Fisher). Serial sections of 80- μ m thickness were obtained using a Microm HM550 cryostat (Mikron).

Fluorescence stereomicroscopy and morphometric analysis of metastases. Bright-field and fluorescent images of skeletal metastases were acquired using a SZX12 Olympus stereomicroscope coupled to an Olympus DT70 CCD color camera. Digital images were analyzed with ImageJ software (<http://rsb.info.nih.gov/ij/>) and calibrated for measurement by obtaining the pixel-to-millimeter ratio. Morphometric evaluation of skeletal tumors was conducted by analysis of serial cryosections, in which the largest representative tumor section for each metastasis was identified. A freehand tool was used to outline the border of each metastatic lesion, and the area was computed using the ImageJ "measure area" function.

Statistics. Data shown in figures are representative of at least two experiments providing similar results. We analyzed the number and size of skeletal metastases between groups using a two-tailed Student's *t* test. *P* $\leq$ 0.05 was considered statistically significant.

Results and Discussion

We have previously shown that the activation of the PI3K/Akt pathway in bone-metastatic prostate cancer cells exposed to human bone marrow aspirates depends mostly on

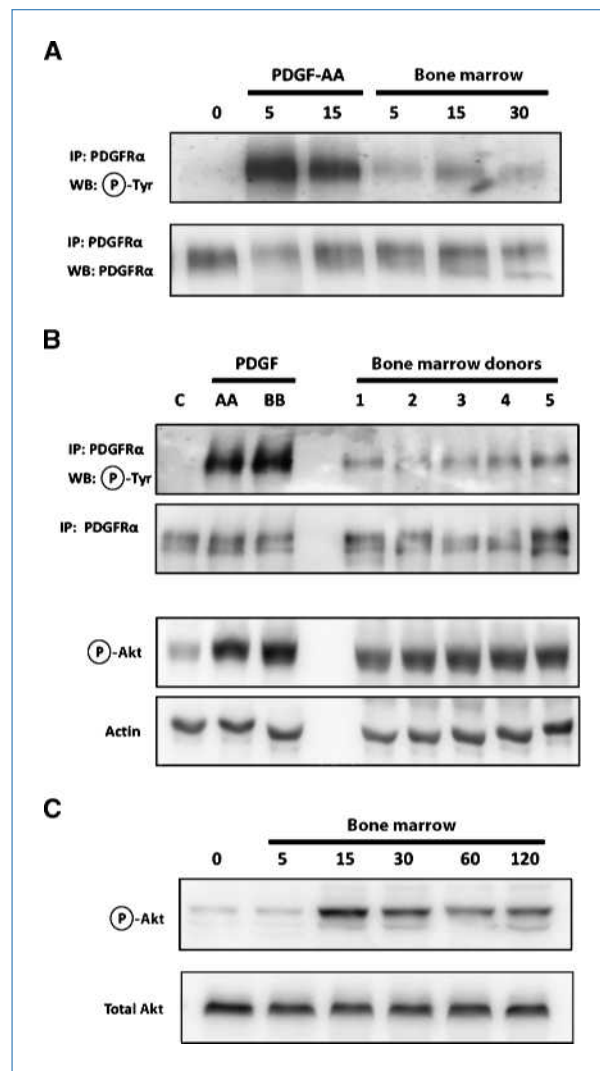


Figure 1. Human bone marrow induces phosphorylation of PDGFR α and activation of downstream Akt pathway in prostate cancer cells. Exposure of PC3-ML prostate cancer cells to acellular human bone marrow induces phosphorylation of PDGFR α , although of a lower magnitude compared with that produced by the proper ligand PDGF-AA (30 ng/mL; A). This effect was consistently reproduced by all human samples tested (B, top). Despite producing a lower extent of PDGFR α phosphorylation than PDGF-AA, bone marrow activated the downstream signaling kinase Akt to an extent almost equivalent to that of the growth factor (B, bottom) and in a time-dependent fashion (C).

PDGFR α signaling (11). Furthermore, prostate cancer cells that lack bone-metastatic potential—and are less responsive to bone marrow—could activate Akt to the same extent as bone-metastatic cells following PDGFR α overexpression (13). Therefore, our first series of experiments sought to ascertain whether the acellular fraction of human bone marrow would induce phosphorylation of PDGFR α , as is the case when this receptor binds and is activated by its proper PDGF ligands (24). As done for our previous studies, we used PC3-ML cells, a subpopulation of the human PC3 cell line originally derived from a skeletal metastasis in a patient affected by prostate adenocarcinoma (25). PC3-ML cells were originally selected for their invasiveness *in vitro* in concert with their bone-metastatic potential in animal models (21). When these cells were exposed to bone marrow, PDGFR α phosphorylation was clearly detected and lasted at least 30 minutes (Fig. 1A). The ability of bone marrow to phosphorylate PDGFR α could be consistently observed using aspirates withdrawn from different donors (Fig. 1B, top).

Structural and functional studies have shown that PDGF binding induces dimerization of PDGFR; this allows the

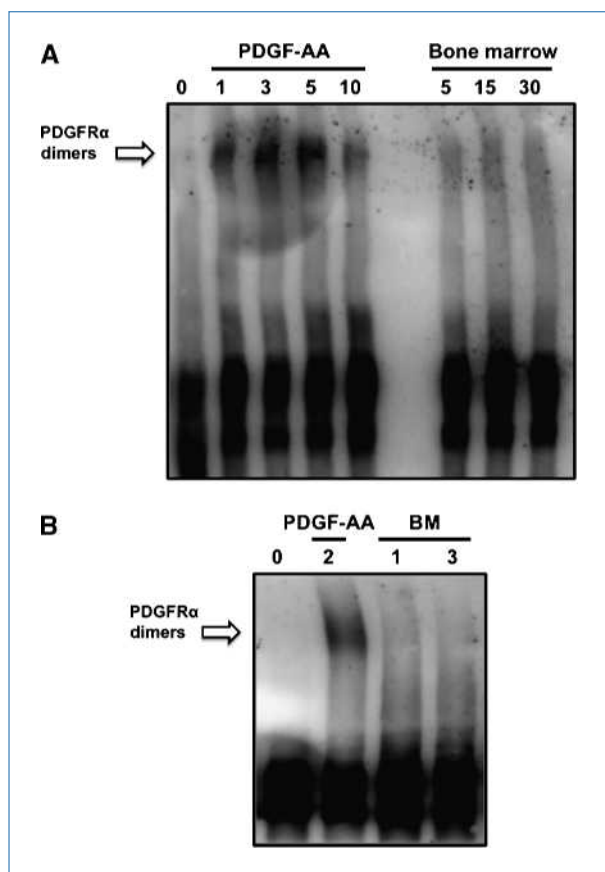


Figure 2. Recruitment of PDGFR α by human bone marrow occurs in the absence of receptor dimerization. Phosphorylation of PDGFR α by PDGF-AA was preceded by receptor dimerization, observed as soon as 1 min after exposure of cells to the ligand. However, bone marrow (BM) did not induce PDGFR α dimerization even in cells that were stimulated for up to 30 min.

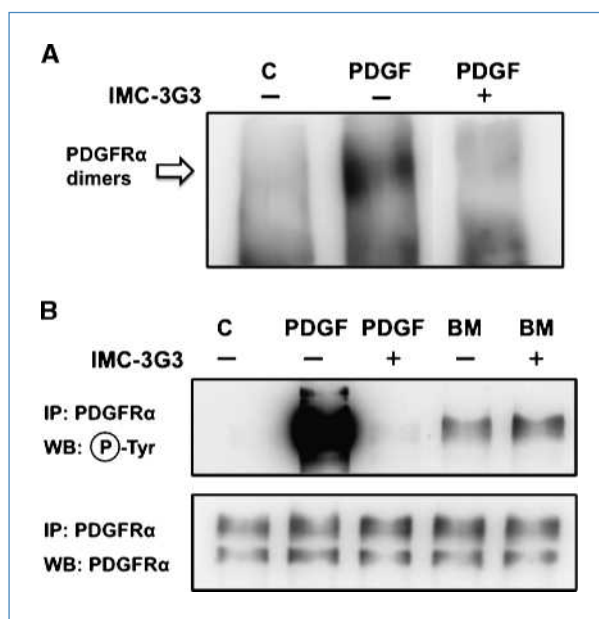


Figure 3. Bone marrow-mediated activation of PDGFR α occurs on blockade of ligand-induced receptor dimerization. Blockade of the ligand binding domain of PDGFR α using the monoclonal antibody IMC-3G3 (20 μ g/mL) prevents receptor dimerization from occurring in response to PDGF-AA ligand (A). As expected, blockade of receptor dimerization prevented receptor phosphorylation in cells exposed to PDGF-AA. However, bone marrow was able to activate PDGFR α despite blockade of PDGFR α dimerization (B).

trans-phosphorylation of several conserved tyrosine residues on the juxtaposed intracellular portion of each receptor (26). In addition to enhancing the catalytic activity of the kinase domain, this event creates docking sites for the binding and activation of signal transduction mediators containing SH2 domains, including members of the PI3K family (27). At least eight tyrosine residues on PDGFR α become phosphorylated on binding of PDGF ligand(s) (28), likely contributing to the strong signal observed in our experiments (Fig. 1A and B).

Interestingly, the considerably lower magnitude of PDGFR α phosphorylation induced by bone marrow, as compared with that induced by PDGF ligand(s), did not translate to a significantly lower activation of the downstream Akt pathway. In fact, remarkably close levels of Akt phosphorylation were observed after exposing PC3-ML cells to either PDGF or bone marrow (Fig. 1B, bottom) and as evidenced by a time-dependent kinetics (Fig. 1C). In addition, we had previously shown that the majority of this observed Akt activation is due solely to PDGFR α signaling (11). This suggests that the activation of PI3K, which is functionally upstream of and mainly responsible for Akt phosphorylation, must be almost equivalent in these two conditions. Thus, we speculate that the majority of PDGFR α phosphorylation caused by bone marrow corresponds to Tyr-731 and Tyr-742 residues, which specifically bind and activate PI3K (24). This would explain the lower magnitude of receptor phosphorylation as compared with stimulation by PDGF

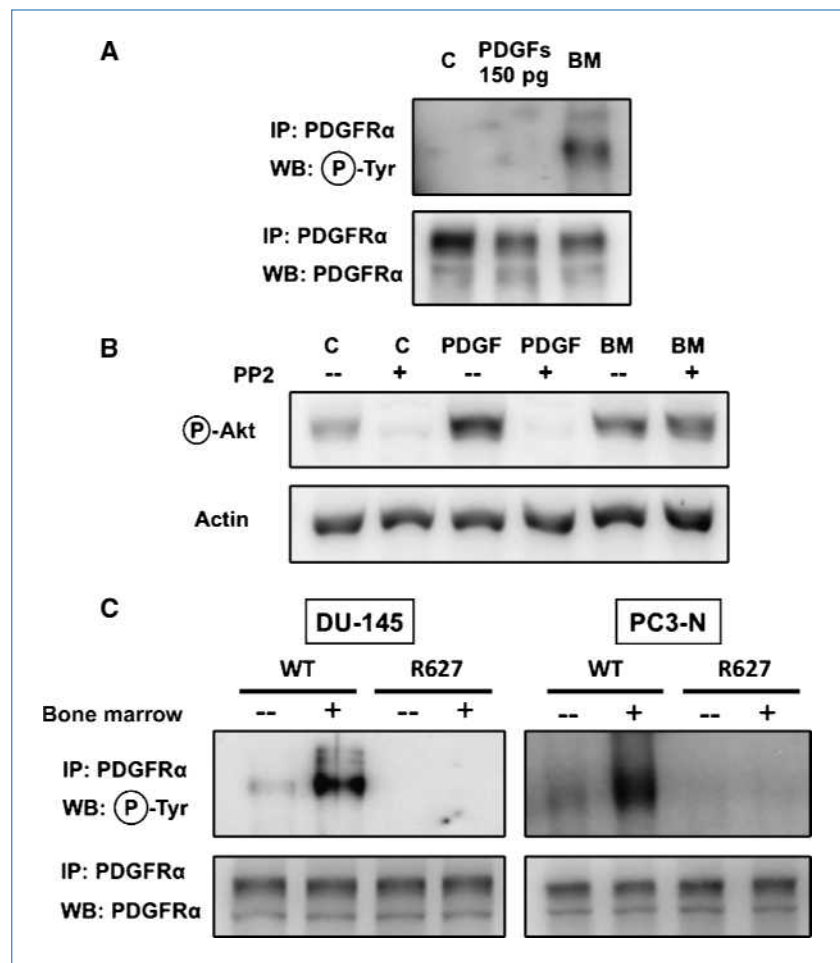
ligands. However, the phosphorylation of only select tyrosine residues could hardly be justified in the event that bone marrow induces PDGFR α dimerization. In fact, whereas dimers could be observed on exposure of PC3-ML cells to PDGF-AA, human bone marrow consistently lacked the ability to induce dimerization of PDGFR α in these cells (Fig. 2A and B).

This initial evidence of an unorthodox mechanism used by bone marrow to initiate PDGFR α signaling was further confirmed by a second set of experiments in which dimerization was blocked using a monoclonal antibody directed against the extracellular portion of the receptor. The humanized antibody IMC-3G3 blocks the ligand-binding domain of PDGFR α (11, 29), thereby inhibiting receptor dimerization following treatment with PDGF-AA (Fig. 3A). By blocking ligand-induced dimerization, IMC-3G3 also prevented PDGFR α trans-phosphorylation (Fig. 3B), which normally occurs by juxtaposition of intracellular kinase domains between receptor monomers (27, 30). However, no differences in PDGFR α phosphorylation were observed when cells were exposed to bone marrow alone or in the presence of IMC-3G3 (Fig. 3B). This result provides compelling evidence that dimerization is not a prerequisite for the activation of

PDGFR α by the soluble fraction of human bone marrow in PC3-ML prostate cancer cells. If this was indeed the case, an active kinase must still phosphorylate the tyrosine residues responsible for recruitment of PI3K to the receptor, as this step should precede downstream activation of Akt by PDGFR α exposed to bone marrow. One likely possibility was the heterodimerization of PDGFR α with other tyrosine kinase receptors. However, this event could be excluded by the absence of any dimer formation following treatment of cells with bone marrow and subsequent cell-surface receptor cross-linking (Fig. 2).

Thus, we hypothesize that PDGFR α is activated without recruitment of its ligand-binding domain and consequent dimerization, possibly by a soluble tyrosine kinase that phosphorylates the monomeric form of the receptor. In fact, this idea is strongly supported by a recent study of proliferative vitreoretinopathy showing that activated Src family kinases (SFK) phosphorylate PDGFR α in mouse embryo fibroblasts exposed to rabbit vitreous humor, the clear viscous fluid that occupies the space between the retina and lens of vertebrates' ocular bulb (31). In these cells, growth factors outside of the PDGF family and present in the vitreous can activate their specific receptor(s), thereby increasing the levels of

Figure 4. PDGF-independent but receptor kinase-dependent phosphorylation of PDGFR α induced by human bone marrow. Exposure of PC3-ML cells to the same concentrations of PDGF ligands detected in human bone marrow fails to achieve receptor phosphorylation (A) or downstream Akt signaling (11). Signaling through PDGFR α in PC3-ML cells exposed to PDGF ligands depends on the activation of SFKs and was blocked with PP2, an inhibitor of this family of kinases. In contrast, bone marrow-induced signaling through PDGFR α was insensitive to PP2 (B). The R627 kinase-dead mutant of PDGFR α was overexpressed in both DU-145 cells (C, left) and PC3-N cells (C, right) and shows that bone marrow-mediated activation of PDGFR α requires the receptor kinase domain for its phosphorylation.



intracellular ROS. This event led to the direct activation of SFKs, which then mediated the phosphorylation of PDGFR α (19). We have previously shown that human bone marrow activates downstream Akt despite containing negligible levels of PDGF ligands (measured at 150 pg/mL by ELISA; ref. 11) and also induces evident PDGFR α phosphorylation (Fig. 4A). Thus, if bone marrow were engaging PDGFR α in prostate cancer cells with a mechanism analogous to vitreous humor in mouse embryo fibroblasts, this would similarly require an increase in intracellular ROS. In this case, either antioxidants or inhibitors of SFKs should then block the activation of PDGFR α . To test this possibility, we treated PC3-ML cells either with 5 mmol/L hydrogen peroxide alone or with bone marrow in the presence of the antioxidant NAC (10 mmol/L). The first condition—adopted to induce intracellular ROS production—was unable to induce PDGFR α phosphorylation. Analogously, NAC did not prevent the activation of the receptor and downstream PI3K/Akt signaling caused by bone marrow (data not shown).

We could also exclude the involvement of SFKs in the activation and signaling of PDGFR α induced by bone marrow by using PP2, a specific inhibitor of this family of kinases

(32, 33). In PC3-ML cells, PP2 did not prevent Akt phosphorylation by bone marrow (Fig. 4B), whereas it completely blocked stimulation of this pathway by PDGF-AA. This finding is in agreement with a described role of SFKs in coordinating ligand-induced recruitment of signaling pathways by tyrosine kinase receptors, by acting upstream of PI3K (34, 35).

Taken together, these results lead us to conclude that human bone marrow activates PDGFR α on prostate cancer cells in a manner that is distinct from the factors found in vitreous humor, supporting the idea that there may be multiple mechanisms whereby this receptor can be activated without the involvement of its canonical PDGF ligands.

It is widely recognized that the activity of the kinase domain in tyrosine kinase receptors is significantly augmented on dimerization and trans-phosphorylation (30). Because we observed a lack of PDGFR α dimerization on exposure of prostate cancer cells to human bone marrow, one would predict that the kinase domain is neither necessary nor responsible for the phosphorylation of monomeric PDGFR α . To effectively investigate this idea, we used two different prostate cancer cell lines that show low levels of this receptor (PC3-N) or completely fail to express it (DU-145; ref. 12)

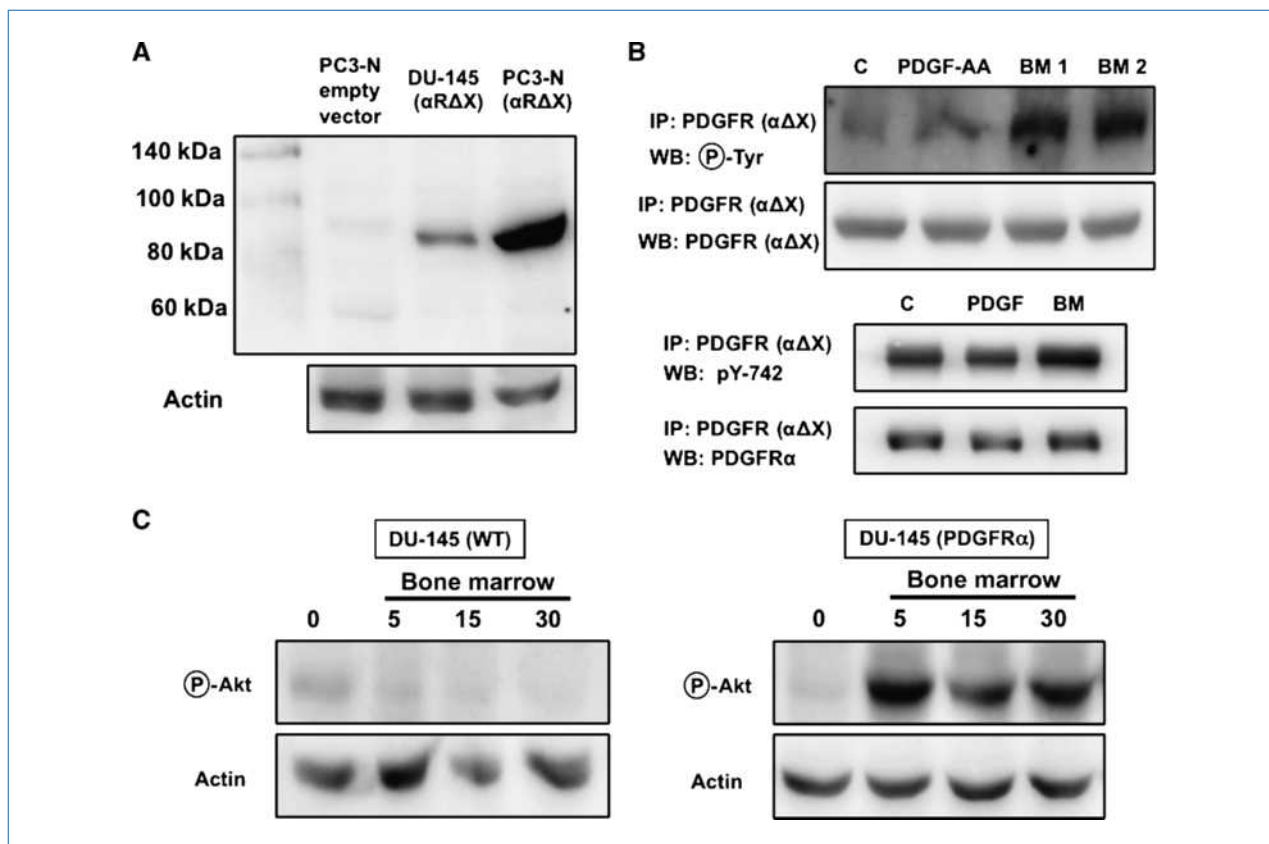


Figure 5. PDGFR α activation by human bone marrow is independent of the extracellular ligand-binding domain. A truncated PDGFR α missing the extracellular binding domain ($\alpha\Delta X$) was stably expressed in DU-145 cells, which normally lack PDGFR α expression (A; ref. 12). As expected, this mutated PDGFR α could not be phosphorylated in cells exposed to PDGF-AA; however, two different samples of human bone marrow elicited a strong receptor phosphorylation (B, top), which involved the Tyr-742 residue, a specific binding site for PI3K (B, bottom). Exposure of wild-type (WT) DU-145 cells to bone marrow did not produce an appreciable activation of the PI3K/Akt pathway, which was conferred by the stable expression of PDGFR α (C).

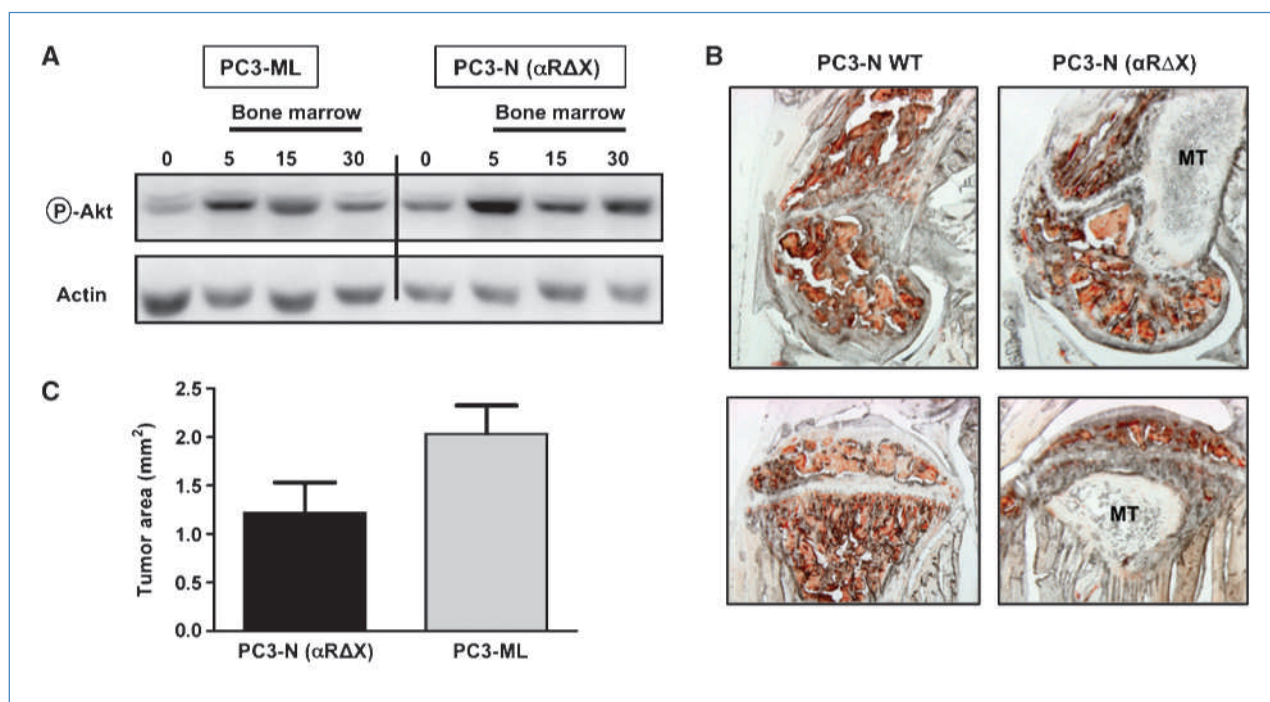


Figure 6. PDGFR(Δ X) induces a bone metastatic phenotype that is indistinguishable from cells expressing full-length PDGFR α . Expression of PDGFR(Δ X) in non-bone-metastatic PC3-N cells results in an increased activation of downstream Akt on exposure to human bone marrow, to an extent comparable to that observed in bone-metastatic PC3-ML cells (A). PC3-N(α Δ X) were able to successfully form skeletal lesions 4 wk after their intracardiac inoculation in SCID mice (B; top, femur; bottom, tibia). MT, metastatic tumor. Importantly, the size of metastases produced by PC3-N(α Δ X) cells did not differ significantly from lesions formed by the metastatic PC3-ML cells, which express substantially higher levels of endogenous, full-length PDGFR α (C). Seven mice were inoculated with PC3-N(α Δ X) cells in two different experiments and presented 13 skeletal tumors in total. Eight mice were inoculated with PC3-ML-(empty vector) cells in two different experiments and presented 22 skeletal tumors in total.

and engineered them to stably express a kinase-inactive PDGFR α mutant (R627; ref. 36). Surprisingly, both R627-expressing cell types failed to show receptor phosphorylation on bone marrow exposure, in contrast to when the same cell types overexpress the wild-type form of PDGFR α (Fig. 4C). Thus, these results implicate the kinase domain in the phosphorylation and signaling of monomeric PDGFR α induced by bone marrow. This observation suggests the possibility of a receptor autophosphorylation event independent of ligand-induced dimerization and most likely affecting the tyrosine residues responsible for PI3K binding and activation. The identification of the mechanism and signaling mediators responsible for this event is the focus of an ongoing investigation in our laboratory.

In previous animal studies, we have shown that PDGFR α can confer bone-metastatic potential to prostate cancer cells (13). Intriguingly, the role exerted by this receptor in the metastatic process may require its indirect activation, likely through the activity of yet unidentified factor. To further investigate this peculiarity of PDGFR α signaling, we stably transfected cancer cells with a truncated receptor mutant (α Δ X) that lacks the extracellular ligand-binding domain (see Materials and Methods for details) and is therefore unable to bind or be activated by proper PDGF ligand(s) or additional molecules that could activate the receptor in a spurious fashion (22).

A first set of experiments was conducted using DU-145 prostate cancer cells, which normally do not express PDGFR α (12) and would therefore not interfere by providing additional signaling through its wild-type full form. The α Δ X expressed in these cells (Fig. 5A) was clearly insensitive to PDGF-AA, as expected due to its lack of binding capability and shown by the absence of any receptor phosphorylation (Fig. 5B, top). However, exposure of DU-145(α Δ X) cells to human bone marrow aspirates induced strong phosphorylation of the truncated receptor, conclusively showing its ligand-independent activation (Fig. 5B, top). This phosphorylation involved the Tyr-742 residue (Fig. 5B, bottom), which on PDGFR α specifically corresponds to the PI3K binding site through its SH2 domain (24, 30), unequivocally linking the soluble fraction of human bone marrow to the downstream activation of the Akt signaling pathway in prostate cancer cells. This event could be observed in cells either constitutively expressing PDGFR α (Fig. 1; refs. 11, 13) or engineered to express the exogenous form of this receptor (Fig. 5C).

A second series of experiments aimed to ascertain whether the α Δ X receptor could reproduce the enhanced capacity of skeletal metastases from prostate cancer cells, as we had previously shown for its full-length form (13). A confirmatory result would convincingly show that PDGFR α could induce a prometastatic phenotype, with pronounced bone tropism, through a ligand-independent transactivation caused by the

acellular fraction of bone marrow. PC3-N cells were originally selected from the parental PC3 cell line for their lack of invasiveness *in vitro* (21). We have previously shown that PC3-N cells express lower levels of PDGFR α and respond weakly to human bone marrow as compared with their bone-metastatic counterpart PC3-ML cells (12, 13). Most importantly, PC3-N cells disseminate to the bone when inoculated in the blood circulation of severe combined immunodeficient (SCID) mice, but fail to grow into macroscopic skeletal metastases unless they are engineered to overexpress full-length PDGFR α (13). When PC3-N cells were stably transduced with a lentiviral vector expressing PDGFR($\alpha\Delta X$) (Fig. 5A) and exposed to bone marrow, Akt was phosphorylated to the same extent observed in either PC3-ML cells (Fig. 6A) or PC3-N cells expressing full-length PDGFR α treated in the same fashion.

To test their bone-metastatic potential, PC3-N($\alpha\Delta X$) cells were then inoculated via an intracardiac route into SCID mice, and the skeletal metastases observed at 4 weeks postinoculation were compared with those detected in mice receiving the highly bone-metastatic PC3-ML cells. When the bone tumors in these two groups of mice were compared, no significant differences in size were observed and the location and bone-destructive properties induced by the $\alpha\Delta X$ and full-length PDGFR α were nearly identical (Fig. 6B and C). These results show that PDGFR α can both increase the responsiveness of human prostate cancer cells to acellular bone marrow *in vitro* and promote their progression in the bone microenvironment in the absence of any involvement of its ligand-binding domain.

Recognizing that PDGFR α could contribute to dissemination and metastatic progression of prostate adenocarcinoma

independently of direct ligand stimulation has significant translational implications. It could be inferred that anticancer therapeutics that are designed to block PDGF ligand binding to PDGFR α would not prevent the activation of downstream signaling pathways in cells that have spread to the bone marrow. In contrast, therapeutic approaches that induce internalization of PDGFR α may provide a better option to inhibit downstream signaling elicited by this receptor in disseminated prostate cancer cells when exposed to the bone marrow microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeting the α Receptor for Platelet-Derived Growth Factor as a Primary or Combination Therapy in a Preclinical Model of Prostate Cancer Skeletal Metastasis

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Abstract

Purpose: Platelet-derived growth factor α (PDGFR α) is highly expressed in primary prostate cancer and associated skeletal metastases. Here, we tested whether targeting this receptor could impair metastatic colonization and progression, as well as prolong survival, either as primary or as combination therapy.

Experimental Design: We used a preclinical animal model of metastasis in which PC3-ML human prostate cancer cells are inoculated directly in the blood circulation. First, the humanized, monoclonal antibody IMC-3G3 was administered to mice bearing established skeletal metastases. Second, we targeted the stromal PDGFR α with IMC-1E10, an antibody specific for the murine receptor. Third, IMC-3G3 and the bisphosphonate zoledronic acid (ZA), administered separately or in combination, were tested on the progression of skeletal lesions and overall survival. In addition, the ability of IMC-3G3 and ZA to impair initial colonization of the bone marrow by prostate cancer cells was investigated.

Results: The blockade of PDGFR α on prostate cancer cells by IMC-3G3 reduces the size of established skeletal metastases, whereas the IMC-1E10 antibody directed against the stromal PDGFR α fails to inhibit metastatic progression. IMC-3G3 and ZA, either separately or in combination, significantly slow tumor growth and seem to prolong survival. Lastly, the blockade of PDGFR α by IMC-3G3 inhibits the initial phase of bone colonization, whereas ZA is ineffective at this stage.

Conclusion: This study presents compelling evidence that targeting PDGFR α with IMC-3G3 delays the progression of early metastatic foci and reduces the size of more established lesions. In addition, IMC-3G3, either alone or in combination with ZA, prolongs survival in animal models. *Clin Cancer Res*; 16(20); 5002–10. ©2010 AACR.

Although skeletal metastases occur with a significantly high rate in patients affected by advanced prostate carcinoma (1, 2), a limited range of options is currently available for the treatment of metastatic bone lesions (3, 4). Equally frustrating is the evidence that the burden deriving from skeletal dissemination represents the main cause of death for these patients, but the current standard of care for advanced prostate cancer produces merely palliative effects (5). Skeletal metastases cause bone resorption and chronic pain and predispose patients to skeletal-related events such as pathologic fractures and spinal cord compression. This skeletal morbidity is presently treated with the administration of bisphosphonates in prophylactic and adjuvant settings (6). Bisphosphonates are inorganic pyr-

rophosphates that inhibit the enzymatic activities of osteoclasts, the cells responsible for the degradation of bone matrix (7, 8). These compounds are effective in alleviating bone loss and pain, as well as reducing the occurrence of skeletal-related events (9, 10). However, bisphosphonates are not without side effects, including renal toxicity, hypocalcemia, and osteonecrosis of the jaw, which may prevent their long-term administration in bone-metastatic prostate cancer patients (11, 12).

In addition to the management of symptoms produced by skeletal involvement and the prevention of skeletal-related event, the use of bisphosphonates in the clinic to target osteoclasts is also aimed to directly inhibit cancer cell growth and survival. This approach is based on evidence that the degradation of bone matrix releases a variety of trophic factors capable of supporting the disseminated cancer cells in their metastatic growth. The resulting increase in tumor mass will recruit and activate a progressively higher number of osteoclasts, thereby generating a self-sustained vicious cycle (13–15). Unfortunately, results from clinical trials have not been encouraging and seem to indicate that, although successful in reducing bone loss and pain, bisphosphonates are ineffective in arresting disease progression or improving survival in patients with metastatic prostate

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Translational Relevance

Skeletal metastases are responsible for significant morbidity and represent the main cause of death in patients with advanced prostate adenocarcinoma. The treatment options currently available for these patients are mostly palliative.

Platelet-derived growth factor α is responsible for conferring bone-metastatic potential to prostate cancer cells. Our studies provide preclinical evidence that the blockade of this receptor is a new and very effective approach to treat skeletal secondary tumors. We targeted platelet-derived growth factor α with IMC-3G3, a humanized monoclonal antibody that is entering phase II clinical trials for advanced solid tumors, including prostate cancer. We showed that this antibody has a broad spectrum of activity because it is effective on initial and established skeletal lesions. In addition, alone and in combination with the bisphosphonate zoledronic acid, IMC-3G3 significantly prolongs survival. This work will expedite the path toward the clinical use of this antibody as a new treatment for skeletal dissemination of prostate adenocarcinoma.

cancer (6, 16). This scenario is likely determined by the fact that disseminated cancer cells may benefit from additional trophic support that does not derive from bone matrix degradation but is readily available in the bone marrow environment and, therefore, not necessarily dependent on osteoclast activity. Indeed, our previous studies have shown that isolated cancer cells and small foci detected shortly after their initial colonization of the bone are either independent of or much less reliant on spatial interactions with osteoclasts than are larger, more advanced lesions (17). Thus, it seems plausible that other compounds used in combination with bisphosphonates, such as those that target survival and/or proliferative pathways in cancer cells, may result in pronounced curative effects.

The α receptor for platelet-derived growth factor (PDGFR α) is a receptor tyrosine kinase that has been correlated with advanced prostate carcinoma and was found associated with skeletal metastases by others (18–20) and us (17, 21). The activation of this receptor in prostate cancer cells is responsible for the recruitment of the PI3K/Akt cell survival pathway. To target the human PDGFR α , we used the humanized monoclonal antibody IMC-3G3 (ImClone, Eli Lilly). This antibody was developed for the treatment of several pathologic conditions and is currently in phase II clinical trials (22). We found that IMC-3G3 suppresses PDGFR α -mediated activation and downstream Akt stimulation by inducing receptor internalization (23). Interestingly, we have also shown that the soluble fraction of human bone marrow is able to transactivate human PDGFR α through a ligand- and dimerization-independent mechanism (23, 24). Thus, IMC-3G3 could

negatively affect the survival of PDGFR α -expressing prostate cancer cells by internalizing the receptor and thereby impeding its transactivation and consequent downstream signaling. Indeed, we have previously observed a strong antimetastatic effect when using IMC-3G3 prophylactically in a preclinical model of bone-metastatic prostate cancer. In this context, the work presented here expands our previous study by addressing any antimetastatic effect of IMC-3G3 administered in a curative protocol, as well as the role played by the simultaneous targeting of PDGFR α on cancer and stromal cells. We also report that IMC-3G3, unlike bisphosphonates, is extremely effective in reducing metastatic growth during the first week following the arrival of cancer cells to the skeleton. Finally, we show that the administration of IMC-3G3, alone or in combination with a bisphosphonate, prolongs the survival of mice bearing skeletal metastases from human prostate cancer cells.

Materials and Methods

Reagents

The IMC-3G3 and IMC-1E10 antibodies were provided by ImClone, Eli Lilly. The bisphosphonate zoledronic acid (ZA) was obtained from Sigma.

Cell lines and cell culture

NIH-3T3 cells were obtained from American Type Culture Collection, whereas the highly bone-metastatic PC3-ML subline was derived from the parental PC-3 cells (American Type Culture Collection) as previously described (25). Cells were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 0.1% gentamicin (Invitrogen). The PC3-ML cells were engineered to stably express enhanced variant of green fluorescent protein (GFP) by using a lentiviral vector from America Pharma Source (17).

In vitro experimental protocol

Cells were washed twice with sterile PBS and then starved from serum for 4 hours before being incubated for 30 minutes with either IMC-1E10 or IMC-3G3 (20 μ g/mL) and successively exposed to PDGF-AA (30 ng/mL).

SDS-PAGE and Western blotting

Cell lysates were obtained and SDS-PAGE and Western Blot analysis were done as previously described (24), with few modifications. Membranes were blotted with antibodies targeting phosphor-Akt (Ser-473; Cell Signaling Technology), PDGFR α (R&D Systems), and total Akt (Cell Signaling). Primary antibody binding was detected using a horseradish peroxidase-conjugated secondary antibody (Pierce). Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce) and were detected with the Fluorochem 8900 imaging system and relative software (Alpha Innotech). Densitometry analysis was done using the UN-SCAN IT software (Silk Scientific).

Animal model of metastasis

Five-week-old, male, immunocompromised mice (CB17-SCRF) were obtained from Taconic and were housed in a germ-free barrier. At 6 weeks of age, mice were anesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine and successively inoculated in the left cardiac ventricle with PC3-ML cells (5×10^4 in a volume of 100 μ L of serum-free DMEM/F12). Although consistently producing skeletal tumors in tibiae and femora of >80% of inoculated mice, PC3-ML cells do not metastasize to any soft-tissue organ with the exception of small tumors produced in the adrenal glands and, very seldom, the liver. Mice were sacrificed at specified time points following inoculation and tissues prepared as described below. For the survival studies, mice were euthanized when in a moribund state as defined by evident and prolonged shivering, extreme prostration, labored breathing, >20% of body weight loss, and/or unresponsiveness to external stimulation. All experiments were conducted in accordance with NIH guidelines for the humane use of animals. All protocols involving the use of animals were approved by the Drexel University College of Medicine Committee for the Use and Care of Animals.

Administration of antibodies for PDGFR α

Animals received a loading dose of 214 mg/kg immediately after inoculation with PC3-ML cells, followed by a maintenance dose of 60 mg/kg every 72 hours thereafter. Doses and administration schedule were selected based on pharmacokinetic and tumor-growth studies (26). Control mice were maintained on an identical dosing schedule and received similar injection volumes of either saline or human immunoglobulins of the IgG₁ subclass as the IMC-3G3 and IMC-1E10 antibodies (26).

Tissue preparation

Tibiae and femora were collected and fixed in 4% formaldehyde solution for 24 hours and then transferred into fresh formaldehyde for additional 24 hours. Bones were decalcified in 0.5 mol/L EDTA for 7 days, followed by incubation in 30% sucrose for cryoprotection or 1% formaldehyde for long-term storage. Tissues were maintained at 4°C during all aforementioned steps and frozen in optimal cutting temperature (OCT) medium (Electron Microscopy Sciences) by placement over dry ice-chilled 2-methylbutane (Fisher). Serial sections of 80 μ m in thickness were obtained using a Microm HM550 cryostat (Mikron).

Fluorescence stereomicroscopy and morphometric analysis of metastases

Bright-field and fluorescent images of skeletal metastases were acquired using a SZX12 Olympus stereomicroscope coupled to an Olympus DT70 CCD color camera. Digital images were analyzed with ImageJ software (<http://rsb.info.nih.gov/ij/>) and calibrated for measurement by obtaining a pixel to millimeter ratio. Morphometric evaluation of skeletal tumors was conducted by analysis of serial cryosections, in which the largest repre-

sentative tumor section for each metastasis was identified. A freehand tool was used to outline the border of each metastatic lesion, and the area was computed using the ImageJ "measure area" function.

Tartrate-resistant acid phosphatase staining

Slides were incubated at 37°C for 5 minutes in a solution containing naphthol 7-Bromo-3-hydroxy-2-naphthoic-*o*-anisidine phosphate and ethylene glycol monoethyl ether (Sigma). Slides were then transferred to a solution containing sodium nitrite and pararosaniline chloride (Sigma) for ~3 minutes.

Statistics

We analyzed number and size of skeletal metastases between groups using a two-tailed Student's *t* test. Statistical significance between multiple groups was conducted using a one-way ANOVA, followed by Tukey's multiple comparison test. A value of $P \leq 0.05$ was considered statistically significant.

Results and Discussion

Previous studies from our group have shown that bone-metastatic human prostate cancer cells express high levels of PDGFR α (21). The exposure of these cells to the soluble fraction of human bone marrow induces phosphorylation of PDGFR α and its downstream activation of the PI3K/Akt survival pathway (23). The antagonism of human PDGFR α achieved with the IMC-3G3 antibody attenuates the signaling events *in vitro* and reduces the number and size of skeletal metastases in preclinical animal models (17). These studies show a prophylactic action of the IMC-3G3 antibody on skeletal metastases; here, we sought to determine whether PDGFR α inhibition could also produce a curative effect on metastatic bone lesions that had sufficient time to establish and progress before IMC-3G3 administration. To this end, we used a model of experimental metastasis that uses PC3-ML human prostate cancer cells previously isolated from the PC3 parental cell line for their high bone-metastatic potential (25). These cells were engineered to stably express an enhanced variant of GFP and directly inoculated in the blood circulation of immunocompromised severe combined immunodeficient mice through the left cardiac ventricle. Metastatic tumors ranging from microscopic foci to macroscopic lesions were identified and measured as previously described (17).

A first set of experiments was conducted with mice inoculated with PC3-ML cells and left untreated for either 7 or 14 days, thus providing a time interval for metastatic tumor growth. Following this first period, treatment with IMC-3G3 began and continued until sacrifice at 4 weeks postinoculation. The dosing protocol consisted of a loading dose followed by maintenance doses administered three times weekly through an i.p. route (17). Control mice were maintained on an identical dosing schedule and received either saline or human immunoglobulins of the same IgG₁ subclass as the IMC-3G3 antibody

(26), with both controls providing identical results. At the end of the study, mice were euthanized and the area of metastatic lesions was measured by fluorescence microscopy of frozen tissue sections. Animals that were administered IMC-3G3 according to these two different curative protocols presented with skeletal lesions in the tibia and femur that seemed significantly reduced in size from those observed in mice from control groups (Fig. 1A). In addition, delaying administration of IMC-3G3 for 1 or 2 weeks following tumor cell inoculation resulted in nearly identical growth inhibition (Fig. 1B). This strongly suggests that prostate cancer cells that express PDGFR α still depend on this receptor for their growth and survival when tumor foci occupy an average area of the bone marrow $<35 \pm 6 \times 10^3 \mu\text{m}^2$ as we determined earlier (17). Interestingly, only at this point in time the bone lesions in our model became spatially associated with the surrounding osteoclasts. In fact, a comparable lack of osteoclast involvement during

the initial stages of bone colonization has been also observed in humans (27). Thus, our data seem to point out to a role for PDGFR α in sustaining metastatic progression in the bone before the onset of osteolysis and induction of the vicious cycle.

Multiple studies have shown that reciprocal interactions between cancer cells and the surrounding stroma play a critical role in tumor growth (28–30). For instance, it has been shown that stromal PDGFR α maintains tumor growth and local angiogenesis when stimulated by PDGF-AA, PDGF-CC, or vascular endothelial growth factor-A secreted by cancer cells (31, 32). Based on this evidence, we sought to establish the contribution of the PDGFR α expressed by stromal cells (i.e., osteoblasts and cells of the mesenchymal compartment of the bone marrow) on the skeletal colonization and metastatic progression of PC3-ML cells. The IMC-3G3 antibody has been shown to be highly selective for human PDGFR α (26). We confirmed this specificity

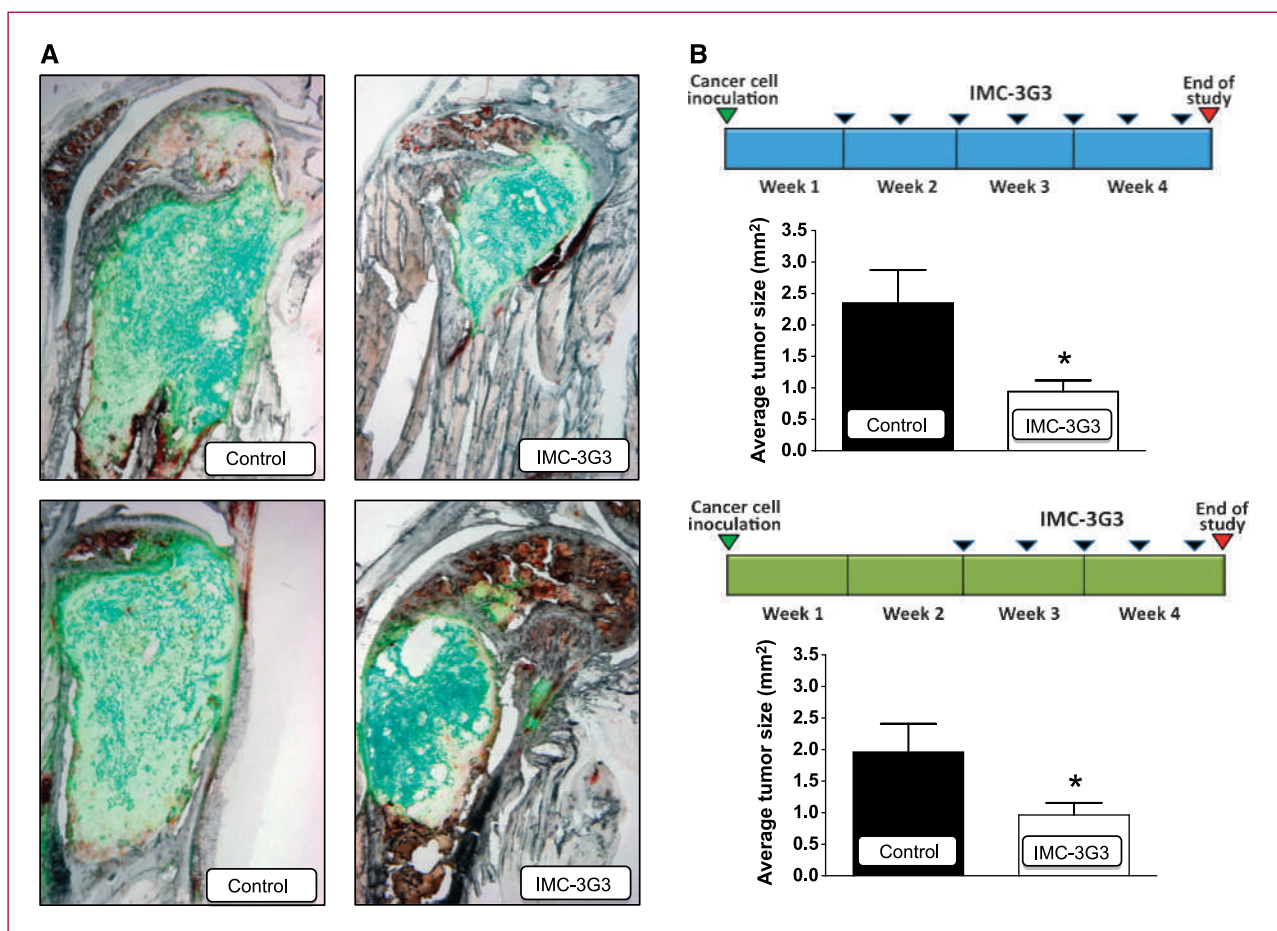


Fig. 1. The monoclonal antibody for human PDGFR α IMC-3G3 reduces the size of established skeletal metastases. Six-week-old male CB17-SCRF mice were inoculated with PC3-ML cells expressing an enhanced variant of GFP through the left cardiac ventricle. Mice had withheld treatment for either 1 or 2 weeks, after which they were maintained on IMC-3G3 for the remainder of the experiment. After 4 weeks, mice were euthanized, their tibiae and femora were cryosectioned, and metastatic skeletal lesions were identified by fluorescence stereomicroscopy and measured. Mice that received IMC-3G3 showed a significant reduction in the average size of bone tumors in 1-week (A) and 2-week (B) treatment-withheld conditions as compared with controls. One-week group, controls ($n = 8$) and IMC-3G3 ($n = 7$); 2-week group, controls ($n = 10$) and IMC-3G3 ($n = 8$). Two different experiments were conducted for each experimental group. *, $P \leq 0.05$.

by exposing NIH-3T3 mouse fibroblasts to PDGF-AA upon pretreatment with IMC-3G3 and noticed that the phosphorylation of Akt was unaffected as compared with control cells (Fig. 2A). Therefore, to effectively target the stroma, we used IMC-1E10, a humanized antibody selected for binding to murine PDGFR α and otherwise sharing an identical structure with IMC-3G3 (33). The species selectivity of this antibody was confirmed by the complete inhibition of Akt phosphorylation observed in NIH-3T3 cells stimulated with PDGF-AA (Fig. 2A).

In the ensuing studies, mice were inoculated with PC3-ML cells and randomly assigned to three experimental groups, which received saline solution (control group), the IMC-1E10 antibody alone, or IMC-1E10 and IMC-3G3 antibodies in combination. Mice were treated according to a prophylactic protocol we described previously (i.e., loading dose administered immediately following cell inoculation) and maintained on a three times weekly dosing schedule until sacrifice after 4 weeks (17). We show that solely targeting stromal PDGFR α by administration of IMC-1E10 had no effect on reducing the size of metastatic lesions in either the tibiae or femora of treated mice (Fig. 2B). On the other hand, administration of IMC-1E10 in combination with IMC-3G3 retained the ability of IMC-3G3 alone to impair metastatic growth (Fig. 2B). Despite that the activity of 1E10 on stromal PDGFR α was not directly assessed, these results suggest that targeting PDGFR α of the stromal cellular compartment is ineffective in decreasing bone metastatic growth and also fails to provide a synergistic effect when coupled with inhibition of the PDGFR α expressed by metastatic prostate cells. Notably, we found that the 4-week antagonism of stromal PDGFR α did not cause toxicity because animals maintained a healthy weight throughout the experiments and failed to show signs of overt organ damage at the necropsy. These preclinical findings are supported by the recent completion of phase I clinical trials reporting the absence of significant adverse effects by the prolonged administration of IMC-3G3 (22). The absence of toxicity observed in these trials may be explained by the minor physiologic role exerted by PDGFR α in fully developed organisms. Although PDGFR α plays a crucial role in embryonic development, in the adult, its function is mostly overshadowed by that of PDGFR β in angiogenesis, wound healing, tissue homeostasis, and other systemic effects (34–36). In fact, there is evidence from genetic replacement experiments that PDGFR β signaling can fully compensate for PDGFR α signaling, but not vice versa (37). The predominant function of PDGFR β in human physiology strongly supports the rationale of safely targeting PDGFR α to counteract cancer cells that depend on this receptor for growth and survival (22). This seems particularly relevant to the field of prostate cancer because the therapeutic options currently available in the clinic for patients with skeletal metastatic disease are extremely limited, present a limited therapeutic window, and are only minimally effective.

Thus, the next series of experiments aimed to compare IMC-3G3 with a current standard of care for patients pre-

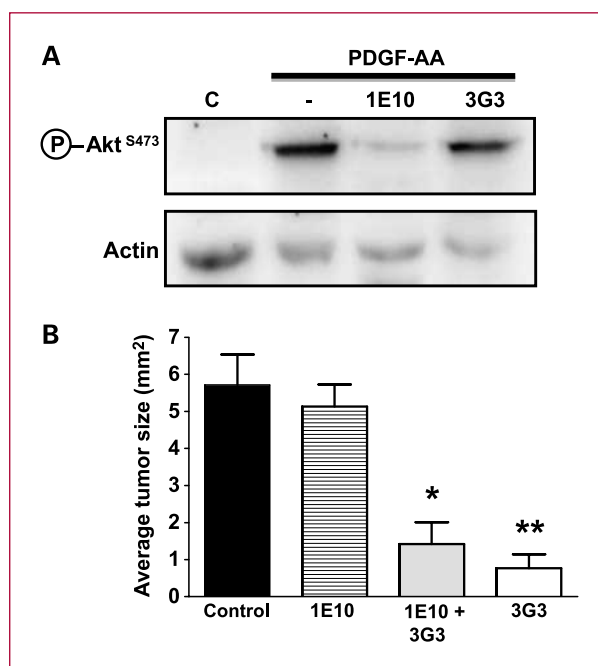


Fig. 2. Targeting of stromal PDGFR α by the mouse-specific IMC-1E10 antibody fails to reduce the size of skeletal metastases. Specificity of the IMC-1E10 antibody (20 μ g/mL) for the mouse PDGFR α was confirmed by the blockade of Akt phosphorylation induced by PDGF-AA (30 ng/mL) in NIH-3T3 murine fibroblasts. In contrast, in the same cells treated with the human-specific IMC-3G3 antibody, the extent of Akt phosphorylation was unchanged as expected (A). Mice were inoculated with PC3-ML cells through the left cardiac ventricle to induce skeletal metastases and treated with either IMC-1E10, IMC-3G3, or a combination of the two antibodies. B, after 4 weeks, mice were sacrificed and the size of metastases in tibiae and femora was measured. Animals that received IMC-1E10 showed no decrease in tumor size versus controls, whereas mice treated with IM-3G3 either alone or in combination with IMC-1E10 showed a significant reduction in the size of skeletal tumor foci. Five animals were used for each experimental group. *, $P < 0.01$; **, $P < 0.001$. C, control.

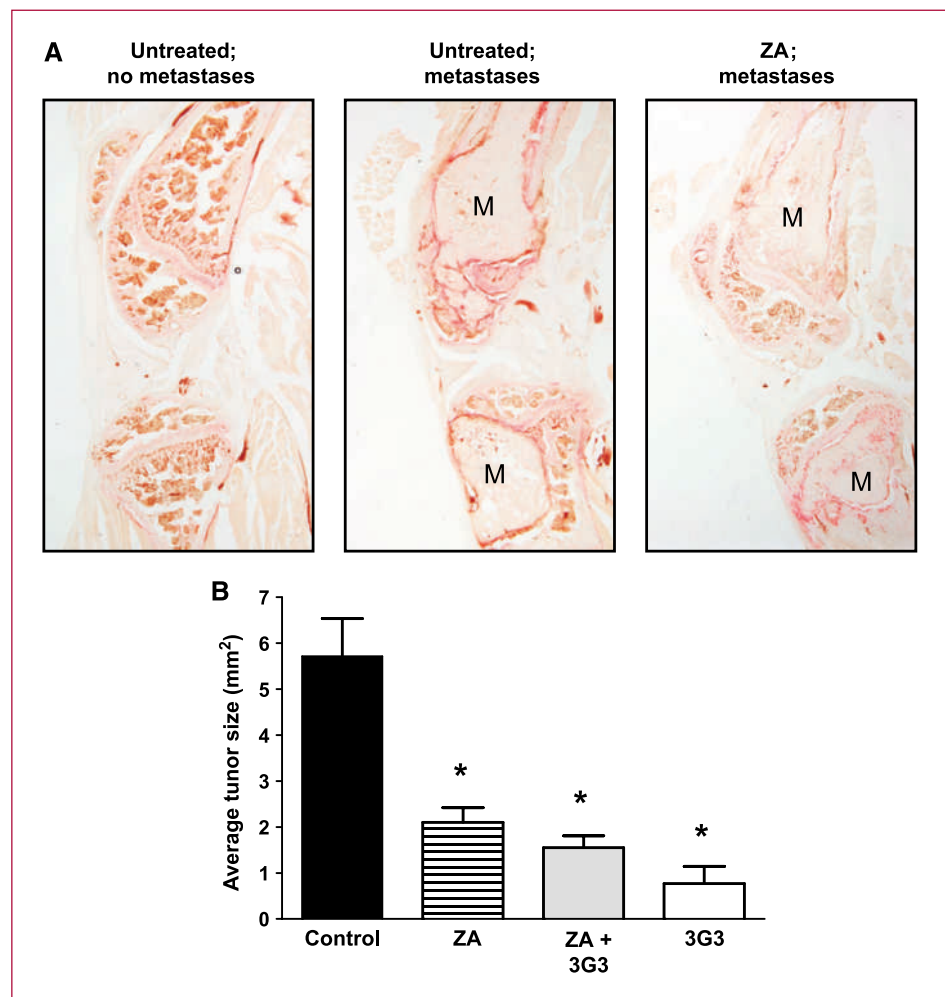
senting with dissemination of prostate cancer to the bone. The presence of significant pain in these patients is caused by alterations in bone turnover deriving from the considerable recruitment and activation of osteoclasts at the site of metastasis (1, 38, 39). The resorption of mineralized bone matrix by these cells followed by deregulated deposition of sclerotic bone by osteoblasts is responsible for the mixed lytic-blastic appearance of metastatic lesions from prostate cancer (40). These histopathologic alterations almost invariably correspond to systemic clinical conditions such as hypercalcemia of variable degrees, as well as skeletal-related event such as spinal nerve compression and pathologic bone fractures (41). Because of the resulting morbidity and reduced quality of life, the administration of inhibitors of osteoclast activity is currently standard of care for patients with bone-metastatic dissemination, including that from prostate cancer (7). ZA is a bisphosphonate with a potent analgesic effect, which has shown significant activity in delaying the time to skeletal-related event in clinical trials

conducted with prostate cancer patients (16). Therefore, we decided to investigate whether metastatic bone lesions would respond to the combined administration of ZA and IMC-3G3. For these studies, mice were assigned to groups receiving ZA or IMC-3G3 alone, as well as ZA plus IMC-3G3 combination therapy, and compared with control groups. IMC-3G3 was dosed as reported above, whereas ZA was administered by weekly s.c. injections of 100 μ g/kg. At the end of the 4-week study, tissues obtained from sacrificed mice were examined for staining of tartrate-resistant acid phosphatase, a widely used histologic indicator of osteoclast activity (42, 43). As expected, mice that received weekly injections of ZA showed a marked reduction in the number and activity of osteoclasts at the tumor-stroma border (Fig. 3A). We also found that mice receiving either ZA or IMC-3G3 alone had a significant decrease in the size of skeletal lesions. However, there was no synergistic reduction of tumor size when the two therapies were combined (Fig. 3B).

Although ZA and IMC-3G3 were equally effective in delaying the growth of metastatic prostate cancer cells in the

bone, we decided to ascertain whether these similar outcomes were the result of different mechanisms of action. This possibility seemed to be supported by the initial evidence presented above that blockade of PDGFR α by IMC-3G3 is particularly effective previous the onset of osteoclast-mediated degradation of the bone matrix, which is the main event inhibited by ZA. To address this point, mice inoculated with PC3-ML cells received either ZA or IMC-3G3 and were sacrificed after only 1 week of bone-metastatic tumor growth. We found that, although both groups of mice had comparable numbers of bone metastases, the lesions in IMC-3G3 treated mice were significantly smaller than the tumors detected in animals treated with ZA, which were indistinguishable from those measured in controls (Fig. 4). These results provide compelling evidence that the antagonism of PDGFR α , achieved in this study with IMC-3G3, effectively impairs the initial colonization of the skeleton by prostate cancer cells. This phase of metastatic progression seems refractory to ZA, which relies on the inhibition of osteoclasts and therefore targets later-stage tumors.

Fig. 3. Inhibitory effect on skeletal metastases exerted by ZA alone or in combination with IMC-3G3. Mice inoculated with PC3-ML cells were treated with a weekly administration of ZA or with IMC-3G3 three times weekly, either separately or in combination. After 4 weeks, mice were sacrificed and inspected for skeletal metastases. Inhibition of osteoclast activity by ZA was confirmed by the uniform reduction of tartrate-resistant acid phosphatase staining, showing fewer and less active osteoclasts recruited to the tumor periphery (A). Animals that received either ZA or IMC-3G3 administered separately showed comparable reduction in the size of metastatic bone tumors. The concurrent inhibition of osteoclast activity by ZA and PDGFR α by IMC-3G3, administered as a combination therapy, did not produce any synergistic effect on tumor growth (B). Five animals were used for each experimental group. *, $P \leq 0.0001$. M, metastatic lesion.



A conclusion that could be drawn from these experiments is that targeting either early metastases with IMC-3G3 or more advanced lesions with ZA leads to a similar outcome in terms of controlling the size of bone metastatic tumors from prostate cancer cells. However, preclinical models of metastatic breast cancer (44) and clinical trials conducted with advanced prostate cancer patients indicate the inability of bisphosphonates to improve overall survival from bone metastatic disease (16). Recent studies with animal models using radiologic and tumor imaging approaches show that reducing the extent of bone destruction and invasion may not fully prevent metastatic breast cancer cells from growing, for example, by infiltrating surrounding soft tissues (44). Based on the evidence that IMC-3G3 reduces metastatic progression by directly impairing cancer cell growth without affecting osteoclasts or bone degradation, we tested the effect on survival of

IMC-3G3 and ZA administered either separately or as a combination therapy.

To carry out these studies, mice were inoculated with PC3-ML cells and randomly assigned to one of four groups: control, IMC-3G3, ZA, or IMC-3G3 plus ZA, as described above, mice received IMC-3G3 three times weekly following the initial loading dose, whereas ZA was administered weekly. Animals were kept in the study until moribund. Necropsic examination did not detect visceral metastases by PC3-ML cells. The only exception was represented by enlarged adrenal glands, frequently bilaterally, consistent with what we have previously reported (45). These small adrenal tumors were only detected by fluorescence stereomicroscopy, always found contained within the gland parenchyma, and therefore very unlikely to cause lethal tumor burden. As shown in Fig. 5, mice treated with ZA alone showed an increase in survival as compared

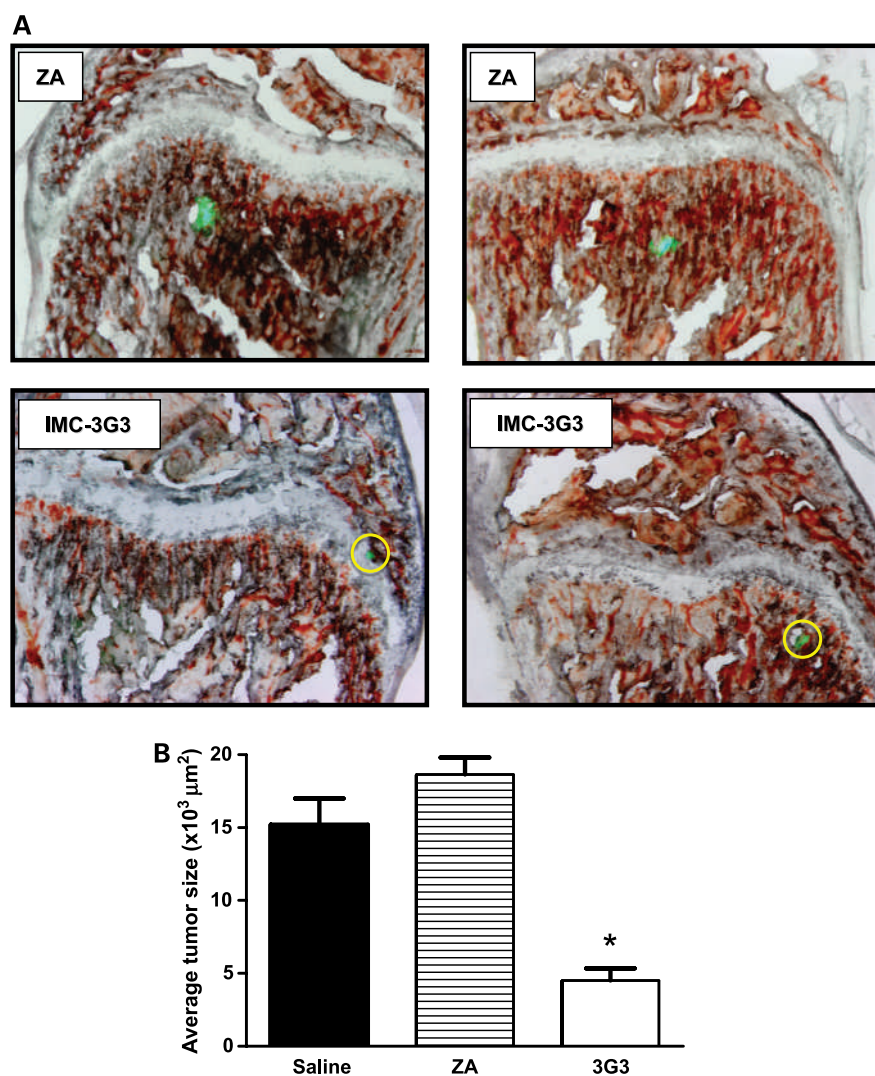


Fig. 4. Blockade of PDGFR α by IMC-3G3 inhibits the early stages of bone-metastatic tumor growth. Animals were injected through an intracardiac route with PC3-ML cells stably expressing enhanced variant of GFP, treated with either ZA or IMC-3G3, and sacrificed 1 week later. Early bone-metastatic lesions were identified by fluorescence stereomicroscopy (A). Tumor foci in the two groups of animals were compared with those detected in control saline-treated animals. The treatment with IMC-3G3 significantly reduced tumor size, whereas the lesions detected in mice that received ZA were comparable in size to those measured in control animals (B). Control, 5 mice and 13 metastases analyzed; ZA, 10 mice and 22 metastases analyzed; IMC-3G3, 10 mice and 26 metastases analyzed. *, $P < 0.0001$. Circles indicate small metastatic foci.

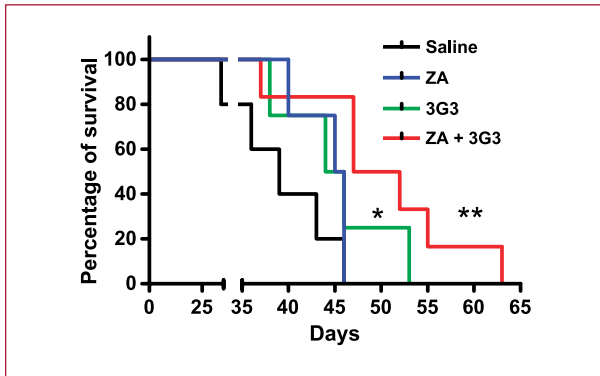


Fig. 5. The IMC-3G3 antibody, alone or in combination with ZA, prolongs survival in animals with skeletal metastases. Mice were inoculated with PC3-ML cells and maintained on a treatment consisting of either IMC-3G3 or ZA administered separately or as a combination. Controls received equal volumes of saline solution. Animals were sacrificed when moribund. The Kaplan-Meier graph shows that IMC-3G3 alone and in combination with ZA was able to prolong survival. Between 4 and 6 animals were used for each group. *, $P < 0.05$; **, $P < 0.01$.

with the control group, although this effect did not reach statistical significance most likely because of the small number of mice used. However, the main conclusion of this set of experiments is that IMC-3G3 used alone prolonged survival and this effect was even more pronounced when this antibody was administered in combination with ZA. Taken together, these results clearly indicate that the blockade of PDGFR α through administration of IMC-3G3 delays the growth of preexisting skeletal metastases from prostate cancer cells and significantly extends survival in animals with bone-metastatic dissemination. These effects were exerted

independently from the inhibition of osteolysis, which is the main mechanism of action of bisphosphonates such as ZA. Remarkably, the administration of ZA resulted in an enhanced survival benefit when combined with the direct antitumor action of IMC-3G3 on prostate cancer cells. This indicates that the mobilization of growth factors from the bone matrix by osteolytic events is likely to play a role in supporting prostate cancer growth at the skeletal level. A strategy aimed to contain bone degradation combined with a direct interference of PDGFR α functioning can significantly prolong survival in preclinical models of metastatic prostate cancer.

Disclosure of Potential Conflicts of Interest

A. Fatatis is a consultant/advisory board member of ImClone.

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Review

Implication of platelet-derived growth factor receptor alpha in prostate cancer skeletal metastasis

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Abstract

Metastasis represents by far the most feared complication of prostate carcinoma and is the main cause of death for patients. The skeleton is frequently targeted by disseminated cancer cells and represents the sole site of spread in more than 80% of prostate cancer cases. Compatibility between select malignant phenotypes and the microenvironment of colonized tissues is broadly recognized as the culprit for the organ-tropism of cancer cells. Here, we review our recent studies showing that the expression of platelet-derived growth factor receptor alpha (PDGFR α) supports the survival and growth of prostate cancer cells in the skeleton and that the soluble fraction of bone marrow activates PDGFR α in a ligand-independent fashion. Finally, we offer pre-clinical evidence that this receptor is a viable target for therapy.

Key words Platelet-derived growth factor receptor alpha, metastasis, prostate cancer, organ tropism

Eighty-five percent of patients are routinely diagnosed with prostate cancer in the absence of secondary tumors. However, depending on initial therapy, histologic grading and residual disease after surgery, many of these patients will eventually present cancer dissemination to bone. Skeletal metastases are responsible for a significant reduction in the quality of life and represent the main cause of death in patients with advanced prostate adenocarcinoma. Treatment for bone metastasis is mostly palliative and is unable to prevent skeletal dissemination or eradicate prostate cancer cells that colonize the bone microenvironment^[1].

Metastasis is a process that requires the successful execution of several sequential steps by cancer cells^[2,3]. Many tumors show a propensity to colonize specific

tissues in the body, a feature defined as organ-tropism^[4]. It is widely recognized that the identification of factors responsible for promoting the adaptation of malignant prostate cells to the bone microenvironment will lead to more effective therapeutic strategies for advanced prostate cancer. However, the molecules and mechanisms determining the organ-tropism of cancer cells are vaguely defined^[5]. Paget^[6] assimilated the compatibility between migrating cancer cells and colonized organs to the required affinity between a seed and the specific soil. In support of this idea we have to date considerable evidence indicating that migration of cancer cells into a foreign tissue needs favorable conditions to survive and proliferate^[5]. Cancer cells failing to receive appropriate support may remain dormant or undergo cell death^[7], thereby exerting negligible clinical impact on the patient. This general paradigm has been proposed for skeletal metastasis and appropriate trophic factors in the bone appear to be crucial for initial cell survival, growth into small foci, and subsequent progression into macroscopic metastases^[8-10]. Thus, disseminated cancer cells expressing the appropriate receptor arsenal for the trophic factors locally produced by the bone marrow stroma will have a major advantage in supporting their survival and growth into clinically evident tumors.

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Expression of PDGFR α by Prostate Epithelial Cells

The presence of platelet-derived growth factor receptors (PDGFRs) and their ligands in the prostate were initially described by Fudge *et al.*^[11]. More recently, Chott *et al.*^[12] reported that primary prostate cancers and their skeletal metastases are positive for PDGFR expression, with PDGFR α being most represented. We confirmed this observation by showing that normal prostate expresses low levels of PDGFR α , which significantly increase upon malignant transformation. We detected a strong signal for PDGFR α in approximately 70% of tissue cores of human prostate and all the samples from skeletal metastases by immunohistochemistry^[13]. Interestingly, a significant number of specimens showed dishomogeneous distribution of PDGFR α on the epithelial compartment, suggesting that cellular phenotypes with different expression patterns for PDGFR α co-exist in the same gland, a scenario replicated by the human prostate cancer cell lines commonly available. For instance, we found that PDGFR α is detectable only in cells derived from skeletal metastases, such as the widely used PC3 cell line. In contrast, cells obtained from lymph node metastases (LNCaP) or brain metastases (DU-145) in prostate cancer patients fail to express either the alpha or beta isoforms of PDGFR^[14].

PDGFR α Expression and Bone-Metastasis Potential

A more direct correlation between PDGFR α expression levels and the propensity of prostate cancer cells to colonize the skeleton was derived from experiments that were conducted using two sub-lines originally obtained from the PC3 parental population. Employing an *in vitro* invasion assay, Wang *et al.*^[15] obtained two sub-lines of invasive and non-invasive PC3 cells. These cells were subsequently evaluated for their metastatic potential in immunocompromised SCID mice through tail vein inoculation. The invasive cells demonstrated bone-metastatic abilities and are currently named PC3-ML, whereas the non-invasive cells also failed to produce macroscopic bone tumors and are currently named PC3-N. We decided to complement these initial experiments using an animal model of metastasis combining fluorescence stereomicroscopy, histologic analysis and digital imaging. We employed prostate cancer cells engineered to stably express enhanced green fluorescent protein (eGFP). The resulting emitted fluorescence facilitated their identification both at the single-cell stage and when growing as metastatic foci of progressively larger size in the skeleton of the inoculated mice^[13]. When inoculated

into mice via an intracardiac route, PC3-N and DU-145 cells were unable to produce macroscopic tumors in the skeleton or any other organ examined, whereas PC3-ML cells produced macroscopic tumors at 4 to 5 weeks post inoculation. However, when earlier time-points were investigated, we established that PC3-N and DU-145 cell lines were both capable of spreading to the skeleton through the blood circulation as effectively as the metastatic counterpart PC3-ML cells^[13]. However, PC3-N could generally survive no longer than one week in the bone marrow and only a small number of mice showed small skeletal metastases at three weeks post inoculation. DU-145 cells could survive only for the first 72 h after arriving into the bone and were never detected at one week post inoculation (Figure 1). Thus, the disparity in metastatic potential of these three malignant prostate phenotypes is not related to the extent of their dissemination to bone. Instead, it appears to depend on their ability to survive in the bone marrow, in which they lodge after extravasating from the blood circulation. Interestingly, we have established that PC3-ML cells express significantly higher levels of PDGFR α than do their non-metastatic counterpart, PC3-N cells. If combined with the complete lack of PDGFR α expression observed in DU-145 cells, these results establish a positive correlation between the expression levels of PDGFR α and the progression of malignant prostate phenotypes in the bone marrow, and indicate that the survival of disseminated cells in a foreign microenvironment plays a key role in the overall metastatic potential of a specific prostate cell phenotype.

In light of this established correlation between PDGFR α expression and bone-metastatic potential, we tested whether the exogenous over-expression of receptor in PC3-N and DU-145 cells could sustain initial bone colonization and promote metastatic growth of these two malignant phenotypes. We found that the over-expression of PDGFR α in PC3-N cells conferred a bone-metastatic potential indistinguishable from PC3-ML cells in terms of number and size of skeletal tumors detected four weeks after inoculation. Interestingly, the ability of DU-145 cells to produce bone-metastatic tumors was unaffected by the expression of PDGFR α ^[13], suggesting that the pre-existing genetic background of a malignant phenotype may ultimately dictate the pro-metastatic role exerted by PDGFR α in prostate cancer cells.

Further studies revealed an unorthodox mechanism by which PDGFR α recruits downstream signaling pathways in prostate cancer cells. The experiments that fully epitomize this atypical signaling by PDGFR α were conducted by over-expressing a truncated form of the receptor, named α R Δ X, in PC3-N cells. The receptor mutant, obtained from Dr. Kazlauskas and collaborators, lacks the extracellular ligand-binding domain and is therefore unable to bind or be activated by proper PDGF

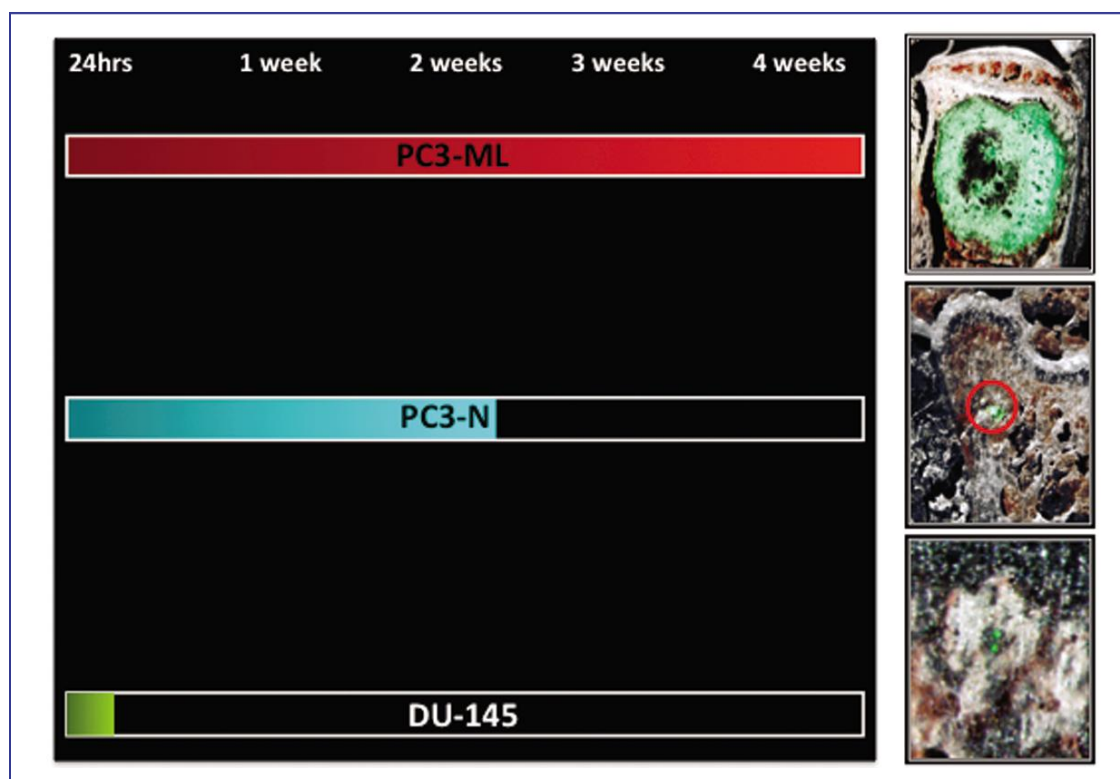


Figure 1. Survival and progression at the skeletal level of prostate cancer cell types expressing different levels of PDGFR α . The PC3-ML sub-line expressed higher levels of the receptor and produced macroscopic skeletal metastases in mice inoculated with cancer cells in the hematogenous circulation via the left cardiac ventricle injection. PC3-N cells expressed lower levels of PDGFR α than did PC3-ML cells and could only survive two weeks in the bone after their dissemination. DU-145 cells were found negative to PDGFR α expression and disappeared from the skeleton between 72 h and one week post inoculation.

ligand(s) or other signaling molecules^[16]. When PC3 (α R Δ X) cells were tested for their metastatic potential in our animal model, they fully emulated the bone-metastatic behavior of both PC3-ML cells and PC3-N cells that over-express the full-length form of the receptor^[17].

Evidence for Transactivation of PDGFR α by Human Bone Marrow

PDGFRs are tyrosine kinases and some of the best-studied growth factor receptors. Two structurally related forms of the receptor are PDGFR α and PDGFR β . Their extracellular portion contains five immunoglobulin-like domains whereas the intracellular part of the molecule contains a kinase domain^[18]. Five PDGF ligands, PDGF-AA, -BB, -AB, -CC and -DD, have been identified, which display different binding affinities for the different receptors^[19]. Since PDGF ligands are dimeric molecules, they bind two receptors simultaneously. Upon binding, the two receptors dimerize, triggering reciprocal phosphorylation at tyrosine

residues located at specific sites on the intracellular portion of each receptor^[20]. This transphosphorylation of PDGFR upon ligand binding serves two important purposes. The phosphorylation of a tyrosine residue in the kinase domain increases its catalytic efficiency. In addition, the phosphorylation of tyrosine residues outside of the kinase domain creates docking sites for signaling molecules. Some of these molecules can function as enzymes, such as phosphatidylinositol 3'-kinase (PI3K), phospholipase C (PLC), the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2, and a GTPase activating protein (GAP) for Ras. Other molecules lack enzymatic activity and function as adaptors, such as Grb2, Nck, Shc and others^[18]. The biological functions of some of these signaling molecules have been characterized and are fundamental for cellular homeostasis. PI3K activates the downstream kinase Akt, which is richly implicated in promoting cell survival^[21].

We initially reported that PDGFR α , in addition to its proper PDGF ligands, could activate downstream signaling pathways, such as PI3K/Akt, when exposed to the soluble fraction of human bone marrow^[22]. Interestingly, the phosphorylation of Akt in PC3-ML cells

exposed to bone marrow could be reduced to less than 40% by AG1296, a putative specific inhibitor of PDGFRs^[23]. More conclusive evidence for a direct activation of PDGFR α was obtained from the detection of its tyrosine-phosphorylation upon the bone marrow treatment of PC3-ML cells^[22].

Considering these results, we decided to measure the concentration of PDGF in the bone marrow aspirates and to identify the isoform(s) of this growth factor responsible for Akt activation in PC3-ML cells. We found that bone marrow aspirates obtained from different donors contained both PDGF-AA and PDGF-BB in concentrations ranging from 400 pg/mL to 2 ng/mL. Our experiments were conducted employing bone marrow diluted twenty fold, thus containing PDGF ligands reaching a maximum concentration of 100 pg/mL. When PC3-ML cells were simultaneously exposed to 100 pg/mL (each) of PDGF-BB and PDGF-AA, the observed activation of Akt was minimal, representing less than 10% of the observed response when exposing these cells to bone marrow. In addition, similar concentrations of PDGF ligands were unable to reproduce tyrosine phosphorylation of the intracellular portion of PDGFR α generated by human bone marrow^[17]. Notably, the phosphorylation of PDGFR α induced by bone marrow was of a lesser magnitude than that generated by the exposure of cells to PDGF-AA. However, the extent of Akt phosphorylation observed under these two conditions was remarkably similar, implying that the phosphorylation caused by bone marrow must predominantly or exclusively affect tyrosine residues on PDGFR α which are responsible for the recruitment and activation of PI3K.

Collectively, these observations strongly suggest that PDGFR α , in addition to being stimulated by PDGF ligands in a widely recognized fashion^[24,25], can also be recruited and activated via ligand-independent mechanisms in a phenomenon termed trans-activation that has been reported for several receptors, including PDGFR^[26-28]. Accordingly, upon activation by the soluble fraction of bone marrow, the canonical dimerization of PDGFR α could not be observed^[17]. This leads to hypothesize that the stimulation of other plasma membrane receptors could successively trigger the phosphorylation and signaling of monomeric forms of PDGFR α via intracellular mediators. As a role for SRC family kinases (SFK) by intracellular reactive oxygen species (ROS) observed in other systems^[16] could be excluded, the implication of other receptor-pathway combinations is likely.

The conclusive evidence for PDGFR α transactivation was obtained using either DU-145 or PC3-N cells stably expressing α RA X. Both cell types responded to human bone marrow with strong Akt activation and tyrosine phosphorylation of α RA X^[17]. These results complement the *in vivo* studies and show

that PC3-N acquired a bone-metastatic potential comparable to that of PC3-ML cells when stably transfected with either the full-length or the truncated form of PDGFR α .

The possibility that the establishment and progression of prostate cancer in the bone could be independently supported by PDGFR α of direct ligand stimulation may have important translational implications. It can be inferred that anti-cancer therapeutics designed to block the ligand-binding domain of PDGFR α may not fully prevent downstream signaling in cells that have spread to the bone marrow. Alternatively, inducing the internalization of PDGFR α may provide a mean to prevent ligand-dependent and -independent activation and provide a better therapeutic option to counteract the growth of prostate cancer cells disseminated to the skeleton.

Targeting PDGFR α to Block Its Downstream Signaling

PDGFR α and PDGFR β are involved in organism development, with PDGFR α playing a greater role during embryogenesis^[29]. In the adult, both receptors cooperate in modulating cellular and physiological processes that largely overlap, including angiogenesis, wound healing and tissue homeostasis^[19,29]. PDGFR β , however, plays a predominant role overall, as demonstrated in mice in which the cytoplasmic domains between PDGFR α and PDGFR β were swapped. These experiments revealed that the PDGFR β intracellular domain could fully substitute for the PDGFR α . In contrast, replacement of the PDGFR β cytoplasmic domain with that of the α -receptor caused abnormalities in vascular smooth muscle cell development and function^[30]. The use of the small-molecule inhibitor STI571 (imatinib mesylate or gleevec) has been reported to block PDGFRs and reduce the expansion of cancer cells within the bone^[31,32]. However, the inhibitory and pro-apoptotic effects of STI571 seem to be exerted prevalently on PDGFR β expressed in endothelial cells of the tumor vasculature rather than directly affecting prostate cancer cells. Alternatively, the toxicity reported in phases I and II clinical trials, which in most cases had to be interrupted^[33,34], may explain the ability of STI571 to comparably block PDGFR α and PDGFR β . In addition, pre-clinical animal studies investigating the survival role of PDGFRs for cancer cells and the effects exerted by STI571 were almost exclusively conducted using animal models in which bone tumors were produced by directly implanting cancer via an intra-osseous route. While this approach significantly shortens the duration of each experiment, it also bypasses the initial stages of lodging and colonization of the bone marrow. Therefore, the peculiar histopathologic features produced by this intra-osseous

approach, as compared to naturally established and progressing skeletal metastases, might also explain the disappointing effects of STI571 in clinical trials.

It seems plausible that the selective inactivation of PDGFR α , employing a monoclonal antibody rather than a broad-range inhibitor such as STI571, could limit the survival of malignant cells that depend on it while causing limited side effects, due to the largely duplicate role exerted by PDGFR β [36]. However, in the event that PDGFR α in prostate cancer cells undergoes transactivation when in the bone marrow microenvironment, an antibody that would target the extracellular ligand-binding domain would fail to completely block signaling. Conversely, an antibody that could induce the internalization of PDGFR α would remove from the plasma membrane an important target for the transactivation of cancer cells exerted by the bone marrow. With this goal in mind, we decided to test IMC-3G3, a humanized monoclonal antibody against PDGFR α . This antibody has been extensively characterized both *in vitro* and *in vivo* and was shown to block both PDGF-AA and PDGF-BB from binding PDGFR α , with a K_d of 40 pmol/L. Also, the binding kinetic of IMC-3G3 to human PDGFR α was defined by BIAcore analysis as well as flow cytometry employing human cells. A significant neutralizing activity of IMC-3G3 against PDGFR α was also observed in mitogenic and phosphorylation assays and this antibody inhibited subcutaneous xenografts in nude mice [36].

In experiments in which PC3-ML cells were exposed to bone marrow, IMC-3G3 was consistently able to reduce downstream Akt phosphorylation in a time-dependent manner. Interestingly, cell-surface biotinylation experiments showed that the inhibitory effect of IMC-3G3 on PDGFR α downstream signaling was tightly correlated to the internalization of this receptor. This event affected more than 80% of the initial levels of PDGFR α after two hours of IMC-3G3 incubation [22]. Furthermore, by using experimental conditions that halt receptor internalization while preserving IMC-3G3 neutralization of the ligand-binding domain of PDGFR α , we could block Akt phosphorylation by PDGF-AA but not by bone marrow [22].

Hence, evidence strongly suggests that IMC-3G3 could be effective in our pre-clinical model of bone metastases to counteract the survival and progression prostate cancer cells disseminated to the skeleton.

Targeting PDGFR α Effectively Counteracts Skeletal Metastases in Animal Models

We initially confirmed the species-specificity of IMC-3G3 *in vitro*, observing that the antibody blocked signaling by human PDGFR α while leaving the mouse

form of the receptor unaffected [17]. Following, we used IMC-3G3 according to a *prophylactic protocol*, in which SCID mice were inoculated in the blood circulation with PC3-ML cells and received a first loading dose of IMC-3G3 followed by subsequent maintenance doses of the antibody bi-weekly, all administered by intraperitoneal injection. When animals were euthanized four weeks later, the number of bone-tumors per mice as well as the number of animals presenting with skeletal metastases in the IMC-3G3-treated groups were significantly lower than those in the saline-treated groups [13]. Similar results were obtained when animals were euthanized two weeks post inoculation, in which bone metastases were reduced by 70% as compared to control groups [13].

Successively, we employed a curative protocol in which mice were inoculated with PC3-ML cells and left untreated for either 7 or 14 days, thus providing a time interval for metastatic tumor growth. Following this first period, treatment with IMC-3G3 began as previously described and continued until the fourth week post inoculation. We found that the skeletal lesions in animals administered IMC-3G3 were significantly reduced in size than those in the control groups receiving either saline or human immunoglobulins of the IgG1 subclass as the IMC-3G3 antibody [37] (Figure 2).

Since it had been previously reported that stromal PDGFR α could support tumor growth and local angiogenesis when stimulated by locally produced PDGF ligands (i.e. PDGF-AA and PDGF-CC), we decided to investigate the contribution of PDGFR α expressed by stromal cells (osteoblasts and mesenchymal bone stromal cells) on the skeletal colonization and metastatic progression of PC3-ML cells. Thus, we used IMC-1E10, a humanized monoclonal antibody selected for binding to mouse PDGFR α and otherwise sharing an identical structure with IMC-3G3 [38]. IMC-3G3, IMC-1E10, or a combination of the two antibodies was used to treat mice that had been inoculated with PC3-ML for 4 weeks. We found that the animals treated with IMC-1E10 showed no decrease in tumor size as compared to control, whereas mice treated with IMC-3G3 either alone or in combination with IMC-1E10 showed a significant reduction in the size of skeletal tumor foci [37].

The current standard of care for patients with advanced metastatic prostate cancer includes the administration of bisphosphonates [39]. These molecules are very effective inhibitors of bone-matrix degradation caused by osteoclasts located in skeletal metastatic lesions [40]. The resorption of mineralized bone and consequent mobilization of growth factors has been shown to support cancer cell growth and survival, while also causing significant pain to patients and increasing the risk for skeletal-related events (SREs) such as pathological fractures and spinal-cord compression [41]. Zoledronic acid (ZA) shows a potent analgesic effect that can significantly delay the time to SREs [42]. However,

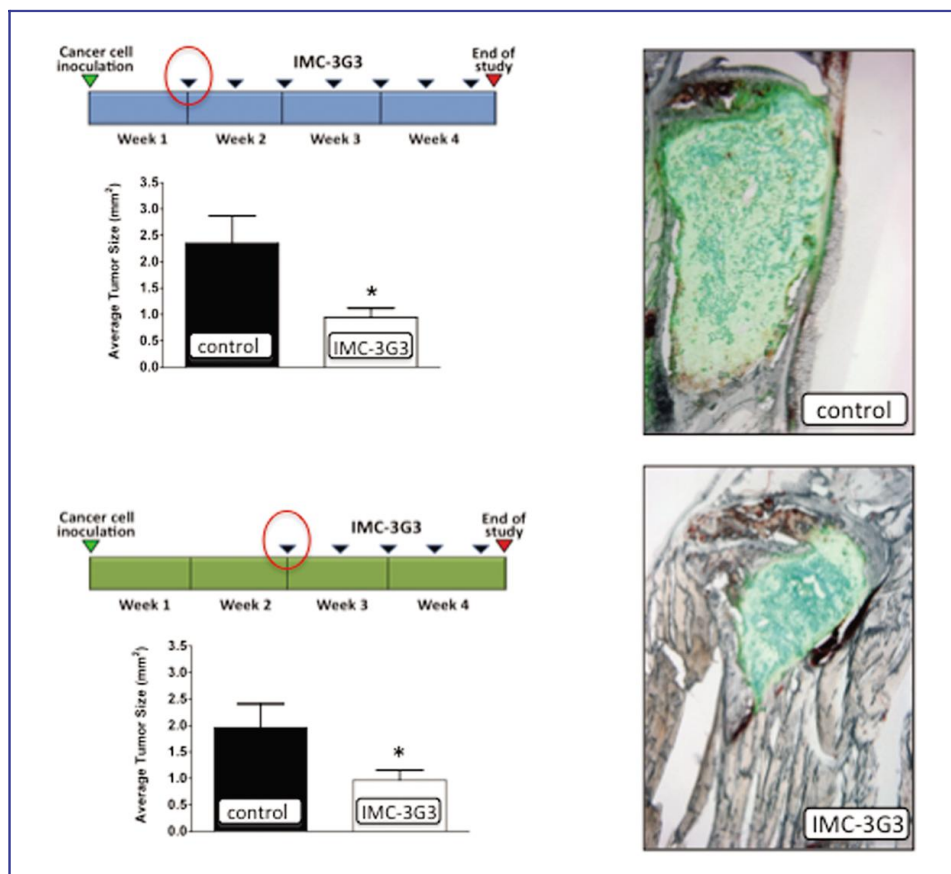


Figure 2. The monoclonal antibody for human PDGFR inhibited skeletal tumor growth. IMC-3G3 is effective in counteracting the progression of established skeletal metastases. After mice were inoculated with prostate cancer cells, treatment was withheld from mice for either one or two weeks, after which treatment of IMC-3G3 was maintained for the remainder of the experiment. When mice were euthanized at four weeks, their tibiae and femora showed a significant reduction in the average size of bone tumors as compared to controls^[37]. This figure is reprinted with permission from Russell *et al.*^[37], *Clinical Cancer Research*, 2010, 16 (2):5002–5010. Copyright © 2010 by American Association for Cancer Research.

while bisphosphonates are credited for a significant palliative role, a recent clinical trial in which ZA was compared to placebo in 422 advanced prostate cancer patients failed to show significant differences in disease progression, performance status and quality of life among the groups^[43]. Similar results were provided by pre-clinical studies in which the progression of the bone metastatic disease from breast cancer cells was transiently delayed, and at later stages the total tumor burden per animal became equivalent to that in vehicle-treated animals^[44,45].

To understand whether the palliative effect exerted by bisphosphonates could be complemented by the anti-metastatic role of IMC-3G3, we investigated whether animals with metastatic bone lesions would respond to the combined administration of ZA and IMC-3G3 with an increase in overall survival. We found that the treatment with IMC-3G3 alone and more significantly in combination with ZA was able to extend survival^[37] (Figure 3).

Conclusions

The series of studies presented here strongly

support an important role of PDGFR α in facilitating the initial lodging and subsequent progression of prostate cancer cells in the bone microenvironment. In addition to an expected stimulation by PDGF ligands, the effect of PDGFR α is exerted through transactivation events initiated by activating signaling molecules present in the soluble fraction of human bone marrow. Importantly, the selective targeting of PDGFR α with monoclonal antibodies such as IMC-3G3, while still allowing PDGFR α to exercise its numerous physiological roles, can effectively counteract the growth of prostate cancer cells at the skeletal level. Finally, based on the positive results obtained with IMC-3G3 in combination with ZA in animal survival studies, a similar combination therapy approach could be envisioned in the clinic for prostate cancer patients.

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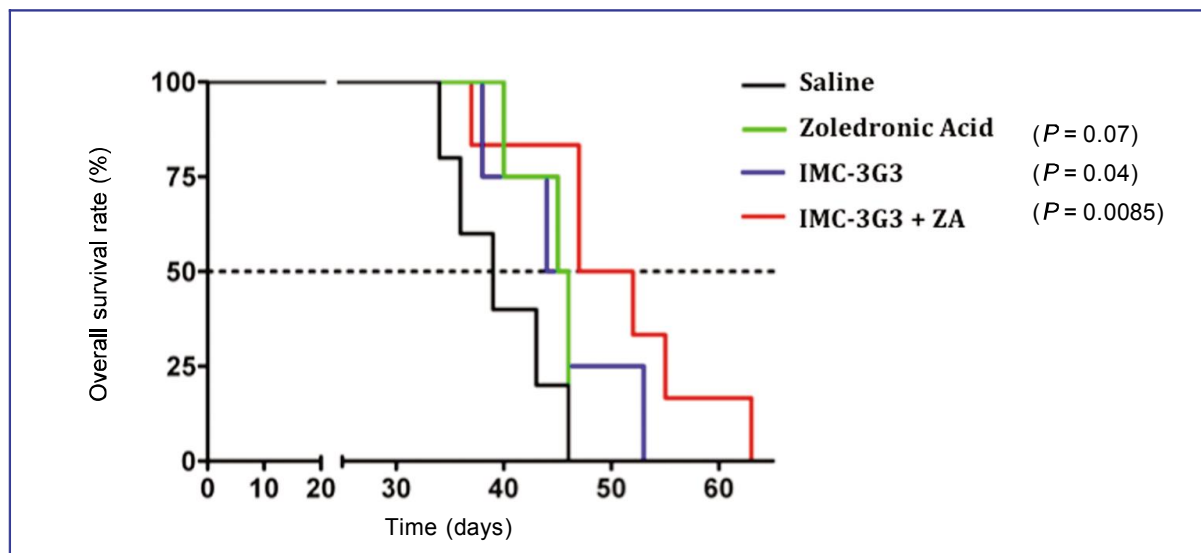


Figure 3. Survival curves for various groups of animals with prostate cancer. The Kaplan-Meier graphs show that targeting PDGFR α with IMC-3G3 induced a significant extension of overall survival in mice inoculated with prostate cancer cells, either alone or administered in combination with zoledronic acid (ZA). In contrast, ZA alone failed to prolong mean survival time^[37]. This figure is reprinted with permission from Russell *et al.*^[37], *Clinical Cancer Research*, 2010, 16 (2):5002–5010. Copyright © 2010 by American Association for Cancer Research.

contributions to our studies.

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