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**14. ABSTRACT**
In this second year of funding we have shown that: a) We have established the time frame during which PDGFRα support the survival and progression of PCa cells disseminated to the skeleton. The results deriving from our pre-clinical animal model of metastasis indicate that this receptor is of paramount importance during the initial phases of bone colonization. This information presents high value for the stratification of patients to be included in phase-III clinical trials for the antibody IMC-3G3 (Olaratumab). Thus monoclonal, fully human antibody is directed to target PDGFRα and being currently evaluated in phase-II trials for a variety of solid tumors, including PCa. b) We have identified a new 3-gene expression signature correlated with the ability of PCa cells to colonize the skeleton in animals. The implications for human pathology need to be fully evaluated; however, the evidence accumulated in our pre-clinical model is compelling. Interestingly, all the encoded protein products are soluble and secreted proteins. This seems to indicate that PCa cells with metastatic abilities modify the bone microenvironment in a paracrine fashion and that surrounding stromal cells may reciprocate by producing survival and growth factors. If this model is confirmed, our results may help identifying novel molecular targets for anti-metastatic therapies.

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INTRODUCTION

The dissemination of prostate cancer to the skeleton is the main cause of death from this form of tumor. We first reported the association between bone-metastatic potential of human prostate cancer cell lines in human and animal models and the expression of the alpha-receptor for Platelet-Derived growth Factor (PDGFR-α). This study was conceived to: a) conclusively establish the role of PDGFRα 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 the signal transduction pathways recruited by PDGFR-α to promote bone-metastatic phenotypes in prostate cancer cells.

BODY

In this second year of funding, we have further extended the studies that last year had been reported in two high-profile publications (Cancer Research and Clinical Cancer Research) as well as presented at the 101st Annual Meeting of the American Association for Cancer Research and by invited lectures at the Kimmel Cancer Center in Philadelphia and Shands Cancer Center at the University of Florida.

As required, our research accomplishments are associated with each task outlined in the SOW, as follows:

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

Different fluorescent prostate cancer cell lines expressing ectopic PDGFRα are tested in our animal model or experimental metastases. One hundred-eighty mice will be used for these experiments (Experimental group 1).

In our first annual report, we noted that 22RV1 and C4-2 cells could not be seen homing to the skeleton of inoculated animals in our model of experimental metastasis (for technical details on our pre-clinical model please refer to the paper we published in Oncogene 2009). We were also planning to test in similar conditions the cell lines VCAP and LAPC-4, obtained from the laboratory of Dr. Karen Knudsen at the Kimmel Cancer Center in Philadelphia. The inclusion of these four additional human prostate cancer (PCa) cell lines would be extremely beneficial because, in contrast to PC3 cells, these cells express either wild type or mutated Androgen Receptor (AR) and display different degrees of castration-resistant status. This will ultimately allow us to investigate the role played by the AR in both initial homing and subsequent progression of skeletal metastases.

Following these initial setbacks, we performed additional experiments in which the preparation of 22RV1 cells for injection was conducted using various conditions. We have then obtained evidence that 22RV1 cells are indeed capable of homing to the skeleton and lungs when delivered into the arterial circulation of SCID mice, but eventually fail to survive and produce macroscopic lesions in either bone or soft tissues. Experiments aiming to establish the time frame during which 22RV1 cells stop colonizing the bone are currently ongoing and include also the other three PCa cell lines mentioned above. Interestingly, we found that all these cells are negative for PDGFR-α expression and therefore represent an ideal model to further establishing the role exerted by this receptor in promoting a PCa bone-metastatic phenotype.

Homing of 22RV1 human prostate cancer cells to the skeleton. At 6-8 weeks of age, mice were anesthetized with the combined administration of ketamine (80mg/kg) and xylazine (10mg/kg) administered by intraperitoneal route and then inoculated in the left cardiac ventricle with 22RV1 cells stably expressing GFP. Animals were sacrificed and tissues were fixed, decalcified in 0.5M EDTA if necessary and frozen in O.C.T. embedding medium (Electron Microscopy Sciences, Hatfield, PA. Serial tissue sections of 80µm in thickness were obtained using a Microm HM550 cryostat (Mikron, San Marcos, CA). Sections of each hind leg were transferred on glass slides, stored at -20 ºC and examined for cancer cells using either an Olympus IX70 fluorescence inverted microscope or an Olympus SZX12 fluorescence stereomicroscope. Bright field and fluorescence images were acquired with an Olympus DT70 CCD color camera connected to a NUANCE imaging system (CRI).
Specific Aim 2: The effects of antibody-mediated targeting of PDGFRα on skeletal metastasis -

This aim has been completed during the first year of funding.

Specific Aim 3: The changes in protein expression and signaling pathways induced by PDGFRα.

Months 24-28: Changes in gene expression induced by ectopic PDGFRα in prostate cancer cells showing the acquisition or potentiation of bone-metastatic capabilities are evaluated and compared to their isogenic, wild type counterparts (Experimental group 6).

Our second year of funding has focused mainly on this part of the proposal. 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 PDGFRα 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 (see figure). 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 are directly up-regulated by PDGFRα expression and correlate 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-1beta (IL-1β), the chemokine CXCL6 (GPC-2) and the peptidase inhibitor 3 (PI3 or SKALP). This observation seems to suggest that, in order to successfully survive and progress into macroscopic metastases, PCa cells alter the bone microenvironment in a paracrine manner.

We are currently systematically over-expressing each gene included in our signature into PCa cells normally lacking bone-metastatic potential to ascertain their role either separately or in combination. To date, we have obtained extremely encouraging data showing that non-metastatic PC3-N cells over-expressing IL-1β produce bone metastases three weeks after arriving to the skeleton. Similar experiments are now being conducted using DU-145 cells, in which PDGFRα did not induce any gene included in our signature and failed to convert them into a bone-metastatic phenotype. Furthermore, this approach will be adopted for both CXCL6 and PI3, to conclusively characterize the newly identified bone-metastatic gene signature.

Months 28-32: Changes in protein expression and activation induced by ectopic PDGFRα in prostate cancer cells showing the acquisition or potentiation of bone-metastatic capabilities are evaluated and compared to their isogenic, wild type counterparts (Experimental group 6).

Months 32-36: additional experiments necessary to confirm and validate the results from DNA and antibody microarrays are conducted.

— The third year of funding will also address the two sub-aims listed above. It is expected that, due to the compelling results that identify IL-1β, CXCL6 and PI3 as possible culprits for bone-metastatic potential of PCa, our study will mostly focus on the proteins encoded by these three genes.
KEY RESEARCH ACCOMPLISHMENTS

In this second year of funding we have shown that:

**Aim 1** 22RV1 human prostate cancer cells are capable of homing to the bone when inoculated directly into the arterial circulation of mice via the left cardiac ventricle. We also found that these cells do not express PDGFRα and do not survive to produce macroscopic metastatic lesions. These cells will be engineered to exogenously express PDGFRα to establish whether this receptor confers metastatic potential similarly to what we have shown for PC3-N.

**Aim 3** A new 3-gene expression signature is correlated with the ability of PCa cells to colonize the skeleton in animals. The implications for human pathology need to be fully evaluated; however, the evidence accumulated in our pre-clinical model is compelling. Interestingly, all the encoded protein products are soluble and secreted proteins. This seems to indicate that PCa cells with metastatic abilities modify the bone microenvironment in a paracrine fashion and that surrounding stromal cells may reciprocate by producing survival and growth factors. If this model is confirmed, our results may help identifying novel molecular targets for anti-metastatic therapies.

REPORTABLE OUTCOMES

**Papers.**


**Abstracts**

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.

**Presentations**

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)

**Ph.D. awarded**

None for this second year of funding.

QingXin (Cindy) Liu is a third-year Ph.D. student directly involved in this project. She will present her thesis proposal to the members of her committee for approval on December 2011. Her thesis defense is expected to be held sometime in 2013.

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.

A main conclusion that emerges from our work and has been fostered by our second year of funding is that the expression of PDGFRα by malignant prostate epithelial cells promotes their dissemination to the skeleton. More importantly, we have obtained initial evidence that the pro-metastatic effect of this receptor is exerted by inducing the overexpression of three genes that encode for secreted proteins. We are currently in the process of functionally validating each of these newly identified potential targets in our pre-clinical model of metastasis.

Effective treatments for metastatic prostate cancer will be conceived only when we are able to recognize and neutralize the local factors supporting the survival and growth of cancer cells in the bone. The results from our study could reveal up to three novel candidates for targeted therapies against advanced, bone-metastatic PCa.
Background and objectives.
Skeletal metastases cause significant morbidity and are the primary cause of death from prostate cancer. The therapies currently available for these bone lesions are mostly palliative; thus, the identification and understanding of the mechanisms that promote the colonization of the skeleton by cancer cells will lead to more effective therapeutic strategies for advanced prostate adenocarcinoma. Skeletal metastases in prostate cancer patients are frequently positive for the alpha-receptor for Platelet-Derived Growth Factor (PDGFR$\alpha$) and we have shown that only prostate cancer cells that express this receptor can produce macroscopic bone lesions in animal models by dissemination through the blood circulation. Here we first investigated the mechanism by which the soluble fraction of human bone marrow activates PDGFR$\alpha$. Then, we tested whether the humanized, monoclonal antibody IMC-3G3 against PDGFR$\alpha$ could impair metastatic colonization and progression as well as prolong survival, either as primary or combination therapy.

Methodologies. We employ a pre-clinical animal model of metastasis in which fluorescent human prostate cancer cells are inoculated directly in the blood circulation of SCID mice via the left cardiac ventricle. Using a combination of fluorescence stereomicroscopy, histological analysis and digital imaging we can identify isolated cells, small tumor foci and macroscopic metastases at the skeletal level.

Results. In the first part of this study, we conclusively established that human bone marrow activates PDGFR$\alpha$ on prostate cancer cells in an unorthodox fashion, which does not require the ligand-binding domain or receptor dimerization. We have previously shown that the IMC-3G3 antibody can induce PDGFR$\alpha$ internalization, thereby impairing its downstream signaling. Thus, in the second part of the study we tested IMC-3G3 in our model of experimental metastasis. First, the IMC-3G3 antibody was administered to mice simultaneously to the inoculation of prostate cancer cells and showed a prophylactic effect by reducing the number of bone metastatic lesions per animals observed at four weeks post-inoculation by 72%. Successively, IMC-3G3 was administered to animals that were bearing skeletal metastases established one or two-week earlier. We found that the antibody exerted also a curative effect, by significantly reducing the size of skeletal tumors as compared to saline- or control IgG-treated animals.

IMC-3G3 was then administered alone or in combination with the bisphosphonate Zoledronic Acid (ZA) and was able to prolong overall survival. Finally, we tested IMC-3G3 and ZA on the initial phase of bone colonization and found that IMC-3G3 - but not ZA - inhibits early skeletal tumors from prostate cancer cells.

Conclusion: We have identified a novel mechanism for PDGFR$\alpha$ activation by the soluble fraction of human bone marrow, which likely play a role in the survival of prostate cancer cells in the skeleton. Our study also
presents compelling evidence that targeting PDGFRα with IMC-3G3 both delays the progression of early metastatic foci and reduces the size of more established lesions. Also, IMC-3G3 either alone or in combination with ZA, prolongs survival in animal models of metastasis.
Implication of platelet-derived growth factor receptor alpha in prostate cancer skeletal metastasis

Qingxin Liu1, Danielle Jernigan1, Yun Zhang1 and Alessandro Fatatis1,2

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 (PDGFRα) supports the survival and growth of prostate cancer cells in the skeleton and that the soluble fraction of bone marrow activates PDGFRα 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[8]. 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.
Expression of PDGFRα 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 PDGFRα being most represented. We confirmed this observation by showing that normal prostate expresses low levels of PDGFRα, which significantly increase upon malignant transformation. We detected a strong signal for PDGFRα 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 heterogeneous distribution of PDGFRs on the epithelial compartment, suggesting that cellular phenotypes with different expression patterns for PDGFRα co-exist in the same gland, a scenario replicated by the human prostate cancer cell lines commonly available. For instance, we found that PDGFRα 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 PDGFRα [14].

PDGFRα Expression and Bone-Metastasis Potential

A more direct correlation between PDGFRα 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 [18]. 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 PDGFRα than do their non-metastatic counterpart, PC3-N cells. If combined with the complete lack of PDGFRα expression observed in DU-145 cells, these results establish a positive correlation between the expression levels of PDGFRα 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 PDGFRα 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 PDGFRα 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 PDGFRα [13], suggesting that the pre-existing genetic background of a malignant phenotype may ultimately dictate the pro-metastatic role exerted by PDGFRα in prostate cancer cells.

Further studies revealed an unorthodox mechanism by which PDGFRα recruits downstream signaling pathways in prostate cancer cells. The experiments that fully epitomize this atypical signaling by PDGFRα were conducted by over-expressing a truncated form of the receptor, named αRΔX, 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.
ligand(s) or other signaling molecules. When PC3 (αRΔX) 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.

Evidence for Transactivation of PDGFRα 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 PDGFRα and PDGFRβ. Their extracellular portion contains five immunoglobulin-like domains whereas the intracellular part of the molecule contains a kinase domain. Five PDGF ligands, PDGF-AA, -BB, -AB, -CC and -DD, have been identified, which display different binding affinities for the different receptors. 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. 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. 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.

We initially reported that PDGFRα, 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. 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 PDGFRα[23]. More conclusive evidence for a direct activation of PDGFRα was obtained from the detection of its tyrosine-phosphorylation upon the bone marrow treatment of PC3-ML cells[23].

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 PDGFRα generated by human bone marrow [17]. Notably, the phosphorylation of PDGFRα 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 PDGFRα which are responsible for the recruitment and activation of PI3K.

Collectively, these observations strongly suggest that PDGFRα, in addition to being stimulated by PDGF ligands in a widely recognized fashion[24-26], 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[25-28]. Accordingly, upon activation by the soluble fraction of bone marrow, the canonical dimerization of PDGFRα 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 monomorphic forms of PDGFRα via intracellular mediators. As a role for SRC family kinases (SKF) 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 PDGFRα transactivation was obtained using either DU-145 or PC3-N cells stably expressing αRΔX. Both cell types responded to human bone marrow with strong Akt activation and tyrosine phosphorylation of αRΔX [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 PDGFRα.

The possibility that the establishment and progression of prostate cancer in the bone could be independently supported by PDGFRα 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 PDGFRα may not fully prevent downstream signaling in cells that have spread to the bone marrow. Alternatively, inducing the internalization of PDGFRα 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 PDGFRα to Block Its Downstream Signaling**

PDGFRα and PDGFRβ are involved in organism development, with PDGFRα 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[19,20]. PDGFRβ, however, plays a predominant role overall, as demonstrated in mice in which the cytoplasmic domains between PDGFRα and PDGFRβ were swapped. These experiments revealed that the PDGFRβ intracellular domain could fully substitute for the PDGFRα. In contrast, replacement of the PDGFRβ 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 PDGFRβ 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 PDGFRα and PDGFRβ. 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 PDGFRα, 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 PDGFRβ. However, in the event that PDGFRα 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 PDGFRα would remove from the plasma membrane an important target for the transactivation of cancer cells exerted by the extracellular ligand-binding domain would fail to completely block signaling. Interestingly, cell-surface biotinylation experiments showed that the inhibitory effect of a neutralization of the ligand-binding domain of PDGFRα by IMC-3G3 could induce the internalization of PDGFRα and was shown to support cancer cell growth and survival, while consequent mobilization of growth factors has been 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.

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 PDGFRα downstream signaling was tightly correlated to the internalization of this receptor. This event affected more than 80% of the initial levels of PDGFRα after two hours of IMC-3G3 incubation. Furthermore, by using experimental conditions that halt receptor internalization while preserving IMC-3G3 neutralization of the ligand-binding domain of PDGFRα, we could block Akt phosphorylation by PDGFR-α but not by bone marrow.

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 PDGFRα 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 PDGFRα while leaving the mouse form of the receptor unaffected. 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. 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.

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.

Since it had been previously reported that stromal PDGFRα 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 PDGFRα 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 PDGFRα and otherwise sharing an identical structure with IMC-3G3, 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.

The current standard of care for patients with advanced metastatic prostate cancer includes the administration of bisphosphonates. These molecules are very effective inhibitors of bone-matrix degradation caused by osteoclasts located in skeletal metastatic lesions. 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. Zoledronic acid (ZA) shows a potent analgesic effect that can significantly delay the time to SREs. However,
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. 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. 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 (Figure 3).

**Conclusions**

The series of studies presented here strongly support an important role of PDGFRα 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 PDGFRα is exerted through transactivation events initiated by activating signaling molecules present in the soluble fraction of human bone marrow. Importantly, the selective targeting of PDGFRα with monoclonal antibodies such as IMC-3G3, while still allowing PDGFRα 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 PDGFRα 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.

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Contributions to our studies.

References


