Human Bone Marrow Activates the Akt Pathway in Metastatic Prostate Cells through Transactivation of the α-Platelet–Derived Growth Factor Receptor

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Abstract

The factors regulating the bone tropism of disseminated prostate cancer cells are still vaguely defined. We report that prostate cancer cells that metastasize to the skeleton respond to human bone marrow with a robust stimulation of the phosphatidylinositol 3-kinase/Akt pathway, whereas prostate cells that lack bone-metastatic potential respond negligibly. The majority of this Akt activation is dependent on α-platelet–derived growth factor receptor (α-PDGFR) signaling, which was shown using the small-molecule inhibitor of PDGFR signaling AG1296. Low concentrations of PDGF-AA and PDGF-BB found in bone marrow aspirates, which were detected by ELISA, do not account for the high levels of α-PDGFR signaling. Additionally, neutralizing PDGF binding using a α-PDGFR–specific antibody (IMC-3G3) failed to produce a significant inhibition of bone marrow–induced Akt activation. However, the inhibitory effect of IMC-3G3 rivaled that of AG1296 when incubation was done under conditions that stimulated α-PDGFR internalization. We conclude that α-PDGFR is activated by multiple soluble factors contained within human bone marrow, in addition to its natural ligands, and this transactivation is dependent on receptor localization to the plasma membrane. Therefore, α-PDGFR expression may provide select prostate phenotypes with a growth advantage within the bone microenvironment. [Cancer Res 2007;67(2):555–62]

Introduction

The stromal microenvironment plays a role in determining the susceptibility of different organs to colonization by migrating cancer cells and the establishment of secondary tumors. Prostate adenocarcinoma shows a propensity to metastasize to the skeleton more than any other tissue in the body. Thus, the bone stroma must contain trophic factors that promote prostate cancer cell growth and survival. The organ-specific nature of the metastatic process depends on multiple selective steps, including adhesion of circulating cancer cells to specific endothelia as well as their migration towards chemoattractant gradients existing within the colonized organs. For instance, the chemokine CXCL12/stromal cell–derived factor-1 has been shown to direct the trafficking of prostate cancer cells to the skeleton, where it is found in high concentrations (1). However, CXCL12/stromal cell–derived factor-1 is also abundant in the optic nerve and cardiac muscle, which are seldom sites of metastasis from this form of cancer. Therefore, it is clear that the tissue-tropism of metastatic cells depends on several factors, including favorable conditions for cell growth offered only by select organs.

The bone marrow surrounds the sinusoids, which are traversed by prostate cancer cells invading the skeleton. In addition to regulating the extravasation process, the marrow could offer a fertile soil to disseminated cells, allowing them to develop into clinically evident metastases. Within the bone marrow, different cell types are regularly characterized by sustained levels of metabolic activity. This normal scenario witnesses periodic bursts of cellular activity due to bone remodeling or repair, inflammation, and reactive hematopoiesis. Bone tissue homeostasis is orchestrated by a plethora of trophic molecules to which prostate cancer cells might also be sensitive. For instance, the role of bone turnover in the preferential location of prostate cancer cells to the skeleton has recently been reported (2).

In this study, we focused on platelet-derived growth factor (PDGF) and its receptors (PDGFR). PDGF exerts a trophic effect on several mesenchymal and epithelial cell types. More specifically, this growth factor regulates both osteoblast and osteoclast activities *in vitro* and its involvement in bone remodeling has been established (3–5). PDGF binds to two isoforms of a tyrosine-kinase receptor, α-PDGFR and β-PDGFR, which can either homodimerize or heterodimerize (6). Tissue samples from normal or nonmalignant hyperplastic prostate glands test negative for both PDGFR isoforms. However, both primary prostate tumors and skeletal metastases from matched subjects tested positive for α-PDGFR expression (7). More importantly, we found that among prostate cells originally obtained from different metastatic sites, the expression of α-PDGFR is detected only in the bone metastasis–derived PC3 cells, whereas LNCaP and DU-145 cells obtained from lymph node and brain metastases, respectively, fail to express α-PDGFR or β-PDGFR (8). In addition, PC3-ML cells, a subline of the parental clone that was selected for *in vitro* invasiveness and *in vivo* metastatic potential (9), express at least three times more α-PDGFR than the subline PC3-N, which is both not invasive *in vitro* and nonmetastatic in immunocompromised mice. High levels of α-PDGFR expression render PC3-ML cells particularly susceptible to the stimulation of the survival phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway by PDGF (8). Interestingly, PC3-ML and PC3-N cells both localize to the skeleton in the first hours immediately following their i.v. injection into immunocompromised mice; however, only the PC3-ML cells could be detected after 72 h and eventually produced evident secondary tumors. A widely accepted paradigm for metastasis

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

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emphasizes the existence of selected bone-metastatic prostate phenotypes, which would be better suited to survive in the bone microenvironment as they can benefit from trophic factors normally supporting the activity of bone-resident cells. This scenario implies that prostate cells metastasizing to organs other than the skeleton are phenotypically different from bone-metastatic cells. Therefore, one could argue that prostate cells that were originally derived from nonosseous secondary tumors are less efficient in recruiting survival signaling pathways, such as PI3K/Akt, as compared with cells from bone metastases, when exposed to the bone microenvironment. This hypothesis supports the model originally proposed by Paget (10), which assimilates the compatibility between migrating cancer cells and colonized organs to the required affinity between seeds and a specific soil.

In our study, we report for the first time the dramatically different effect that human bone marrow aspirates exert on the activity of the Akt survival pathway in bone-derived compared with brain-derived human prostate cells. We also measured the effective concentrations of PDGF-AA and PDGF-BB in human bone marrow aspirates and found that α-PDGFRs in bone-metastatic prostate cells are activated predominantly by transactivation and only marginally by direct ligand binding.

Materials and Methods

Human bone marrow acquisition and processing. Bone marrow samples from normal male donors (ages 18–45 years) were supplied by Cambrex (Walkersville, MD; Poeitics Donor Program). Samples were shipped and maintained at 4°C throughout processing and were subsequently stored at −80°C. Briefly, samples were centrifuged at 1,500 rpm to separate the soluble and cellular phases. Supernatant was removed and filtered using 0.8- and 0.22-μm filters in succession. Fifty microliters of processed bone marrow were administered to cells in 1 mL of experimental medium for a final 1:20 dilution.

Animals. Four- to six-week-old male severely immunocompromised mice (CB17 SC-M) were obtained from Taconic and maintained in a germ-free animal facility. All experiments were done in accordance with NIH guidelines for the humane use of animals. All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine.

Reagents and chemicals. PDGF-BB, PDGF-AA, and AG1296 were purchased from Calbiochem (San Diego, CA). IMC-3G3 was provided by ImClone Systems, Inc. (Brunchburg, NJ). The plasmid pEGFP-N1 encoding enhanced green fluorescent protein (EGFP) was obtained from BD Biosciences (San Jose, CA).

Cell culture. PC3 ML cells were isolated and expanded from parental PC3 [American Type Culture Collection (ATCC, Rockville, MD)] as previously reported (9). DU-145 cells were purchased from ATCC. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin. Cells were stored in liquid nitrogen and used for no more than 10 passages after thawing.

***In vivo* metastasis model.** Mice were inoculated with 1×10^5 cells in a volume of 100 μL DMEM via the left cardiac ventricle. Necropsy was done after the animals were sacrificed 5 weeks postinjection. Tumors were identified under a fluorescence stereomicroscope (Olympus SZX12) and subsequently processed for histochemistry (Wax-it Histology Services).

***In vitro* experimental protocol.** For experiments done in the presence of serum, cells were treated with fresh DMEM supplemented with 10% FBS and 50 μg/mL gentamicin 24 h before exposure to bone marrow. In contrast, experiments in the absence of serum (starved cells) were done 4 h after washing cells twice with PBS and replacing growth medium with serum-free DMEM. AG1296 was administered to cultures 30 min before exposure to bone marrow. IMC-3G3 was administered to cells at the stated pretreatment times.

SDS-PAGE and Western blotting. Cell lysates were obtained and SDS-PAGE and Western blot analysis done as previously described (11) with few modifications. Membranes were blotted with antibodies targeting phospho-Akt (Ser^{473}; Cell Signaling Technology, Beverly, MA), α-PDGFR (R&D Systems, Minneapolis, MN), and actin (Sigma, St. Louis, MO). Primary antibody binding was detected using horseradish peroxidase–conjugated Protein A or Protein G (Sigma). Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce, Rockford, IL) and detected with the Fluorochem 8900 imaging system and relative software (Alpha Innotech, San Leandro, CA). Densitometry analysis was done using the UN-SCAN IT software (Silk Scientific, Orem, UT). 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.

Immunoprecipitation of tyrosine-phosphorylated proteins. Following bone marrow treatment, 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). Lysates were cleared by centrifugation and tyrosine-phosphorylated proteins were isolated using the Catch and Release Phosphotyrosine, clone 4G10 Immunoprecipitation Kit (Upstate, Lake Placid, NY) in accordance with the manufacturer's instructions. Non-tyrosine-phosphorylated proteins were collected as flow-through and served as our internal control.

ELISA. Concentrations of PDGF-BB, PDGF-AA, and epidermal growth factor (EGF) were measured using their respective Quantikine Human Immunoassay kits (R&D Systems). Assays were done according to the manufacturer's instructions.

Cell-surface biotinylation. Following treatment with IMC-3G3 or PDGF-BB, cells were washed twice with ice-cold PBS and cell-surface proteins were biotinylated using Sulfo-NHS-SS-Biotin (Pierce Biotechnology). Cell lysates were cleared by centrifugation and the biotinylated membrane fraction was separated from the cytosolic fraction using the Pinpoint Cell Surface Protein Isolation Kit (Pierce Biotechnology) in accordance with the manufacturer's instructions.

Statistical analysis. Data are presented as means ± SEs. Student's t test was used to compare data between groups. Statistical significance was assumed at $P \leq 0.05$.

Results and Discussion

The main goal of our study was to test whether prostate cells that were collected from distinct secondary organs and show different metastatic potential and tissue tropism respond differently to bone marrow, which is the soluble environment they must inhabit on reaching the skeleton.

The PC3-ML subline was derived from the PC3 cell line, which was originally obtained from a skeletal metastasis in a patient with primitive prostate adenocarcinoma (12). PC3-ML cells were selected for their ability to both invade *in vitro* and generate skeletal metastases in severe combined immunodeficient (SCID) mice (9). Indeed, when we inoculated SCID mice with PC3-ML cells expressing EGFP, 15 of 18 injected animals presented with single or multiple bone secondary tumors when sacrificed at 5 weeks following injection (Fig. 1). However, when similar experiments were conducted using DU-145 cells, which were originally obtained from a brain metastasis, none of the injected SCID mice presented with skeletal metastases. Thus, the distinct phenotypes of PC3 and DU-145 cells could explain their different sites of metastasis in humans and determine their organ-tropism when administered to animals.

The "seed and soil" hypothesis emphasizes the importance of tissue-specific conditions in supporting the survival and growth of disseminated cancer cells. This paradigm suggests that cancer cells targeting distinct organs differ in their responsiveness to

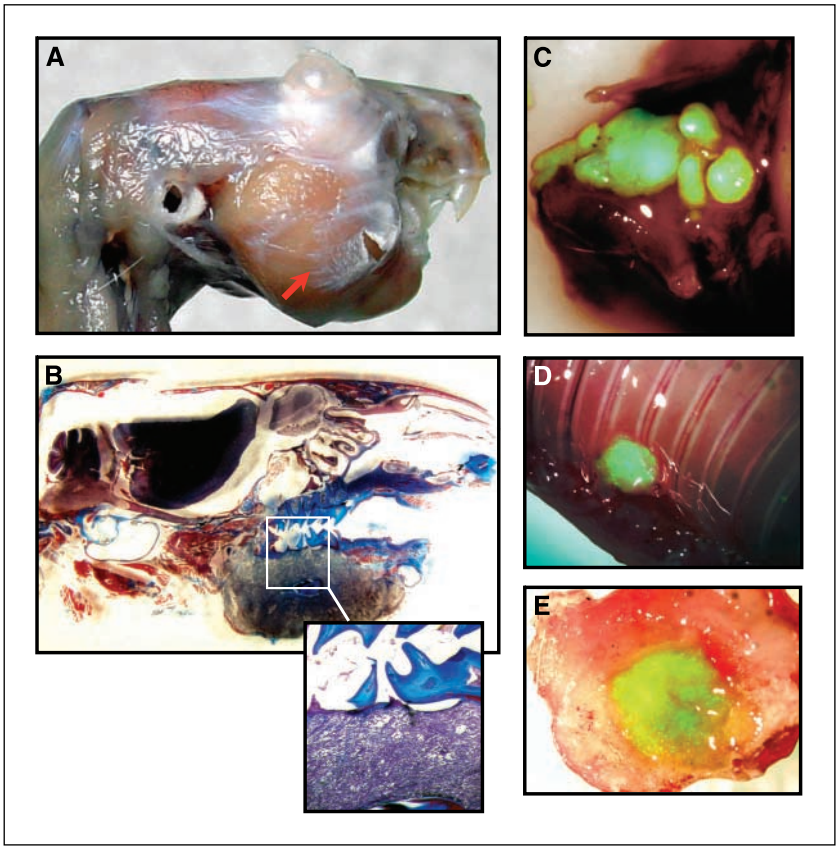

Figure 1. Secondary tumors induced by PC3-ML bone-metastatic prostate cells. *A,* location to the mandible was observed in most of the injected animals. *Arrow,* tumor mass. *B,* histologic analysis of the bone secondary tumor shown in (*A*), done using Masson's staining. *Inset,* tumor mass subverting the normal anatomic organization of teeth (magnification, ×10). *C* to *E,* identification of secondary tumors at the lumbar vertebra, ribs, and tibia (transverse section) caused by metastatic prostate cells expressing a fluorescent tag.

molecules produced by a specific tissue microenvironment. Thus, we hypothesize that DU-145 cells lack bone tropism because they are unable to effectively use the resources present in the bone marrow, whereas the PC3-ML phenotype excels at this task.

Among all the signaling pathways that could be differently regulated by the bone microenvironment in prostate cancer cells, we focused on the PI3K/Akt pathway because of its crucial involvement in promoting cell survival and growth of both normal and malignant cells (13, 14). We therefore exposed PC3-ML and DU-145 cells to human bone marrow aspirates diluted in culture medium and tested the activation state of Akt by Western blotting. Experiments were done following a 4-h incubation period in serum-free medium, a procedure we have shown to down-regulate Akt phosphorylation in both cell types (8). As shown in Fig. 2*A*, a robust and time-dependent phosphorylation of Akt was detected in PC3-ML cells, whereas DU-145 cells were minimally responsive. The activation of signaling pathways by growth factors *in vitro* is commonly investigated in the absence of serum to isolate the stimulatory effects of the molecules under investigation. However, such a procedure might also overemphasize the activation of a signaling pathway in the absence of additional and competing stimulatory molecules, which are normally present in animal sera. Therefore, comparable experiments were done using cells kept in the presence of serum; consistently with the results obtained with serum-starved cells, PC3 ML cells robustly activated Akt upon exposure to bone marrow, whereas DU-145 elicited only a modest response (Fig. 2*B*). These results illustrate that bone-metastatic PC3-ML cells are better suited to recruit the prosurvival Akt kinase in the presence of bone marrow compared with non–bone-metastatic DU-145 cells (Fig. 2*C*).

We have previously shown that PC3-ML cells express α-PDGFR and activate the PI3K/Akt pathway when exposed to PDGF, whereas DU-145 cells lack this receptor and Akt activation in response to PDGF. This growth factor is important for a variety of cell types and regulates bone metabolism and turnover, among other physiologic processes. Osteoblasts and osteoclasts secrete both PDGF-AA and PDGF-BB *in vitro* (3, 15) and, consequently, these growth factors are assumed to be present in the soluble milieu of the bone marrow. To ascertain whether the responsiveness of PC3-ML cells to bone marrow aspirates could be related to the expression of α-PDGFR, we used AG1296, a small synthetic inhibitor specific for PDGFRs (16). We initially determined the minimum effective dose of AG1296 capable of completely inhibiting the Akt phosphorylation induced by 30 ng/mL PDGF-BB in PC3-ML cells. Subsequently, PC3-ML cells were exposed to bone marrow in the absence or presence of 20 μmol/L AG1296, which was established to be the minimum effective dose in our system (Supplementary Data 1*A*). Cells were exposed to bone marrow for 30 min, which corresponds to the peak of Akt phosphorylation observed in the experiments described above and shown in Fig. 2. We found that AG1296 reduced the activation of Akt to <40% of what was observed in cells treated with bone marrow alone (Fig. 3*A* and *D*), thus suggesting that α-PDGFR signaling was responsible for the majority of Akt activation on exposure of PC3-ML cells to bone marrow. Further support to this idea was provided by the evidence for α-PDGFR time-dependent tyrosine phosphorylation in the presence of bone marrow (Fig. 3*B*).

We were then interested in measuring the concentration of PDGF in the bone marrow aspirates as well as identifying the isoform(s) of this growth factor responsible for Akt activation in

PC3-ML cells. However, bone marrow aspirates are rich in platelets, which store PDGF in α-granules along with other growth factors, including EGF (17). Platelets can degranulate in response to physiologic signals or mechanical stimuli, including blood and marrow withdrawal. Therefore, EGF concentrations were also measured to rule out the possibility that the PDGF measured in the marrow derived from platelet degranulation rather than local production by bone cells. Four of six aspirates tested negative for EGF, showing a lack of this growth factor in bone marrow as well as eliminating the possibility of platelet degranulation, and these samples were used for concentration analysis and the experiments described in this study. We found that bone marrow aspirates obtained from three different donors contained both PDGF-AA and PDGF-BB in concentrations ranging from 400 pg/mL to 2 ng/mL. Because we did our experiments after diluting aspirates 20-fold for practical reasons, cells were in fact exposed to concentrations of PDGF between 20 and 100 pg/mL (Supplementary Data 1*B*). To establish whether these seemingly low concentrations of PDGF could be responsible for the considerable magnitude of α-PDGFR activity and Akt phosphorylation induced by bone marrow, we exposed PC3-ML cells to serum-free medium containing both PDGF-BB and PDGF-AA (100 pg/mL for each isoform). The stimulation of Akt we observed in these conditions was minimal, representing <10% of that obtained by exposing these cells to bone marrow (Fig. 3*C*), and did not correspond to the 60% inhibition of Akt phosphorylation produced by AG1296 (Fig. 3*D*). Due to this disparity, we considered the possibility that, in addition to the minimal direct stimulation by PDGFs, α-PDGFR was being predominantly activated by mechanisms alternative to ligand binding.

To further investigate this hypothesis, we used the neutralizing monoclonal antibody IMC-3G3, which is specific for human α-PDGFR (18). The ability of IMC-3G3 to inhibit the phosphorylation of Akt induced by human bone marrow in PC3-ML cells was compared with that of AG1296. The IMC-3G3 antibody acts extracellularly by blocking the ligand binding domain, whereas the AG1296 inhibitor acts intracellularly by interfering with α-PDGFR kinase signaling. Therefore, these two tools with different mechanisms of action are ideal to discriminate between ligand-induced and ligand-independent activation of α-PDGFR.

We first tested the efficacy of IMC-3G3 and the dose dependency of its effect in our system and found that a preincubation time of 15 min and a concentration of 20 μg/mL were sufficient to neutralize the stimulatory effect of 30 ng/mL PDGF-BB (Fig. 4*A*). We predicted that the antibody would induce only a minor inhibitory effect on Akt phosphorylation, in agreement with the negligible stimulation observed when PDGF-AA and PDGF-BB were added to the cells simultaneously and in concentrations similar to those we detected in the bone marrow (Fig. 3C and Supplementary Data 1*B*). In contrast, the effect of IMC-3G3 was significantly stronger than expected, showing ~40% inhibition for Akt phosphorylation compared with untreated cells (Fig. 4*B* and *C*). An interesting observation was made when PC3-ML cells were incubated with IMC-3G3 for different time periods before being exposed to bone marrow. Using this approach, we found that the extent of the inhibitory activity of the IMC-3G3 antibody on Akt phosphorylation was dependent on the duration of the preincubation, with the incubation time of 120 min being significantly more effective than 15 min used for the experiments described above (Fig. 4*D*). These results could not be explained by the slow

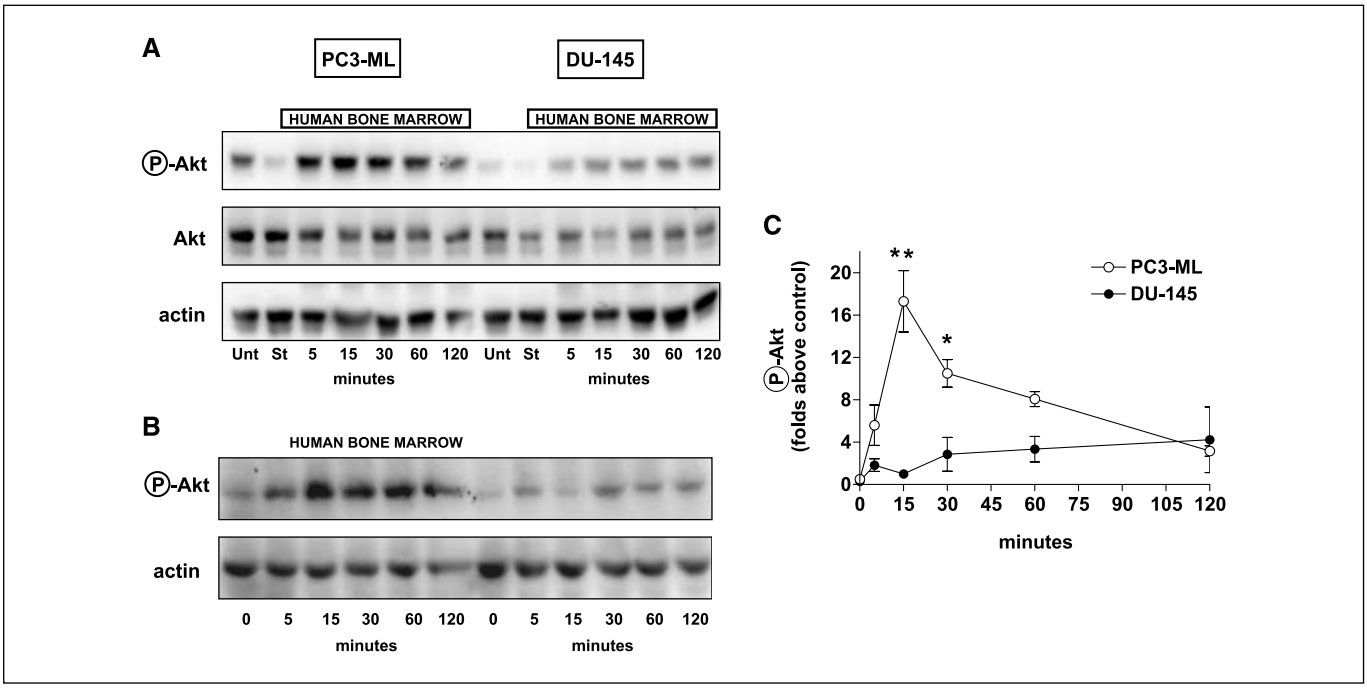

Figure 2. Effects of human bone marrow aspirates on Akt activity in prostate cancer cells, detected by Western blotting analysis. *A,* bone-metastatic and bone metastasis–derived PC3-ML prostate cells were exposed to bone marrow in the absence of serum and showed a strong stimulation of Akt phosphorylation. In contrast, non–bone-metastatic and brain-derived DU-145 prostate cells showed only minimal Akt activation in similar conditions. *B,* the exposure of PC3-ML and DU-145 cells to human bone marrow in the presence of serum confirms the difference in Akt responsiveness between these two cell phenotypes. Samples were run on the same gels for effective comparison of intensity levels. Total Akt levels showed complete correspondence to actin levels. Thus, measurement of actin expression was used in the following experiments. *C,* graphic representation of the experiments described in (*A*). Values are expressed as folds of stimulation above control cells (untreated), which were not exposed to bone marrow. **, $P < 0.01$; *, $P < 0.05$, versus DU-145 values at corresponding time points. *Bars,* SD.

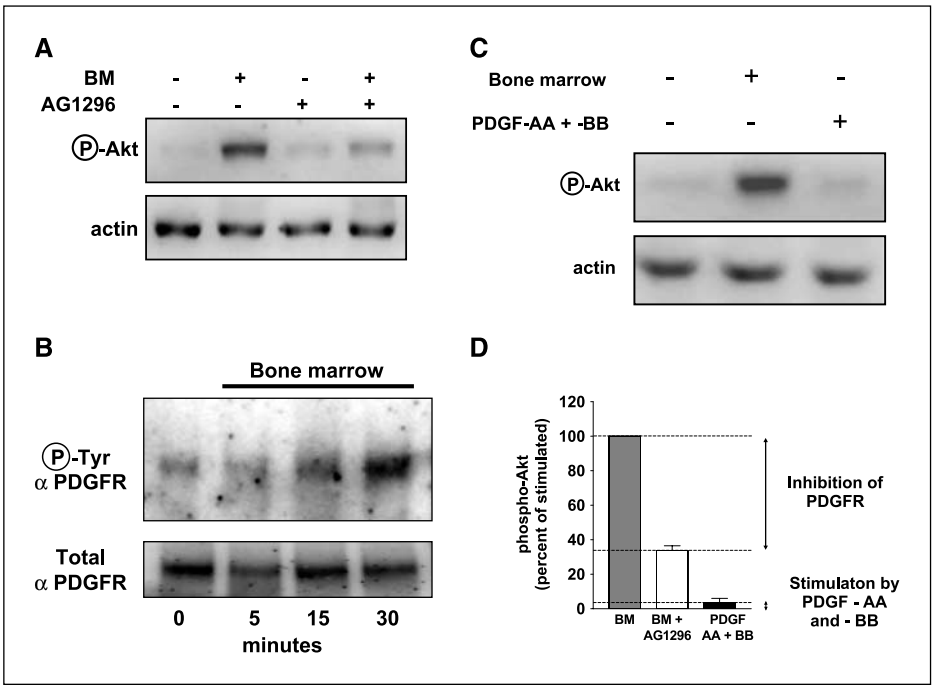

Figure 3. Involvement of α-PDGFR in Akt phosphorylation induced in PC3-ML cells, detected by Western blotting analysis. *A,* AG1296 significantly inhibits Akt stimulation induced by bone marrow in PC3-ML cells. *B,* time-dependent tyrosine-phosphorylation of α-PDGFR upon exposure to human bone marrow. *C,* PDGF-AA and PDGF-BB used in concentrations similar to those detected in human bone marrow only minimally stimulate Akt phosphorylation in PC3-ML cells. *D,* comparison between the effects of AG1296 on bone marrow exposure and PDGF-AA and PDGF-BB added simultaneously on Akt stimulation in PC3-ML cells. *Bars,* SD.

kinetics of IMC-3G3 binding to α-PDGFR, as this antibody was able to completely block the Akt stimulation induced by 30 ng/mL PDGF after 15 min of preincubation (Fig. 4*A*). Based on this evidence, we postulated that IMC-3G3 was inducing the internalization of α-PDGFR and, therefore, its inhibitory effect on Akt signaling was related to the disappearance of the PDGFR from the plasma membrane, in addition to the mere blocking of its binding site. Confirmation of this hypothesis would indicate that, in bone-metastatic prostate cells, human bone marrow induces a ligand-independent activation of α-PDGFR, which leads to the recruitment of the Akt survival pathway. Therefore, the next experiments were aimed to investigate a potential correlation between α-PDGFR internalization kinetics induced by IMC-3G3 and its ability to inhibit bone marrow stimulation of Akt. We initially established that PDGF treatment induced the rapid and complete internalization of α-PDGFR within 5 min (not shown). Although with slower kinetics, IMC-3G3 was also able to induce α-PDGFR internalization (Fig. 5*A*), which involved almost 80% of the initial receptor levels after 2 h, and was tightly correlated to the time dependency of IMC-3G3 inhibitory effect on Akt phosphorylation induced by bone marrow (Fig. 5*B*). These results indicate that α-PDGFR is internalized on binding IMC-3G3 and this mechanism significantly down-regulates the activation of the PI3K/Akt signaling pathway by human bone marrow in bone-metastatic prostate cells.

However, at this point of the study, we needed conclusive evidence that blocking the ligand-binding site of α-PDGFR, in the absence of receptor internalization, would cause only a discreet inhibition of bone marrow–induced Akt phosphorylation. This would corroborate the idea that the PDGF-AA and PDGF-BB we detected in the bone marrow aspirates provide a negligible contribution to the activation of α-PDGFR in bone-metastatic prostate cells, and therefore confirm the concept of α-PDGFR transactivation. The neutralization of α-PDGFR in the absence of internalization would also exclude that additional isoforms of PDGF (i.e., PDGF-AB and PDGF-CC) could be present in the bone marrow aspirates and activate α-PDGFR in PC3-ML cells as well as rule out the possibility of an autocrine stimulation loop, which has previously been shown as a mechanism of α-PDGFR transactivation (19).

Thus, additional experiments were conceived to halt or significantly reduce the internalization of α-PDGFR while preserving the ligand-blocking properties of the IMC-3G3 antibody. To this end, PC3-ML cells were incubated with IMC-3G3 at a temperature of 4°C, a condition recognized to inhibit cytosolic trafficking and receptor internalization, while maintaining antibody binding properties (20). Indeed, at this temperature, α-PDGFR internalization was entirely prevented (Fig. 6*A*), whereas IMC-3G3 successfully blocked Akt phosphorylation caused by PDGF (Fig. 6*B, top*). This experimental approach was further validated by the observation that by transferring PC3-ML cells incubated with IMC-3G3 at 4°C to the temperature of 37°C, we could induce a time-dependent internalization of α-PDGFR (Fig. 6*A*). Based on this evidence, we administered bone marrow to PC3-ML cells previously incubated with IMC-3G3 at 4°C to block the ligand-induced activation of α-PDGFR while still allowing a potential transactivation event to occur. In these conditions, IMC-3G3 only marginally inhibited Akt phosphorylation compared with when its preincubation was done at 37°C (Fig. 6*B, bottom*—compare with Fig. 4*B*). As would be predicted, the inhibitory effect of IMC-3G3 could be recovered by transferring the cells incubated at 4°C to the temperature of 37°C before exposing them to bone marrow, a condition that allows IMC-3G3 to internalize α-PDGFR and therefore preclude its transactivation (Fig. 6*C* and *D*).

Several conclusions can be drawn from our study. First, the soluble fraction of human bone marrow activates the PI3K/Akt pathway in PC3-ML prostate cells, which were initially isolated from a skeletal metastasis and consistently target the skeleton when injected in SCID mice (Fig. 1). In contrast, bone marrow induces only a negligible activation of Akt in DU-145 prostate cells, which were initially isolated from a brain metastasis and do not form skeletal tumors when injected in SCID mice. The different

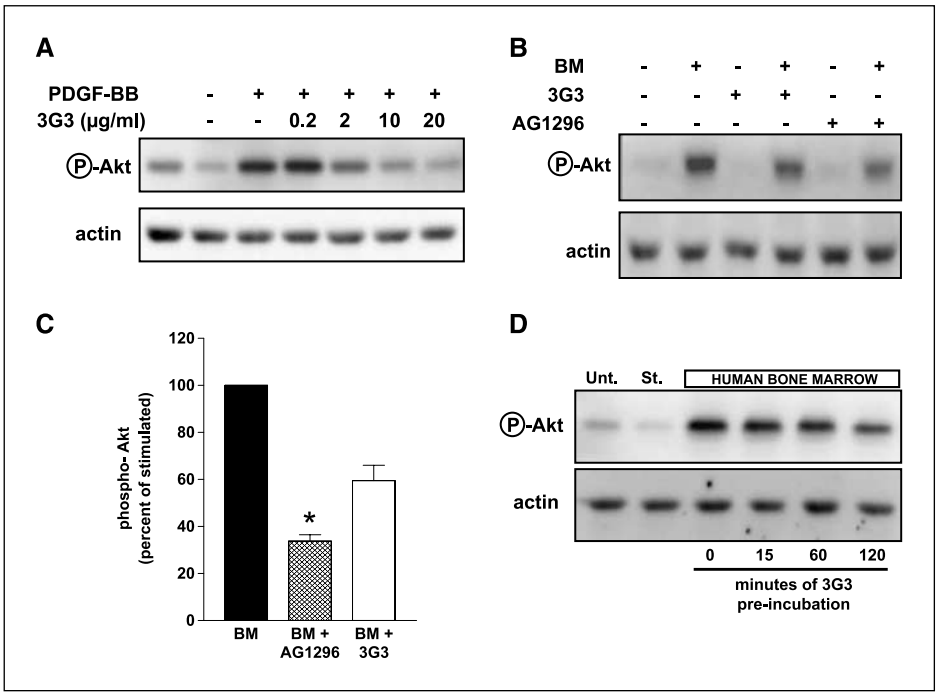

Figure 4. Effect of the IMC-3G3 antibody on α-PDGFR signaling investigated by Western blotting. *A,* dose-effect of IMC-3G3 on Akt phosphorylation induced by 30 ng/mL PDGF-BB in PC3-ML cells. *B* and *C,* comparison between IMC-3G3 and AG1296 inhibitory effects on Akt phosphorylation induced by bone marrow exposure in PC3-ML cells. *, $P < 0.05$. *Bars,* SD. *D,* the inhibitory effect of IMC-3G3 on Akt phosphorylation depends on the preincubation time.

propensity of these two prostate phenotypes to grow in the bone might be related to their diverging abilities to activate Akt, an essential signaling pathway that could promote and sustain their survival in the bone marrow microenvironment. However, conclusive evidence for this hypothesis would be only obtained by appropriate studies conducted in animal models, which are currently ongoing in our laboratory.

Although a supporting role of the bone marrow stroma on metastatic prostate cells has repeatedly been proposed (21), this is the first study in which prostate cells with different bone tropism were exposed to human bone marrow and their activation of the PI3K/Akt pathway was investigated.

A second important conclusion of our study is that Akt activation induced by bone marrow in bone-metastatic prostate cells is largely dependent on α-PDGFR signaling. The expression of PDGFRs by prostate cells located in human skeletal metastases has previously been reported, whereas we have recently shown that normal epithelial as well as malignant prostate cells taken from non-bone-metastatic sites lack PDGFR expression (8). Our study now shows a direct link between the signaling of this receptor and the Akt responsiveness to human bone marrow in prostate cells with high bone-metastatic potential. As the core of the widely accepted seed and soil hypothesis highlights the affinity between selected cancer phenotypes and the microenvironment of different organs, we then propose that the expression of α-PDGFR provides prostate cells that show high propensity to metastasize to the skeleton with exceptional receptiveness to the bone marrow stroma. Finally, we describe for the first time in prostate cancer cells an alternate and ligand-independent activation of α-PDGFR that requires receptor localization to the plasma membrane. PC3-ML cells respond to PDGF concentrations of 30 ng/mL with a robust activation of PI3K/Akt (Supplementary Data 2*A*). However, when these cells were exposed to bone marrow containing PDGF-AA and PDGF-BB in the lower picomolar range, α-PDGFR still induced a strong phosphorylation of Akt, which could not be explained by ligand binding (Supplementary Data 2*B*). In fact, we show that the blockade of α-PDGFR binding site exerted only a negligible effect on the downstream signaling, whereas receptor internalization inhibited the vast majority of Akt activation (Supplementary Data 2*C*). Transactivation of multiple tyrosine

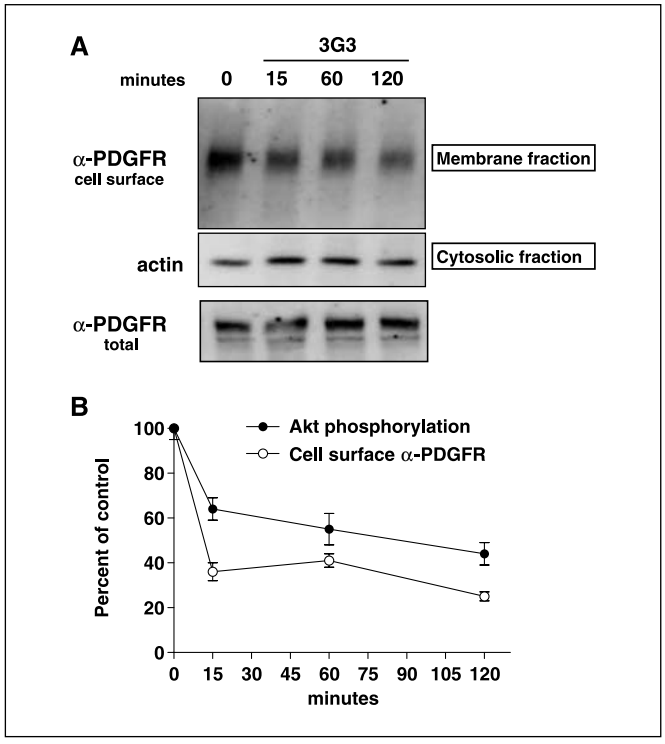

Figure 5. Internalization of α-PDGFR. *A,* the IMC-3G3 antibody induces the time-dependent internalization of α-PDGFR. *B,* correlation between α-PDGFR internalization and inhibition of Akt phosphorylation induced by IMC-3G3 in PC3-ML cells exposed to bone marrow. *Bars,* SD.

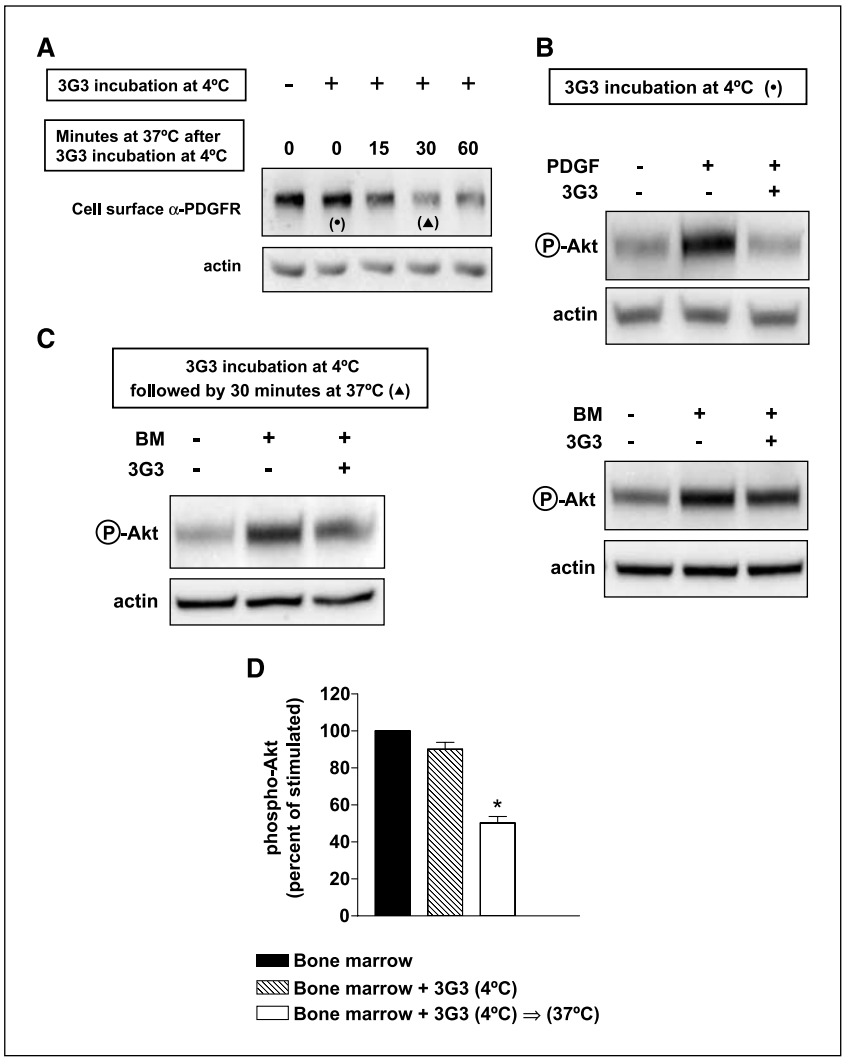

Figure 6. Evidence for bone marrow–induced α-PDGFR transactivation in PC3-ML cells. *A,* effects of preincubation with IMC-3G3 at 4°C alone or followed by incubation at 37°C on α-PDGFR internalization in PC3-ML cells. *B,* preincubation with IMC-3G3 at 4°C (block of the binding domain) inhibits Akt phosphorylation induced by PDGF-BB but not that induced by human bone marrow. *C,* preincubation with IMC-3G3 at 4°C followed by incubation at 37°C (inducing α-PDGFR internalization) inhibits the effect of bone marrow on Akt phosphorylation. *D,* graphic comparison of Akt phosphorylation induced by bone marrow in PC3-ML cells on blocking ligand binding versus blocking ligand binding in addition to inducing α-PDGFR internalization using IMC-3G3. *, $P < 0.05$. *Bars,* SD.

kinase receptors, including PDGFR (22), has been described in a wide range of normal and malignant cell types (23–26). Here we provide the first evidence that the soluble fraction of human bone marrow activates the PI3K/Akt pathway in bone-metastatic prostate cells predominantly through α-PDGFR transactivation.

The possibility of receptors activating prosurvival signaling pathway through transactivation might amplify the range of potential targets for therapeutic intervention. A current challenge in cancer therapy is the acquisition of secondary resistance to antibodies or small-molecule inhibitors directed against specific receptors (27, 28). In such circumstances, combination drug treatments against multiple plasma membrane receptors involved in transactivation could effectively target a common downstream signaling pathway while reducing the likelihood of drug resistance (29–32).

Our laboratory is currently investigating the receptor(s) responsible for transactivating α-PDGFR and the bone marrow molecule(s) involved. The identification of organ-specific stromal factors that recruit survival signaling pathways could give insight to the tissue tropism exhibited by a number of advanced stage malignancies. Characterizing their molecular mechanism of action may provide new targets and therapeutic strategies aiming to overcome metastatic disease.

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