Hypoxia Stabilizes GAS6/Axl Signaling in Metastatic Prostate Cancer

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Abstract

The receptor tyrosine kinase Axl is overexpressed in a variety of cancers and is known to play a role in proliferation and invasion. Previous data from our laboratory indicate that Axl and its ligand growth arrest–specific 6 (GAS6) may play a role in establishing metastatic dormancy in the bone marrow microenvironment. In the current study, we found that Axl is highly expressed in metastatic prostate cancer cell lines PC3 and DU145 and has negligible levels of expression in a nonmetastatic cancer cell line LNCaP. Knockdown of Axl in PC3 and DU145 cells resulted in decreased expression of several mesenchymal markers including Snail, Slug, and N-cadherin, and enhanced expression of the epithelial marker E-cadherin, suggesting that Axl is involved in the epithelial–mesenchymal transition in prostate cancer cells. The Axl-knockdown PC3 and DU145 cells also displayed decreased in vitro migration and invasion. Interestingly, when PC3 and DU145 cells were treated with GAS6, Axl protein levels were downregulated. Moreover, CoCl2, a hypoxia mimicking agent, prevented GAS6-mediated downregulation of Axl in these cell lines. Immunohistochemical staining of human prostate cancer tissue microarrays showed that Axl, GAS6, and hypoxia-inducible factor-1α (Hif-1α; indicator of hypoxia) were all coexpressed in prostate cancer and in bone metastases compared with normal tissues. Together, our studies indicate that Axl plays a crucial role in prostate cancer metastasis and that GAS6 regulates the expression of Axl. Importantly, in a hypoxic tumor microenvironment Axl expression is maintained leading to enhanced signaling. Mol Cancer Res; 10(6); 1–10. ©2012 AACR.

Introduction

Cancer metastasis remains one of the most challenging clinical problems. In the United States alone, more than 350,000 people die each year due to skeletal complications associated with bone metastasis common in many cancers such as prostate and breast cancer (1). The 5-year survival rate of primary prostate cancer is nearly 100%, however after the detection of bone metastasis the 5-year survival rate declines to 33% (2). In order for cancer cells to metastasize they must at first detach from the primary tumor, invade the surrounding extracellular matrix (ECM), and cross the endothelium to enter the blood stream (3). Cancer cells must then move across the endothelium and invade the ECM before migrating and taking up residence in distant organs such as bone. Understanding this multistep process is therefore crucial for elucidating the molecular events that produce a lethal phenotype.

Axl is one member of a tyrosine kinase receptor family (receptor tyrosine kinase, RTK), which also includes Sky (Tyro3) and Mer. Together, Axl, Sky, and Mer or the TAM (Tyro3-Axl-Mer) family of RTKs are characterized by an extracellular domain containing 2 immunoglobulin-like domains and 2 fibronectin type 3–like domains. Axl encodes a 140 kD protein and was originally identified as a transforming gene in patients with chronic myelogenous leukemia (4, 5) and in chronic myeloproliferative disorders (6). Growth arrest–specific 6 (GAS6), is a vitamin K–dependent ligand for Axl, Sky, and Mer (7–9), so named because it was initially discovered as a protein which is overexpressed in growth-arrested cells (10). Binding of GAS6 leads to Axl receptor dimerization and autophosphorylation on its tyrosine residues, which triggers a cascade of intracellular signaling (11). Several studies have indicated that Axl plays a role in tumor invasion and metastasis in a number of cancers, including breast, pancreatic, glioblastoma, hepatocellular, and renal carcinomas (12–18). The expression level of Axl is directly proportional to tumor grade and predicts poor patient survival rate (19–25).

At present, less is known as to the biologic role played by Axl in prostate cancer progression. Previous findings from our laboratory have shown that Axl is highly expressed in metastatic prostate cancer and suggested that the interaction of GAS6 and TAM RTKs may play a role in establishing tumor dormancy in the bone marrow microenvironment (26). In this present report, we show that Axl, which is highly
expressed in metastatic prostate cancer cells, promotes migration and invasion of prostate cancer cells in vitro and plays an important role in regulating expression of genes involved in epithelial–mesenchymal transition (EMT) of cancer cells. Our studies also reveal that Axl expression levels are negatively regulated by its ligand GAS6. However, in hypoxic environments such as in a tumor or in bone metastases, downregulation of Axl expression is prevented. Together, these studies show that Axl plays a crucial role in prostate cancer metastasis, and its expression is regulated by both GAS6 and hypoxia.

Materials and Methods

Cell culture

PC3 (CRL-1435), DU145 (HTB-81), and LNCaP (CRL-1740) prostate cancer cell lines were obtained from the American Type Culture Collection. All prostate cancer cell lines were cultured in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen) and 1% (v/v) penicillin-streptomycin (Invitrogen) and maintained at 37°C, 5% CO₂, and 100% humidity.

Antibodies and reagents

Anti-Axl antibody (catalog no. 4939), anti-Slug antibody (catalog no. 9585), anti-Snail antibody (catalog no. 3879), anti-E-cadherin antibody (catalog no. 3195), anti-N-cadherin antibody (catalog no. 4061), and anti-β-actin antibody (catalog no. 4970) were purchased from Cell Signaling (Beverly, MA). Anti-Axl antibody (catalog no. AF885; R&D Systems) was detected using a Zenon Alexa Fluor 555 goat IgG labeling kit (Invitrogen). Staining with an anti-Axl antibody (catalog no. AF154; R&D Systems) and anti-human hypoxia-inducible factor-1α (Hif-1α; catalog no. 610958; BD Biosciences) were detected using a Zenon Alexa Fluor 488 mouse IgG1 labeling kit (Invitrogen). Human recombinant GAS6 was purchased from R&D Systems. CoCl₂ (catalog no. C8861) was purchased from Sigma-Aldrich.

Axl knockdown by lentiviruses

GIPZ Lentiviral shRNA mir vectors containing either Axl shRNA or nonsilencing (scrambled) shRNA were purchased from Open Biosystems. Lentiviruses were constructed at the Vector Core in University of Michigan (Ann Arbor, MI). Stable Axl knockdowns in DU145 and PC3 cells were generated by lentiviral infection followed by 1 week of selection with 1 μg/mL of puromycin. Viral infection efficiency was assessed by determining the percentage of GFP-positive cells. Axl knockdown in these cells was confirmed by both real-time PCR and Western blot analysis.

RNA extraction and real-time PCR analysis

Real-time PCR was carried out using standard techniques. Briefly, total RNA was isolated by RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized in a 20 μL reaction volume using 0.4 μg of total RNA. Reverse transcription products were analyzed by real-time PCR in TaqMan Gene Expression Assays of several target genes including GAS6, Axl, Sky, Mer, Slug, Snail, N-cadherin, E-cadherin, and β-actin (Applied Biosystems). Real-time PCR analysis was conducted with 15.0 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 1.5 μL of TaqMan Gene Expression Assay, 1 μL of cDNA, and RNase/DNase-free water in a total volume of 30 μL. All sample concentrations were standardized in each reaction to exclude false-positive results. Reactions without template and/or enzyme were used as negative controls. The second-step PCR (95°C for 30 seconds and 60°C for 1 minute) was run for 40 cycles after an initial single cycle of 95°C for 15 minutes to activate the Taq polymerase. The PCR product was detected as an increase in fluorescence using an ABI PRISM 7700 sequence detection system (Applied Biosystems). RNA quantity (Cₚ) was normalized to the housekeeping gene β-actin control by using the formula: Cₚ = 2¹⁰⁻⁴₀ₐₗₜ sample⁻¹⁰⁻⁴₀ₐₗₜ control. The threshold cycle (Cₜ) is the cycle at which a significant increase in fluorescence occurs.

Western blot analysis

Prostate cancer cells (1 × 10⁶ cells per well) were cultured in 6-well plates in RPMI medium. For Axl knockdown and EMT studies, whole-cell lysates were made from cells grown in media containing serum. For GAS6 regulation studies, prostate cancer cells were serum starved overnight, after which they were either left untreated or treated with 100 ng/mL of GAS6 for 6 hours and overnight, or with 100 μmol/L of cobalt chloride (CoCl₂) for 6, 7 hours, and overnight. Whole-cell lysates were prepared from cells, separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with 5% milk for 1 hour and incubated with primary antibodies overnight at 4°C. Primary antibody was used a 1:1,000 ratio with 5% dry milk. Blots were incubated with peroxidase-coupled secondary antibodies (Promega) for 1 hour at a ratio of 1:10,000, and protein expression was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Membranes were reprobed with polyclonal anti-β-actin antibody (1:1,000; Cell Signaling) to control for equal loading.

In vitro migration and invasion assays

In vitro migration and invasion assays were done using the Corning Cell migration, chemotaxis, and invasion assay protocol. PC3 and DU145 cells were serum starved overnight. A total of 2 × 10⁵ cells were plated in the upper chamber of a 24-well transmembrane permeable support with 8 μm pore size (catalog no. 3422; Corning). For the invasion assay only, 100 μL of basement membrane extract (BME)-coating solution (catalog no. 3455-096-02; Trevigen) was placed in the top chamber before cell plating. GAS6 (100 ng/mL) in serum-free media or serum-containing media were used as chemotactants in the bottom chamber. After 24 hours, the cells which had migrated or invaded were dissociated using a cell dissociation buffer (catalog no. 3455-096-05; Trevigen). Cells were then stained with
Calcein AM fluorescent dye (catalog no. C3100MP; Molecular Probes; Invitrogen) for 1 hour and placed in a 96-well solid black microplate (catalog no. 3916; Corning). At the end of the experiments, the fluorescent intensities of bottom chambers were read by a fluorescent plate reader at 485 nm excitation and 520 nm emission. A standard curve was made by staining known numbers of cells with Calcein AM. Fluorescent data from migration and invasion assay experiments were converted to cell numbers based on this standard curve.

**Tumor tissue microarrays and immunostaining**

Human tumor microarrays were provided by the Prostate SPORE Tissue Core Laboratory, Urology Department, University of Michigan, for immunofluorescence staining. Tissue microarray (TMA) sections were unmasked with pepsin solution (Lab Vision) at 37°C for 15 minutes and pH balanced with PBT (PBS plus 0.2% Triton X-100) at room temperature for 5 minutes. Sections were blocked with Image-iT FX signal enhancer (Invitrogen) for 30 minutes before incubating with fluorescence-labeled primary antibodies at room temperature for 2 hours in the dark. Sections were washed with PBS, fixed with 10% formalin (Sigma-Aldrich) for 10 minutes, mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen), and covered with cover glass. Images were taken with Olympus FV-500 confocal microscope. For data analysis, Software NIS Elements BR3.2 64-bit (Nikon) was used in conjunction with 20× DIC M (0.19 um/px) for all 20× images using an automated mass field measurement. For single color, GAS6 or Axl 555 mono wavelength channels were used, 488 and 555 wavelengths were used to quantify colocalization with Hif-1α. Data are presented as percent positive of each 20× field.

**Statistical analysis**

All *in vitro* experiments were carried out at least 3 times with similar results. Results from representative assays are shown. Numerical data are expressed as mean ± SD. Significance of the difference between 2 measurements was determined by unpaired Student *t* test. Values of *P* < 0.05 were considered significant.

**Results**

**Metastatic prostate cancer cells express high levels of Axl**

Our earlier study indicated that Axl was highly expressed in prostate cancer cell lines (26). In that study, immunostaining of Axl in TMA samples revealed that Axl expression had a positive correlation with tumor grade. To confirm our earlier observations, real-time PCR was carried out to detect mRNA expression levels of the GAS6 receptors Axl, Sky, and Mer in the metastatic prostate cancer cell lines PC3 and DU145 and in a nonmetastatic cell line LNCaP. As expected, Axl was highly expressed in metastatic cancer cells PC3 and DU145, whereas very low levels of Axl were detected in the nonmetastatic cell line LNCaP (Fig. 1A). Little or no expression of Sky and Mer was observed in all cell lines (Fig. 1A). These results corroborate our earlier data and strongly indicate that Axl plays a role in metastasis in prostate cancer cells. To further explore the biology of Axl expression in prostate cancer cells, Axl expression was downregulated using a lentivirus vector harboring either scrambled shRNA (sc.shRNA) or Axl shRNA. Real-time PCR analysis confirmed Axl shRNA-induced knockdown of Axl mRNA in PC3 and DU145 compared with controls (Fig. 1B and C).

**Axl regulates EMT in prostate cancer cells**

The process of EMT allows a cancer cell to undergo biochemical changes to assume a mesenchymal phenotype. This results in cancer cells which have enhanced migratory capacity, increased invasiveness, enhanced resistance to apoptosis, and which exhibit an ability to degrade the ECM components (27). To determine whether Axl plays a role in regulating the process of EMT, Western blot analysis was conducted to compare the protein levels of molecules...
involved in the process of EMT. As shown in Fig. 2A, B, and D, PC3 and DU145 cells in which Axl expression is downregulated expressed diminished levels of the mesenchymal markers Snail, Slug, and N-cadherin and enhanced levels of the epithelial marker E-cadherin. Whereas there were changes in the protein levels of these EMT markers, no changes in mRNA levels were seen following Axl knockdown in PC3 cells (Fig. 2C) and DU145 (not shown), suggesting that Axl regulates EMT marker expression at the protein level.

Knockdown of Axl in prostate cancer cells leads to inhibition of migration and invasion in vitro

To determine whether expression of Axl plays a functional role in prostate cancer metastasis, in vitro migration and invasion assays were conducted. Following overnight serum starvation, migration across a Boyden chamber towards serum or GAS6 decreased significantly for PC3 and DU145 cells in which Axl was knocked down, as compared control cells (Fig. 3A and C). Similarly, in vitro invasion assays were also conducted to determine the invasion potential of Axl knockdown PC3 and DU145 cells. Similar to the migration assay, PC3 and DU145 cell invasion across a Boyden chamber was suppressed when Axl expression was reduced (Fig. 3B and D). Together these in vitro data suggest that Axl plays a role in promoting migration and invasion of prostate cancer cells.

Exogenous treatment with GAS6 reduces Axl protein expression in prostate cancer cells

To determine whether GAS6 regulates the expression of its receptor Axl, PC3 and DU145 cells were treated with GAS6 at different time points following overnight serum starvation. Axl protein levels in both cell lines were decreased at 6 hours following GAS6 treatment. Moreover, Axl protein levels remained low even after overnight treatment with GAS6 (Fig. 4A and B). While Axl protein levels went down upon GAS6 treatment, there were no significant changes in Axl mRNA levels (Fig. 4C and D). This indicates that GAS6 regulation of Axl expression is mediated at the protein rather than the mRNA level.

Hypoxia prevents GAS6-mediated downregulation of Axl in prostate cancer cells

Several studies have indicated that the tumor microenvironment is hypoxic in nature (28). CoCl2, a reagent which mimics a hypoxic environment when added in vitro (29), was used to create a hypoxic environment for PC3 and DU145 cells in culture. Interestingly, CoCl2 treatment prevented GAS6 downregulation of Axl protein expression in both PC3 and DU145 cells (Fig. 5A and B). To verify CoCl2’s role as a hypoxia mimicking agent, Hif-α levels were analyzed in PC3 and DU145 cells after CoCl2 treatment. It was observed that Hif-α levels increased within 6 hours of
treatment with CoCl₂ treatment of both PC3 and DU145 cells (Fig. 5C). Our results indicate that hypoxia stabilizes Axl protein and prevents its degradation via GAS6.

GAS6 and Axl colocalize with Hif-1α in both primary tumors and bone metastasis in human prostate TMA samples

Our in vitro studies indicate that GAS6 negatively regulates Axl protein levels, yet in hypoxic conditions Axl protein levels do not change upon in response to GAS6 stimulation. It was therefore critical to explore whether this in vitro result is also true in the hypoxic regions of tumors (as defined by Hif-1α staining; ref. 30). Consequently, human prostate cancer TMAWs were probed with antibodies that target GAS6 and Hif-1α or Axl and Hif-1α to determine their colocalization. Overall, Hif-1α levels were higher in prostate tumors tumor than in the normal prostate (Fig. 6). It was also observed that GAS6 is highly expressed in regions positive for Hif-1α in both primary tumor and in bone metastasis (Fig. 6). Similarly it was seen that Axl was expressed in regions positive for Hif-1α in both primary tumors and significantly in bone metastasis (Fig. 7). Thus, both GAS6 and Axl are expressed within hypoxic regions of a tumor and significantly in metastases. Together these results show that GAS6 negatively regulates Axl under normoxic conditions (Figs. 4 and 5), but not under hypoxic conditions in vitro, and within a hypoxic tumor Axl expression remains high (Fig. 7) despite the presence of abundant GAS6 expression (Fig. 6). These data suggest that the continuous GAS6-Axl signal in regions of hypoxia may contribute significantly to migration, invasion, and metastasis.

Discussion

In this study, we evaluated the role of Axl in prostate cancer progression. We observed that reducing the expression of Axl in prostate cancer cell lines significantly decreased both the in vitro migration and the invasion of cancer cells across a Boyden chamber toward either GAS6 or serum. Reducing Axl expression also resulted in the downregulation of several mesenchymal markers including Snail, Slug, and N-cadherin whereas at the same time enhanced expression of the epithelial marker E-cadherin. Not surprisingly, the expression of Axl is negatively regulated by the presence of its ligand. However under hypoxic conditions, such as those in the bone marrow or in a tumor setting, GAS6 does not downregulate Axl expression, which may result in tumor progression toward an EMT state.

The physiologic role of Axl and its ligand GAS6 has been extensively studied in vascular smooth muscle cells and in...
neurons. GAS6 stimulation of Axl plays a role in chemotaxis in these cells (31, 32). Recently, the role of Axl and GAS6 has been studied in a variety of cancers (12). Enhanced Axl expression correlates with increased lymph node metastasis and poor clinical outcomes in oral squamous cell carcinoma (19). Downregulation of Axl in hepatocellular carcinoma attenuates proliferation and migration in vitro and peripheral lymph node metastasis in vivo (15). Axl was found to be overexpressed in 70% of stage II pancreatic ductal adenocarcinoma (PDA) samples. GAS6 and Axl overexpression in PDA samples are associated with poor prognosis in patients with stage II PDA (12), and Axl expression in pancreatic cancer is significantly associated with lymph node metastasis (12). Axl knockdown prevents migration and invasion in pancreatic cancer cell lines and results in decreased amounts of activated (GTP-bound) GTPase proteins Rho and Rac; significant downregulation in transcript levels of the EMT-associated transcription factors Slug, Snail, and Twist; and significant decrease in matrix metalloproteinase (MMP)-9 mRNA levels (21). The presence of Axl in primary breast cancers predicts reduced overall survival. In mammary epithelial cells Axl is induced by EMT and Axl knockdown prevents the spread of highly metastatic breast carcinoma cells to lymph nodes and several major organs (17). Overexpression of Axl in lung adenocarcinomas and gliomas also predicts a less than favorable outcome (24, 25). Several strategies are being developed to inhibit GAS6 signaling in cancer (33). Using mouse models it has been shown that a small-molecule inhibitor of Axl kinase, R428, significantly reduces metastasis of breast cancer and extends survival (34).

Little is known about how Axl expression is regulated in cancer cells. In breast cancer cells, the intermediate filament and mesenchymal marker vimentin positively regulates Axl expression (13). In colorectal and cervical carcinoma cell lines, myeloid zinc finger protein 1 (MZF1) binds to the Axl promoter, transactivates promoter activity, and enhances Axl-mRNA and protein expression in a dose-dependent manner (14). In Axl-transformed NIH-3T3 cells, Axl is posttranslationally regulated by proteolytic-mediated degradation, and half life of Axl is around 2 hours (4). This indicates that Axl protein is highly unstable and is rapidly degraded under certain conditions. Studies in human lens
epithelial cells have indicated that GAS6 promotes down-regulation of Axl protein by inducing phosphorylation and ubiquitination of Axl and the interaction of Axl with the ubiquitin ligase c-Cbl (35).

In this report, we used a hypoxia mimicking agent, CoCl₂, to show that GAS6 secretion is not able to negatively regulate expression Axl in PC3 and DU145 cells under hypoxic conditions. Quite likely Axl protein expression is stabilized in a hypoxic microenvironment as we observed very little alteration in Axl mRNA expression in response to GAS6 signaling. Regions of the tumor microenvironment are known to be very hypoxic (28). In a primary tumor, hypoxia induces an EMT, whereas in the bone hypoxia may increase the expression of transcription of factors which can further promote skeletal metastasis of the tumor (36). HIF-1α is a highly unstable protein that under normoxic conditions is degraded by proteolytic-mediated degradation. Hypoxia stabilizes HIF-1α protein by preventing its degradation (37). We observed that Axl expression under hypoxic conditions may no longer be regulated by the GAS6 ligand and therefore may remain available for signaling or for the promotion of an EMT-like state which may drive progression of metastasis.

Earlier, we showed that GAS6 is produced by primary human osteoblasts (26, 38). We have shown that in acute lymphoblastic leukemia cells migrate toward the bone using the GAS6/Mer axis. In prostate cancer we have shown that GAS6 production by osteoblasts influences tumor dormancy and thereby protects tumor cells from chemotherapy-induced apoptosis. There have been other studies using melanoma, breast, pancreatic, and colon cancer cell lines in which cancer cells were shown to promote their own growth by educating infiltrating leukocytes to upregulate the production of GAS6 (39). These observations indicate that GAS6 production is high in the bone marrow microenvironment and that the presence of a tumor further promotes GAS6 production. Yet GAS6 can either be produced by osteoblasts (paracrine loop) or by the tumor cells themselves (autocrine loop). The hypoxic microenvironment in the bone may facilitate the high expression of Axl by the tumor cells despite high GAS6 expression. This results in increased production of both the ligand and receptor, which boosts GAS6/Axl signaling within the bone microenvironment that in turn drives tumorigenesis (Model in Fig. 8). A similar phenomenon can be imagined in a primary prostate cancer microenvironment, which is also highly hypoxic in nature. 

It is well known that normal cells control the length and intensity of RTK signaling by receptor downregulation. This pathway involves ligand-induced internalization by endocytosis, followed by degradation in lysosomes, as seen in EGF receptors (EGFR) and in platelet-derived growth factor receptors (PDGFR; refs. 40–42). In cancer cells, however, some of these deactivation pathways are compromised, leading to overexpression of RTKs (43, 44). It is highly
likely that the regulation of Axl expression is disrupted in a similar manner in cancer, particularly in a hypoxic tumor microenvironment, and our group is focusing on this in our ongoing studies.

In conclusion, our data provide evidence for the role played by Axl and GAS6 in prostate cancer tumorigenesis and also offer insights into how GAS6/Axl signaling is regulated in prostate cancer. While we still do not know how Axl expression is stabilized during hypoxia, a further understanding of this process may provide significant therapeutic possibilities, especially when coupled with the knowledge that GAS6 signaling may regulate prostate cancer dormancy in the marrow (26). In fact, it will be interesting to explore whether other RTK inhibitors which are being considered for cancer therapeutic testing will alter these pathways, and whether they may be more effective with agents that prevent receptor stabilization (45, 46). Clearly, as evidence continues to mount that dormant microscopic tumors are prevalent in healthy individuals, understanding the molecular mechanisms that regulate dormancy will become increasingly important as our population ages.

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

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References


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