Matriptase Activation and Shedding through PDGF-D-mediated Extracellular Acidosis

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Running Title: PDGF D-mediated acidosis leads to matriptase activation.

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ABSTRACT

Activation of β-PDGFR (platelet-derived growth factor receptor) is associated with prostate cancer (PCa) progression and recurrence after prostatectomy. Analysis of the β-PDGFR ligands in PCa revealed association between PDGF-D expression and Gleason score as well as tumor stage. During the course of studying the functional consequences of PDGF ligand-specific β-PDGFR signaling in PCa, we discovered a novel function of PDGF-D for activation/shedding of the serine protease matriptase leading to cell invasion, migration, and tumorigenesis. The present study showed that PDGF-D, not PDGF-B, induces extracellular acidification which correlates with increased matriptase activation. A cDNA microarray analysis revealed that PDGF-D/β-PDGFR signaling upregulates expression of the acidosis regulator carbonic anhydrase IX (CAIX), a classic target of the transcriptional factor HIF-1α. Cellular fractionation displayed a strong HIF-1α nuclear localization in PDGF-D expressing cells. Treatment of vector control or PDGF-B expressing cells with the HIF-1α activator, CoCl₂ led to increased CAIX expression accompanied by extracellular acidosis and matriptase activation. Furthermore, the analysis of the CAFTD cell lines, variants of the BPH-1 transformation model, showed that increased PDGF-D expression is associated with enhanced HIF-1α activity, CAIX induction, cellular acidosis and matriptase shedding. Importantly, shRNA-mediated knockdown of CAIX expression effectively reversed extracellular acidosis and matriptase activation in PDGF-D transfected BPH-1 cells and in CAFTD variants that express endogenous PDGF-D at a high level. Taken together, these novel findings reveal a new paradigm in matriptase activation involving PDGF-D-specific signal transduction leading to extracellular acidosis.

Keywords: PDGF-D; Matriptase; Acidosis; HIF-1α; CAIX
INTRODUCTION

Prostate cancer (PCa) is the most diagnosed non-cutaneous cancer and second leading cause of cancer-related death in American men (35). This carcinoma is very much amenable to treatment at the localized stage; however, once metastasized, PCa is not as responsive to therapeutic intervention and this is reflected by the relatively poor survival rate of PCa patients with metastatic disease (35). Therefore, it is imperative to understand the molecular mechanism underlying the transition of PCa from *in situ* to invasive disease. Platelet-derived growth factor (PDGF) signaling has been implicated in the development and progression of PCa (23, 24, 44). This family is composed of 2 receptors (α-PDGFR and β-PDGFR) which form homo- or heterodimeric complexes that can be activated by their cognate ligands, PDGF-A, B, C or D. α-PDGFR is activated by PDGF-A and PDGF-C while β-PDGFR is bound by PDGF-B and PDGF-D (46). PDGF-A and B are secreted as active homo- or heterodimers while PDGF-C and D are released into the extracellular milieu as inactive homodimers that can be proteolytically activated by serine proteases such as matriptase and uPA (28). In PCa, β-PDGFR is upregulated in approximately 50% of bone metastatic cancer cases and is part of a 5-gene signature predicting PCa recurrence post radical prostatectomy (21, 36). While, the classic β-PDGFR ligand, PDGF-B, has not been often detected in clinical PCa specimens, our group has demonstrated that PDGF-D correlates with PCa Gleason score and tumor stage and induces β-PDGFR transformative potential (43). Furthermore, our recent study demonstrated that PDGF-D drives a more invasive program in prostate epithelial cells as compared to PDGF-B, supporting the functional significance of the aforementioned clinical and preclinical findings (24). Interestingly, the PDGF-D-specific invasive phenotype was dependent on the activation of the serine protease, matriptase.

Matriptase is an epithelial-specific type II transmembrane serine protease, shown to be upregulated in a many cancers including PCa (19). Matriptase’s repertoire of substrates includes hepatocyte growth factor (HGF), PDGF-C, PDGF-D, uPA and extracellular matrix (ECM) components such as Collagen IV (11, 42, 43). Consequently, matriptase regulates proteolytic signaling networks as
well as ECM remodeling, and thus being considered as a classic promoter of invasive growth of tumors (7, 8, 41, 42). Matriptase activation is a dynamic process involving autolysis, conformational change, and then complex formation with HGF activator inhibitor-1 (HAI-1) (18). As a 70-kDa transmembrane zymogen, matriptase once activated quickly carries out its enzymatic activity before being inhibited by its endogenous inhibitor HAI-1 and shed into the extracellular milieu as 110 and 95-kDa complexes (18). While the signal transduction pathways leading to matriptase activation are largely unknown, sphingosine-1-phosphase and suramin are classic activators of matriptase in various cell types (16). Particularly in PCa cells, dihydrotestosterone and ErbB-2 signaling are also implicated in matriptase activation (12, 45).

In the present study, we report a novel PDGF-D/β-PDGFR autocrine signaling loop that mediates matriptase activation. The PDGF-D-specific signaling cascade induces HIF-1α nuclear localization and transcriptional activation of carbonic anhydrase IX (CAIX) which in turn results in extracellular acidosis and matriptase activation leading to enhanced cell invasion.
MATERIALS AND METHODS

Cell culture. Vector control (Hygro), PDGF-B and PDGF-D expressing BPH cells (PDGF-B and PDGF-D BPH-1 cells, respectively) were previously described (24) and cells maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, 0.5% penicillin-streptomycin, and 200ug/mL hygromycin (Life Technologies, Carlsbad, CA). Parental BPH-1 cells and BPH-1 CAFTD-3 and CAFTD-4 were a generous gift of Dr. Simon W. Hayward at Vanderbilt University and grown in RPMI 1640 supplemented with 5% fetal bovine serum, 1% L-glutamine, 0.5% penicillin-streptomycin (Life Technologies).

shRNA-mediated downregulation of CAIX. PDGF-D BPH-1 or BPH-1 CAFTD-4 cells, grown to 90% confluence, were transfected with scrambled (Open Biosystem RHS4346) or two CAIX shRNA expression vectors (Open Biosystem RHS4430-100993056 and RHS4430-100996131) using Lipofectamine 2000 (Life Technologies) per manufacturer’s protocol. PDGF-D BPH-1 and BPH-1 CAFTD-4 cells were selected with 4µg/mL and 0.5µg/mL puromycin, respectively. shRNA efficacy was determined by immunoblot analysis using whole cell lysates prepared by lysing cells for 30 minutes in 1X RIPA lysis buffer (Millipore, Billerica, MA) supplemented with 100mM PMSF, 200mM NaVO₃, 1M NaF and 8% 50X protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration was determined using the Pierce BCA protein quantitation assay (Pierce Biotechnology, Rockford, IL).

Reagents. The anti-active matriptase (M69) antibody recognizes an epitope specific to the activated chain of matriptase while the total matriptase (M32) antibody recognizes both latent and active forms of matriptase and the HAI-1 antibody detects free and matriptase-complexed HAI-1 as described in (17). Anti-PDGF-B and Histone H1 antibodies were from Millipore, and the anti-PDGF-D antibody was custom designed against the growth factor domain (GD) amino acids 254-272 of PDGF-D and affinity purified (Zymed Biomedical, San Francisco, CA) as described in (44). Anti-CAIX, PTEN, phospho- and
total Akt and β-PDGFR were obtained from Cell Signaling (Boston, MA). Anti-HIF-1α was from Novus Biologicals (Littleton, CO) and anti-β-actin was from Sigma.

*Extracellular pH analysis and detection of shed/activated matriptase.* Cell lines were grown to confluence, washed with warm PBS, then treated with a HEPES-buffered Ringer solution containing (mmol/L): 122.5 NaCl, 5.4 KCl, 0.8 MgCl₂, 1.2 CaCl₂, 1.0 NaH₂PO₄*2H₂O, 5.0 glucose, 10 HEPES (37). At the indicated time points, conditioned media was collected and the cell debris removed by centrifugation at 2000 RPM for 5 minutes. The pH of the conditioned media was measured using a Beckman Φ300 Digital pH Meter and pH readouts were subtracted from the pH at time zero to determine changes in extracellular pH which were further normalized to the live cell numbers (10⁶ cells). Experiments were read in triplicates and performed in at least three separate experiments. To detect shed/activated matriptase, cells were plated and treated for 24-hours as described above and conditioned media was then concentrated 50-fold using Amicon Ultra-4 centrifugal filters (Millipore) for immunoblot analysis of matriptase under non-reducing conditions.

*Microarray analysis and RT-PCR validation.* Vector (Hygro), PDGF-B and PDGF-D BPH-1 cells, grown to confluence, serum starved for 48 hours then RNA harvested using the Qiagen RNeasy mini kit (Valencia, CA) and subjected to the Illumnia HumanHT-12v4 array analysis. Resultant expression data was background subtracted but not normalized. A strategy was needed to determine which probes were differentially expressed between experimental conditions. We assume that the mean expression level for each probe for each experiment was normally distributed, using the respective bead mean and standard error to define the normal distribution parameters. We then generated 1,000,000 deviates for each experimental condition. P-values were computed as the proportion of the resultant ratios of deviates that were greater than 1.5 or less than 2/3 (2 one-sided tests). Significant probes (p-value<0.05 for either of the 2 one-sided tests) and estimated effect sizes for each planned comparison were then imported into Ingenuity for pathway analysis.
RT-PCR validation was performed using primers in Supporting Table 1. Real time RT-PCR was performed using QPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. Relative values of gene expression were normalized to β-actin and calculated using the 2^{-ΔΔCt} method.

Acidic pH and CoCl₂ treatments. Vector (Hygro) and PDGF-B BPH-1 cells were grown to confluence, washed with PBS then treated with pH7.4 (control) or pH6.0 phosphate buffer. Conditioned media was collected and concentrated for immunoblot analysis. For HIF-1α activation, vector (Hygro) and PDGF-B BPH-1 cells were grown to confluence, washed with PBS, then treated with 100µM CoCl₂ (Sigma, St. Louis, MO) in serum free media for 24 hours. Conditioned media were harvested, concentrated and utilized for matriptase analysis. Total RNA was collected and cell lysates were subjected to subcellular fractionation.

Subcellular fractionation. Cells were first lysed for 30 minutes in cell lysis buffer containing 1M HEPES (pH7.9), 1M KCl, 0.5M EDTA, 0.1M EGTA, 10% NP-40, 0.1M DTT, 100mM PMSF and 2% 50X protease inhibitor cocktail (Roche). Cells were then spun in a Sorvall 4°C tabletop microcentrifuge for 2 minutes at 13,000RPM and the cytoplasmic fraction collected. Cell pellets were washed with ice-cold PBS and the remaining pellets were lysed for 30 minutes in nuclear extraction buffer containing 1M HEPES (pH7.9), 5M NaCl, 0.5M EDTA, 0.1M EGTA, 0.1M DTT, 100mM PMSF and 2% 50X protease inhibitor cocktail (Roche), followed by centrifugation in a Sorvall 4°C tabletop microcentrifuge at 13,000RPM for 10 minutes for the collection of the nuclear fraction. Protein concentration was determined using the BCA protein quantitation assay (Pierce Biotechnology).

Matrigel cell invasion. Live cells were counted using a Trypan Blue exclusion assay and 75,000 cells were placed in a Matrigel coated 8µm transwell (BD Biosciences, San Jose, CA). Complete growth media was used as a chemoattractant and invasion permitted for 16 hours with BPH-1 PDGF-D cells and 8 hours
with BPH-1 CAFTD-4 cells. The top surface of the transwells were then cleaned with a Q-tip, stained in 0.9% crystal violet then migrated cells were quantitated using a Nikon TMS-F inverted microscope at 100X. Five high-power fields (HPF) were analyzed and plotted as average cell number ± standard deviation. Experiments were conducted in triplicates and performed in at least three separate experiments.

Cell Proliferation. 2,500 cells were plated in a 96-well plate overnight to allow for cells attachment. Cells were then switched to serum free media and cell proliferation was measured using the colorimetric WST-1 assay (Roche) per manufacturer’s recommendations. Experiments were conducted in replicates of 6 and performed in at least three separate experiments.

Intracellular pH analysis. Vector (Hygro), PDGF-B, and PDGF-D BPH-1 cells were grown to confluence, washed with warm PBS and either treated with complete growth media (baseline) or serum free media for 16 or 24 hours. At the indicated time point, cells were trypsinized and resuspended in fresh serum free media and the membrane surface charge (zeta potential) was read using Malvern Zetasizer Nano ZS (Worcestershire, UK) as described in (34). To monitor intracellular pH using the fluorescent probe BCECF (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein; Life Technologies), vector (Hygro), PDGF-B, and PDGF-D BPH-1 cells were grown in 35mm glass bottom dishes (MatTek, Ashland, MA) to confluence, and washed with PBS before being loaded with 10µM BCECF for 10 minutes. Cells were then washed with PBS and fluorescence imaged using an Olympus FSX100 at 40X magnification. Experiments were conducted in triplicates and performed in at least three separate experiments.

PDGF-D transactivation. Conditioned media from parental BPH-1 or BPH-1 CAFTD-4 cells was obtained and concentrated as described above. Media was then incubated with or without 5ng recombinant matriptase (rMat; R&D Systems) for 2 or 6 hours. CM alone was used as a digest control. To test the functional activity of processed PDGF-D, the CM reaction from above were added to NIH3T3
cells and β-PDGF activation assessed. rPDGF-B and serum free media (SFM) alone were used as positive and negative controls, respectively. Numbers under phospho-PDGFβ and Akt are relative densitometric values obtained using NIH Image J.

Statistical analysis. Statistical significance was determined using unpaired Student’s $t$-test, and differences were considered significant when $P$ value was less than 0.05.
RESULTS

PDGF-D-specific signaling supports extracellular acidosis and matriptase activation. Matriptase is a transmembrane zymogen and once activated it is bound by its endogenous inhibitor HAI-1 and shed into the extracellular milieu as 110 and 95-kDa complexes (18). Using the non-malignant prostate epithelial cell line BPH-1, we observed a drastic increase in matriptase shedding/activation in response to PDGF-D overexpression but not PDGF-B (Fig. 1A). Matriptase activation was detected exclusively in the conditioned media using an antibody specific to activated matriptase as well as HAI-1 which demonstrates the presence of the high molecular weight matriptase/HAI-1 complex. Accordingly, total matriptase levels were slightly reduced in cell lysate from PDGF-D BPH-1 cells (Fig. 1A). The mechanism by which matriptase is activated and shed are largely unknown at present. Interestingly, Tseng et al. recently reported that acidosis plays a key role in matriptase activation (40). To determine if this is a possible mechanism underlying PDGF-D-mediated matriptase activation/shedding, we measured the extracellular pH (pHe) of the conditioned media. PDGF-D BPH-1 cells demonstrated a significantly acidic media at both 16 and 24 hours compared to vector (Hygro) and PDGF-B BPH-1 cells (Fig. 1B). Since extracellular acidification could be a result of increased cell proliferation, we analyzed vector (Hygro), PDGF-B and PDGF-D BPH-1 cell proliferation and observed no significant difference in the proliferation rate (Supporting Fig. 1A). Moreover, when extracellular pH was normalized to cell number, PDGF-D BPH-1 cells continued to exhibit a more significant pH drop than vector (Hygro) or PDGF-B BPH-1 cells (Supporting Fig. 1B). To ascertain that extracellular acidification plays an important role for matriptase activation/shedding, vector (Hygro) and PDGF-B BPH-1 cells were exposed to either neutral (pH7.4) or acidic (pH6.0) phosphate buffer for 10 or 30 minutes. We observed a time dependent activation of matriptase/shedding exclusively in the acidic buffer in both cell lines (Fig. 1C). In tumor cells, extracellular acidification is accompanied with a slightly alkaline intracellular pH (25). To monitor intracellular pH, we measured intracellular membrane zeta potential. Cell membrane zeta potential is usually between -20mV to -25mV and becomes less negative when exposed to positively charged particles (i.e. H⁺) and more negative when exposed to negatively charged particle (i.e. OH⁻) (34, 47).
PDGF-D BPH-1 cells demonstrated negative zeta potential readings overtime suggesting the intracellular charge of the cell membrane is more basic in comparison to the control or PDGF-B BPH-1 cells (Fig. 1D). To corroborate the zeta potential findings, we next measured the intracellular pH using the fluorescent probe BCECF which fluoresces more intensely under basic pH due to the unprotonated phenol and carboxylic acid functional groups (14). We observed greater fluorescent intensity in PDGF-D BPH-1 cells as compared to vector (Hygro) or PDGF-B expressing cells (Fig. 1E) indicating a basic intracellular compartment.

To complement our overexpression model of PDGF in BPH-1 cells, we utilized the BPH-1 transformation model described in Hayward et al. (10). Briefly, parental BPH-1 cells were grafted beneath the renal capsule with cancer-associated fibroblasts (CAF) and resulting tumors were cultured to obtain the first generation of BPH-1 CAFTD variants. The first generation cell lines were then re-grafted without CAFs to obtain the second generation of CAFTD variants. Within our study, we chose parental BPH-1, BPH-1 CAFTD-3 and BPH-1 CAFTD-4 as these cells demonstrated progressively transformed phenotypes including gradual increase in tumorigenesis and invasion into surrounding stroma (10). First, we assessed the protein expression profile for PDGF-B and D in the parental BPH-1, BPH-1 CAFTD-3 and-4 variants. Immunoblot analysis showed an increase in PDGF-B expression in BPH-1 CAFTD-3 but a decrease in the more aggressive CAFTD-4 variant (Fig. 2A). It should be noted that PDGF-B was detected in cell lysates of CAFTD variants, while it was barely detected in conditioned media, likely due to its binding to ECM proteins or internalization through autocrine signaling. Unlike PDGF-B that is intracellularly processed and secreted as a biologically active dimer, PDGF-D is secreted as a latent dimer containing the N-terminal CUB domain and the C-terminal growth factor domain. The full length PDGF-D (50kDa) can be processed into the active 18kDa growth factor domain and eventually the inactive 15kDa species by extracellular serine proteases (43). Immunoblot analysis detected full-length PDGF-D in the conditioned media to gradually increase from parental BPH-1 to BPH-1 CAFTD-3 to CAFTD-4 (Fig. 2A). To confirm the 50kDa band detected in the BPH-1 CAFTD variants is indeed the latent PDGF-D proteins, BPH-1 CAFTD-4 conditioned media was incubated with recombinant matriptase, a
well-characterized activator of PDGF-D. Using an anti-PDGF-D antibody raised against the growth factor domain (44), we confirmed that the full-length PDGF-D in CAFTD-4 conditioned media can be processed into the growth factor domain (Supporting Fig. 2A). To test the biological activity of BPH-1 CAFTD-4 derived PDGF-D, recombinant matriptase-treated conditioned media from BPH-1 parental or CAFTD-4 cells was used to stimulate the β-PDGFR in NIH3T3 cells. Undigested BPH-1 CAFTD-4 conditioned media demonstrated higher β-PDGFR activation as compared to parental BPH-1 cells (lane 4 vs. lane 6 in Supporting Fig. 2B). Importantly, recombinant matriptase-mediated proteolytic processing resulted in enhanced β-PDGFR activation, especially in BPH-1 CAFTD-4 samples (lane 3 vs. lane 5 in Supporting Fig. 2B) as expected from matriptase-mediated generation of PDGF-D growth factor domain as shown in Supporting Figure 2A.

Next, we asked whether increased PDGF-D expression correlates with matriptase activation/shedding and/or extracellular acidosis. Consistent with our overexpression model, BPH-1 CAFTD-4 cells, which expressed higher PDGF-D, displayed greater matriptase activation and shedding as well as enhanced extracellular acidosis (Fig. 2B-C), even after the normalization to the cell numbers (Supporting Fig. 3A-B). Similar to our overexpression model, matriptase activation/shedding and acidosis were associated with PDGF-D expression, but not with PDGF-B as BPH-1 CAFTD-3 variant cells, which express higher PDGF-B, did not exhibit high levels of matriptase activation/shedding compared to the parental BPH-1 cells. Previously, we demonstrated that PDGF-D expressing BPH-1 cells were more invasive than their PDGF-B counterparts (24). To assess if this is true in the in vivo derived BPH-1 transformation model cell lines, we performed Matrigel invasion assays and observed greater invasive potential in the BPH-1 CAFTD variants, specifically CAFTD-4, correlating the PDGF-D expression profile of these variant with cell invasion (Fig. 2D and Supporting Fig. 3C). Thus, both ectopic and endogenous PDGF-D expression models support the notion that PDGF-D signaling mediates extracellular acidosis and concomitant matriptase activation supporting cell invasiveness.
PDGF-D-specific signaling enhances CAIX expression. In an effort to determine the molecular mechanisms by which PDGF-D-specific signaling mediates extracellular acidosis, we performed a cDNA microarray analysis of vector (Hygro), PDGF-B and PDGF-D BPH-1 cells, and analyzed the expression levels of different pH regulators (Fig. 3A). Among those, we found that carbonic anhydrase IX (CAIX) expression increased at both the RNA and protein levels in response to PDGF-D overexpression in BPH-1 cells (Fig. 3B-C). In agreement with previous reports (4), CAIX proteins were detected, ranging from 35 to 58 kDa. Next, we wanted to confirm these findings in the BPH-1 transformation model, specifically in BPH-1 CAFTD-4 cells that express PDGF-D at high levels. RT-PCR demonstrated an increase in CAIX expression, and not CAXII, and these findings were corroborated through immunoblot analysis (Fig. 3D-E).

PDGF-D-mediated extracellular acidosis is dependent on CAIX expression. CAIX hydrates metabolically released CO$_2$ into H$^+$ and HCO$_3^-$ while HCO$_3^-$ is imported into the cell by different bicarbonate transporters such as anion exchangers, excess of H$^+$ on the outside of tumor cells result in an acidic extracellular milieu promoting invasive and metastatic behavior (2, 9). To assess the functional role CAIX plays in PDGF-D-mediated extracellular acidosis, we stably downregulated CAIX expression using two separate shRNA constructs then monitored extracellular acidosis and cell invasion. Attenuation of CAIX expression in PDGF-D BPH-1 (Fig. 4 A-B) and BPH-1 CAFTD-4 (Fig. 4C-D) cells significantly abrogated extracellular acidosis and this effect was independent of cell number (Supporting Fig. 4). Importantly, downregulation of CAIX reduced matriptase activation/shedding in cells that express high levels of either ectopic or endogenous PDGF-D (Fig. 5A-B). Moreover, PDGF-D-mediated Matrigel cell invasion was abrogated in response to CAIX downregulation (Fig. 5C-D; Supporting Fig. 5). Taken together, these results support the role of CAIX in PDGF-D-mediated extracellular acidosis, matriptase activation and cell invasion.
PDGF-D supports nuclear translocation of HIF-1α. Since CAIX is a classic HIF-1α target gene (25), we examined whether PDGF-D-specific signaling involves modulation of HIF-1α expression and/or subcellular localization. While there was little change in the mRNA level of HIF-1α (Supporting Fig. 6A), subcellular fractionation showed an increase in nuclear HIF-1α in response to PDGF-D overexpression in BPH-1 cells (Fig. 6A). These findings were corroborated using the BPH-1 transformation model where the more aggressive BPH-1 CAFTD-4 variant with increased PDGF-D expression displayed increased HIF-1α nuclear localization as compared to BPH-1 parental cells (Fig. 6B).

To test the functional significance of nuclear localization of HIF-1α for CAIX expression and cellular acidosis, we treated vector (Hygro) and PDGF-B BPH-1 cells with the HIF-1α activator, CoCl₂. Treatment with CoCl₂ mediated nuclear localization of HIF-1α (Supporting Fig. 6B), upregulated CAIX expression (Fig. 6C) and resulted in extracellular acidosis (Fig. 6D-E) in both cell lines. These effects remained even after normalization for cell number (Supporting Fig. 6C-D). Notably, matriptase activation was increased in response to CoCl₂ exposure in both vector (Hygro) and PDGF-B BPH-1 cells (Fig. 6F). Taken together, we propose a working model that PDGF-D-specific signaling induces HIF-1α activation which in turn upregulates CAIX expression, resulting in extracellular acidosis leading to matriptase activation and invasive phenotype (Fig. 7).
In a growing tumor mass, dwindling nutrient supply and poor oxygen perfusion alter tumor metabolism and induce tumor-associated hypoxia (25), resulting in excessive production of lactate and carbonic acid leading to tumor-associated acidosis (3). While such harsh conditions are detrimental to the cell viability, malignant tumor cells have often adapted to the tissue microenvironment through genetic alterations such as upregulation/activation of hypoxia-induced factor-1 (HIF-1α) (6, 27). Importantly, clinical studies have shown that hypoxia is associated with poor prognosis in many cancers and intratumoral acidosis is linked to chemotherapy resistance (25, 33).

HIF-1α is upregulated in a myriad of cancers including PCa and its expression correlates with cancer metastasis (48). This basic helix-loop-helix (bHLH) transcription factor is regulated by both oxygen-dependent and independent mechanisms (33). While HIF-1α is an unstable protein under normoxic conditions, HIF-1α degradation is inhibited under hypoxic conditions due to inactivation of its negative regulators, prolyl hydroxylase domain-2 (PHD-2) and factor inhibiting HIF-1 (FIH) (1). HIF-1α then heterodimerizes with HIF-1β which functions as a transcription factor and induces expression of angiogenic and cell survival factors as well as regulators of metabolism and acidosis such as GLUT1 and carbonic anhydrase IX (CAIX) (3). The tumor cell’s ability to regulate the cellular pH is critical for its survival, as a decrease in the intracellular pH is detrimental to the cell’s biochemical and biological processes such as enzyme function and membrane integrity (6). CAIX is a transmembrane metalloenzyme whose molecular weight has been reported to range from 35 to 58 kDa (4). Our study corroborates these reports and demonstrates loss of all CAIX species in response to CAIX shRNA (Fig. 4). CAIX is responsible for hydrating metabolically released CO₂ into H⁺ and HCO₃⁻. Anion exchangers cooperate with CAIX activity importing HCO₃⁻ into the cell to stabilize intracellular pH leaving an excess of extracellular H⁺ which leads to extracellular acidification (26, 39). Consistently, clinical studies have shown increased CAIX expression levels in colorectal, ovarian, gastric, pancreatic and breast cancers; however, little is known about its involvement in PCa (3, 22). In vitro and animal studies showed that
CAIX indeed mediates extracellular acidification of colon and ovarian cancer cells and shRNA knockdown of CAIX reduced *in vivo* tumorigenesis (4, 38), demonstrating the functional significance of CAIX in human cancers. Importantly, the present study identified CAIX as a downstream mediator of PDGF-D-specific signaling leading to extracellular acidosis in PCa cells.

Excess $H^+$ on the outside of the tumor cells result in an acidic extracellular milieu promoting invasive and metastatic behavior of cancer cells (9, 20). In fact, exposure of melanoma cells to acidic pH enhanced MMP-2 and MMP-9 activity and increased lung metastatic lesions (31). Interestingly, oral administration of sodium bicarbonate inhibited the metastatic potential of PC-3M PCa cells (30). Furthermore, extracellular pH (pHe) analysis of migrating cells demonstrated a decrease of pHe at the leading edge of cells (37). These studies suggest that extracellular acidosis provide a biochemically favorable environment for protease activity which in turn permits cellular invasion. This may be especially true for matriptase which exhibits enhanced activation at pH6.0 (40). Matriptase is often upregulated during cancer progression including PCa and has been shown to mediate PCa cell migration, invasion and tumorigenesis (7, 8, 41). This type-II transmembrane serine protease acts on an array of substrates ranging from ECM components to growth factors (42-44). Previous work from our laboratory demonstrated that matriptase activates PDGF-D in a sequential two-step proteolytic processing, generating a hemidimer that consists of one full-length PDGF-D subunit and a growth factor domain followed by generation of growth factor domain dimer (Fig. 7). Interestingly, our previous studies showed that matriptase-activated PDGF-D signaling results in matriptase activation (24, 43), demonstrating a unique autocrine positive feedback loop between PDGF-D signaling and matriptase activation. In the current study, we report a novel pathway whereby PDGF-D signaling upregulates nuclear HIF-1α leading to CAIX gene expression which in turn mediates extracellular acidosis responsible for matriptase activation, driving cell invasion and further PDGF-D processing (Fig. 7). These results are critical for understanding the oncogenic actions of PDGF-D as well as the molecular mechanisms underlying the regulation of HIF-1α and its downstream mediators in PCa.
In addition to its regulation by oxygen sensors, HIF-1α can also be modulated in an oxygen-independent manner (33). Growth factor-mediated signaling stimulates protein synthesis, increasing HIF-1α translation (33) while post-translational modification stabilizes HIF-1α levels (6). Both p38 and ERK MAPK family members have been suggested to phosphorylate HIF-1α increasing HIF-1α activity (13, 29). The balance between protein degradation (O2-dependent) and synthesis and stabilization (O2-independent) controls the amount of available HIF-1α. Since our current results were performed under normoxic conditions, it is probable that PDGF-D-mediated increases in nuclear HIF-1α occur through upregulation of either protein synthesis or stabilization. In our preliminary study, vector (Hygro), PDGF-B and PDGF-D BPH-1 cells were treated with cycloheximide. In the absence of protein synthesis for 2 hours, HIF-1α protein was undetected regardless of PDGF expression, consistent with the notion that HIF-1α is an unstable protein. Upon removal of cycloheximide in the presence or absence of the proteasome inhibitor MG132, the levels of cytoplasmic HIF-1α proteins were comparable among cells. In contrast, nuclear HIF-1α levels were significantly higher in PDGF expressing cells. Nuclear HIF-1α was detected as early as 30 minutes post removal of cycloheximide (Supporting Figure 7A, arrow). In the presence of MG132, nuclear HIF-1α was accumulated especially in PDGF-D expressing cells. These preliminary results suggest that PDGF-D-specific signaling may facilitate HIF-1α nuclear transport and perhaps its stability rather than increased translation of HIF-1α mRNA.

The present study helps explain how a growth factor utilizes an intracellular signaling program to create a tissue microenvironment that is favorable for activation of proteolytic cascades promoting tumor cell invasion. These results also have implications for the efficacy of current therapeutic modalities. Since most cytotoxic agents are weak bases, extracellular acidosis neutralizes these agents before they can reach their targets (25). In fact, acidosis has been reported to mediate methotrexate resistance in KHT sarcoma and B16F1 melanoma cells (32). We envision that blocking PDGF-D would not only reverse PDGF-D-mediated cell signaling for the previously characterized PDGF-D functions such as tumor cell survival, motility, and angiogenesis but also reduce intratumoral acidosis, and thereby abrogate matriptase activity and reduce tumorigenic potential. It should be noted that the PDGF-D downstream mediators we
identified in this study, CAIX and matriptase, have been used as noninvasive biomarkers to detect tumors (5, 15). In colon cancer for instance, active matriptase is specifically detected in aggressive disease using a fluorescently labelled matriptase antibody (15) while fluorescently labeled CAIX inhibitors have shown great selectivity in pre-clinical trials detecting hypoxic tumors (5).

Taken together, we have identified a novel PDGF-D-mediated signaling pathway involving HIF-1α for CAIX regulation, resulting in extracellular acidosis, matriptase activation leading to a more invasive cellular program in PCa (Fig. 7), providing valuable information as to PDGF-D isoform-specific functions.
CONFLICT OF INTEREST

C.-Y.L is an inventor on US patents #6,077,938 (Title: Monoclonal antibody to an 80-kDa protease), #6,677,377 (Title: Structure based discovery of inhibitors of matriptase for the cancer diagnosis and therapy by detection and inhibition of matriptase activity) and #7,355,015 (Title: Matriptase, a serine protease and its applications).
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FIGURE LEGENDS

Figure 1. PDGF-D-specific signaling induces extracellular acidosis and matriptase activation/shedding in prostate epithelial cells. A) Immunoblot analysis of matriptase and HAI-1 in conditioned media and total cell lysates from serum-starved vector control (Hygro), PDGF-B or PDGF-D BPH-1 cells. B) Changes in extracellular pH of vector control (Hygro), PDGF-B or PDGF-D BPH-1 cells are plotted. Lines represent the mean ± SD. *p<0.05. C) Immunoblot of active and total matriptase in conditioned media from vector control (Hygro) or PDGF-B BPH-1 cells upon treatment with pH7.4 or pH6.0 phosphate buffer for indicated time period. Intracellular pH of vector control (Hygro), PDGF-B and PDGF-D BPH-1 cells were monitored through membrane zeta potential (D) and BCECF fluorescent (E) measurements. Lines represent the mean ± SD. *p<0.05. aMat, active matriptase; tMat, total matriptase. Ponceau S was used to evaluate equal gel loading of conditioned media.

Figure 2. Enhanced PDGF-D expression in the BPH-1 transformation model correlates with extracellular acidosis, matriptase shedding and increased cell invasion. Immunoblot analysis of PDGF-D and PDGF-B (A) as well as matriptase and HAI-1 (B) in conditioned media or cell lysates from serum-starved parental BPH-1, BPH-1 CAFTD-3 and CAFTD-4 variants. aMat, active matriptase; tMat, total matriptase. Ponceau S was used to evaluate equal gel loading of conditioned media. Changes in extracellular pH [pHe] (C) and Matrigel cell invasion (D) were monitored in BPH-1 parental and BPH-1 variant cells. Values represent the mean ± SD. *p<0.05 between BPH-1 parental and BPH-1 CAFTD-3; **p<0.05 between BPH-1 parental and BPH-1 CAFTD-4.

Figure 3. CAIX expression is enhanced in response to PDGF-D upregulation. A) List of pH regulating genes from a cDNA microarray analysis of vector control (Hygro), PDGF-B and PDGF-D BPH-1 cells. Qualitative and quantitative RT-PCR as well as immunoblot analysis of carbonic anhydrase (CA) family members in vector control (Hygro), PDGF-B and PDGF-D BPH-1 (B-C) and BPH-1 CAFTD variant (D-E) cells.
Figure 4. CAIX downregulation abrogates PDGF-D-mediated extracellular acidosis. PDGF-D BPH-1 (A) or BPH-1 CAFTD-4 (C) cells were transfected with scrambled (shScrm) or two separate CAIX shRNA and knockdown monitored via immunoblotting. Changes in extracellular pH (pHe) were monitored in control and shRNA-mediated CAIX knockdown PDGF-D BPH-1 (B) or BPH-1 CAFTD-4 (D). Bars represent the mean ± SD. *p<0.05 between shScrm and shCAIX-2; **p<0.05 between shScrm and shCAIX-3.

Figure 5. Matriptase shedding and cell invasion is attenuated in response to CAIX knockdown. Immunoblot analysis of matriptase and HAI-1 using conditioned media of PDGF-D BPH-1 (A) and BPH-1 CAFTD-4 (B) cells without or with CAIX knockdown (shScrm and shCAIX, respectively). aMat, active matriptase; tMat, total matriptase. Ponceau S was used to evaluate equal gel loading. Matrigel invasion was monitored in control and shRNA-mediated CAIX knockdown PDGF-D BPH-1 (C) and BPH-1 CAFTD-4 (D) cells. Bars represent the mean ± SD. *p<0.05 between shScrm and shCAIX-2; **p<0.05 between shScrm and shCAIX-3.

Figure 6. PDGF-D signaling upregulates nuclear HIF-1α. HIF-1α localization was monitored in vector control (Hygro), PDGF-B, and PDGF-D BPH-1 cell (A) or in the parental BPH-1 and CAFTD-4 variant (B). Nuc; nuclear fraction. Cyt; cytoplasmic fraction. HistoneH1 and PTEN were used as loading controls for the nuclear and cytoplasmic fractions, respectively. Upon treatment of vector (Hygro) or PDGF-B BPH-1 cells with 100µM CoCl2, carbonic anhydrase (CA) mRNA expression [RT-PCR] (C), changes in extracellular pH (pHe) (D-E) and matriptase activation (F) were monitored. Lines represent the mean ± SD. *p<0.05. aMat, active matriptase; tMat, total matriptase. Ponceau S was used to evaluate equal gel loading.
Figure 7. A working model of PDGF-D-mediated matriptase activation. Matriptase activates full length PDGF-D (FL-PDGF-D) in a bi-phasic manner first yielding a PDGF-D hemidimer (HD-PDGF-D) then growth factor only PDGF-D (GD-PDGF-D). PDGF-D/β-PDGFR signaling mediates HIF-1α nuclear translocation and transcription of CAIX leading to extracellular acidosis (H+) supporting matriptase activation and enhancing further PDGF-D processing and driving a pro-invasive program. L-Mat, latent matriptase; aMat, activate matriptase.
A. BPH-1 CAFTD

B. Media

Lysate

BPH-1 CAFTD

BPH-1 CAFTD

PonceauS

PDGF B

β-actin

C. Change in Extracellular pH

Time (Hrs)

BPH-1

CAFTD-3

CAFTD-4

* * *

D. No. of Invading Cells/HPF

BPH-1

CAFTD-3

CAFTD-4

* * *
Figure 7

Najy, et al.