A Novel Signaling Axis of Matriptase/PDGF-D/β-PDGFR in Human Prostate Cancer

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Abstract

Increasing evidence indicates the significance of platelet-derived growth factor receptor-β (β-PDGFR) signaling in prostate cancer (PCa). Accordingly, preclinical studies suggest the potential of β-PDGFR as a therapeutic target in metastatic PCa. However, a ligand responsible for β-PDGFR activation in PCa was unknown, and recent clinical trials with imatinib mesylate showed limited success due to normal tissue toxicity. Similarly, in spite of mounting evidence indicating the significance of matriptase in PCa, little is known about its substrates or molecular actions during PCa progression. Here, we identified PDGF-D as a ligand for β-PDGFR in PCa and discovered matriptase as its regulator. Matriptase activates PDGF-D by proteolytic removal of the CUB domain in a 2-step process, creating a hemidimer, followed by growth factor domain dimer (GFD-D) generation. Matriptase can deactivate PDGF-D by further proteolytic cleavage within the GFD, revealing its biphasic regulation. Importantly, PDGF-D/matriptase colocalization is accompanied with β-PDGFR phosphorylation in human PCa tissues. This study unveiled a novel signaling axis of matriptase/PDGF-D/β-PDGFR in PCa, providing new insights into functional interplay between serine protease and growth factor signaling networks.

Introduction

Members of the platelet-derived growth factor (PDGF) family are well-known inducers of cell migration, proliferation, and transformation [reviewed in (1)]. The 2 classic PDGF members PDGF-A and -B are secreted as active growth factor homo or heterodimers and readily activate their cognate receptors, α-PDGF and β-PDGF, whereas the newly discovered PDGF family members PDGF-C and PDGF-D are secreted as latent homodimers containing the N-terminal CUB domain and the C-terminal growth factor domain (GFD) (2–4). The removal of the CUB domain is required for the GFD dimers (GFD-D) of PDGF-C and -D to activate α-PDGF and β-PDGF, respectively (5, 6).

Increasing evidence suggests critical roles for PDGF during cancer progression, especially in prostate cancer (PCa). A majority of prostate tumor tissues at both primary and metastatic sites express β-PDGFR at increased levels as determined by immunohistochemical analysis (7–9). A recent study revealed that β-PDGFR is 1 of the 5 genes that predict PCa recurrence after prostatectomy (10), and preclinical studies suggested the potential of β-PDGFR as a therapeutic target in metastatic cancers (11–16). However, little research has been conducted to identify PDGF ligand(s) responsible for β-PDGFR activation during PCa progression. PDGF-B, originally thought to be the sole ligand for β-PDGFR, has been difficult to find in PCa patient specimens (12, 17). This raises the possibility that the newly discovered PDGF ligand PDGF-D may be responsible for the activation of β-PDGFR signaling in human PCa. To study the roles of PDGF-D in PCa, we previously established human PCa cell lines engineered to overexpress PDGF-D (6, 18). We showed that PDGF-D accelerates the early onset of prostate tumor growth and drastically enhances PCa cell invasion into surrounding stroma in a mouse model (18).

In this study, we wished to identify a proteolytic activator of PDGF-D in PCa cells and to validate their expression in human PCa tissues. We found that matriptase is a PDGF-D-inducible protease that regulates PDGF-D activity. Matriptase, also referred to as MT-SP1 (membrane-type serine protease type 1), is a type II transmembrane serine protease often found in complex with its cognate inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1) (19–21). Although mounting evidence showed increased matriptase expression in human tumors of epithelial origin, including prostate (22, 23), breast (24), cervical (25), ovarian (26), and gastrointestinal tract (27),...
little is known about its substrates or its molecular actions during cancer progression at present. Here, we report that matriptase activates PDGF-D in a 2-step process, creating a hemidimer (HD) containing a 50-kDa full-length monomer (FL-M) and an 18-kDa GFD before producing the active dimer consisting of two 18-kDa GFD peptides. Through site-directed mutagenesis, we identified the matriptase cleavage site of PDGF-D at R247/R249 within the hinge region between the CUB and GFD of the full-length PDGF-D dimer (FL-D). Interestingly, we found that the GFD-D is susceptible to further cleavage by matriptase, generating a biologically inactive 15-kDa fragment, revealing its biphasic regulation of PDGF-D activity.

Immunohistochemical analysis of human prostate tumor tissues demonstrated increased expression of both PDGF-D and matriptase. In situ hybridization analysis of PDGF-D mRNA confirmed that PDGF-D is produced predominantly by carcinoma cells. Importantly, confocal microscopic analysis revealed colocalization of PDGF-D and matriptase in human prostate tumor tissues. Consistent with our finding that matriptase is an activator of PDGF-D signaling, increased β-PDGFR phosphorylation was detected in malignant prostate tissues with increased expression of both markers, especially in poorly differentiated prostate carcinoma and the neoplastic glands in which perineural invasion occurs. Taken together, the present study identifies matriptase as a regulator of PDGF-D activity in human PCa and provides molecular insight into matriptase-mediated dynamic proteolytic processing of PDGF-D.

Materials and Methods

Cell lines

Human prostate carcinoma LNCaP cells overexpressing PDGF-D and vector-controlled cells were established previously and maintained in RPMI 1640 medium with 5% fetal bovine serum (FBS) (18). Mouse fibroblast NIH3T3 cells were purchased from ATCC and maintained in DMEM/F12 medium with 10% bovine serum (FBS) (18). Mouse fibroblast FTX1532 cells were obtained from Dr. R. Fridman (Wayne State University, Detroit, MI) and maintained in RPMI 1640 medium with 10% FBS.

Antibodies, proteases, and inhibitors

Generation of custom antibody (8D2) that recognizes GFD of PDGF-D was described previously (18). Anti-matriptase antibodies M32 and S5 were described previously (21). Anti-phosphorylated-β-PDGFR (pY751) antibody was purchased from Cell Signaling Technology (Boston, MA, USA). Anti-HAI-1 ectodomain Ab was purchased from R&D Systems (Minneapolis, MN, USA). Purification of the serine-protease domain of matriptase (rMatriptase) was described previously (28). HAI-1 construct within a pcDNA3.1 vector was a kind gift from Dr. R. Fridman (Wayne State University).

siRNA transfection

LNCaP–PDGF-D cells were transfected with nontargeting or matriptase SMARTPOOL™siRNA (Dharmacon, Inc., Lafayette, CO, USA), using Lipofectamine 2000 according to manufacturer’s protocol. After transfection for 5 hours, cells were maintained in RPMI 1640 medium with 5% FBS for 48 hours before serum starvation. Conditioned media (CM) and cell lysates were collected after culturing in serum-free medium for 48 hours. Cell lysates were harvested with RIPA lysis buffer.

Isolation of the PDGF-D dimer species

Using a duplicate nonreducing Western blot as a guide, the appropriate bands were cut out of the SDS-PAGE gel at specific molecular weights. Excised gel fragments were minced by passing through syringe, followed by eluting with elution buffer (50 mmol/L of Tris-HCl, 150 mmol/L of NaCl, and 0.1 mmol/L of EDTA; pH 7.5) at room temperature overnight. Eluates containing rPDGF-D was analyzed by immunoblotting.

Immunohistochemical analysis of PDGF-D and matriptase

Formalin fixed, paraffin-embedded human PCa specimens were obtained from the Wayne State University Pathology Research Core Facility. Antigen retrieval was performed in sodium citrate buffer. Slides were incubated overnight at 4°C with either anti-PDGF-D Ab (8D2) or anti-matriptase Ab (S-5) and visualized with DAB (Vector Labs, Burlingame, CA, USA). Mayer’s hematoxylin was used to counterstain nuclei. Negative controls were performed without the corresponding primary antibody.

In Situ hybridization

Digoxigenin-labeled antisense probes were synthesized from pCR2.1-PDGF-D by using the DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. PDGF-D sense probes, created with pcDNA3.1-PDGF-D, were used as the negative control. Slides were prehybridized for 1 hour at 65°C followed by incubation with either PDGF-D antisense or sense probes overnight at 65°C. The probe was detected using anti-digoxigenin antibodies and visualized using DAB.

Immunofluorescent double staining of matriptase and PDGF-D in human prostate tissue

Slides were double probed with anti-PDGF-D (8D2) and anti-matriptase antibodies (M32). Texas Red–conjugated antimouse and FITC-conjugated anti-rabbit secondary antibodies were used to detect matriptase and PDGF-D, respectively (Jackson Immunoresearch Laboratories, West Grove, PA). Confocal immunofluorescence microscopic analysis was performed at the Microscopy, Imaging and Cytometry Resources Core at Wayne State University, School of Medicine, using a Zeiss LSM 510 confocal microscopy system equipped with a C-Apochromat (NA = 1.2) 63× korr objective lens (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Results

Identification of matriptase as an activator of PDGF-D in human prostate carcinoma LNCaP cells

We previously showed that LNCaP cells fail to process PDGF-D into the GFD in the presence of the serine protease...
inhibitor aprotinin (6). Furthermore, we showed that recombinant PDGF-D (rPDGF-D) undergoes proteolytic processing more effectively in the presence of PDGF-D-transfected LNCaP (LNCaP–PDGF-D) cells compared with control LNCaP–neo cells (18). These results suggest that a PDGF-D–inducible serine protease is responsible for the processing of PDGF-D in LNCaP cells. To identify this enzyme, RNA samples collected from serum-free culture of LNCaP–PDGF-D and LNCaP–neo cells were analyzed via microarray, screening a gene chip containing 12,814 unique human clones (Agilent Technologies, Santa Clara, CA, USA). Each sample was run in triplicate against the reference RNA sample, and gene expression levels in LNCaP–PDGF-D cells were compared with those in LNCaP–neo cells, with the cutoff fold-change being 2.0-fold ($P < 0.01$). This analysis revealed that the mRNA level of matriptase increased in LNCaP–PDGF-D cells compared with LNCaP–neo cells by 2.5-fold ($P = 0.00435$). Matriptase, also known as MT-SPI1, is an 80-kDa membrane-bound serine protease that was originally isolated from breast and PCa cells (20, 29). Increased matriptase expression in LNCaP–PDGF-D cells was confirmed at the protein level (Fig. 1A). To determine whether LNCaP-produced matriptase is critical for PDGF-D processing, LNCaP–PDGF-D cells were transiently transfected with HAI-1, its endogenous inhibitor, or with siRNA. Generation of the GFD of PDGF-D was reduced following HAI-1 transfection or siRNA-mediated matriptase knockdown, suggesting a role for matriptase in the proteolytic processing of PDGF-D in LNCaP cells (Fig. 1B and C). In a separate experiment, we examined the biological consequence of reduced PDGF-D processing on its ability to activate β-PDGFR and induce cell motility in a paracrine manner. To this end, serum-starved fibroblasts were treated with CM collected from control and matriptase-knockdown LNCaP–PDGF-D cells and analyzed for the status of β-PDGFR activation and for their motility. β-PDGFR was readily activated in NIH3T3 cells following treatment with CM from LNCaP–PDGF-D cells, whereas β-PDGFR activation was far less apparent by treatment with CM from matriptase-knockdown LNCaP–PDGF-D cells. Accordingly, FTX-1532 cell migration was greatly reduced following treatment with CM from matriptase-knockdown LNCaP–PDGF-D cells compared with the control (Fig. 1D, top and middle panels). Finally, we examined the effects of matriptase knockdown on the ability of LNCaP cells to process...

![Image](cancerres.aacrjournals.org)
exogenous PDGF-D protein. Although recombinant full-length PDGF-D dimer (rPDGF-D) was effectively processed into the GFD-D when incubated with LNCaP–PDGF-D cells as previously reported (18), the processing was significantly reduced in the absence of matriptase expression in these cells (Fig. 1D, bottom panel). It should be mentioned that PDGF-D analyzed in Figure 1D (bottom panel) was almost exclusively rPDGF-D because the incubation period was short enough so as not to include a significant amount of PDGF-D secreted from LNCaP cells as we previously demonstrated (18). Taken together, these results demonstrated a critical role for matriptase in the activation of PDGF-D in LNCaP cells.

**Matriptase regulation of PDGF-D activity in a biphasic manner: activation and deactivation**

To test whether PDGF-D is a direct substrate for matriptase, rPDGF-D was incubated overnight with increasing concentrations of recombinant uPA, overnight. Samples were analyzed by immunoblot analysis using anti-PDGF-D antibody. C, serum-starved NIH3T3 cells were stimulated for 10 minutes with matriptase-treated rPDGF-D processed by indicated concentrations of matriptase (as shown in A). β-PDGFR was immunoprecipitated and immunoblotted with antiphosphotyrosine (top) and anti-β-PDGFR antibodies (bottom). D, rPDGF-D was incubated at 37°C with increasing nanomolar concentrations of recombinant uPA overnight. Samples were analyzed by immunoblot analysis using anti-PDGF-D antibody.
suggest that matriptase may be a more effective activator of PDGF-D than uPA.

**Identification of the matriptase cleavage site in the hinge region of full-length PDGF-D and characterization of PDGF-D hemidimers**

Previous sequence analysis of the amino terminus of the GFD of PDGF-D revealed the proteolytic cleavage site at R247 or R249 within the hinge region by unknown proteases (4). Like most serine proteases, matriptase cleaves at a lysine or an arginine in the P1 position of the substrate, with preference to arginine (21). Further analysis of matriptase cleavage sites, using phage display libraries and computer modeling, predicted that glycine/phenylalanine/small residue are at the P2 site and that P3/P4 sites are in a basic/nonbasic, or vice versa, relationship (30). Comparison of the R247/R249 region of PDGF-D with the known cleavage sites of other matriptase substrates yielded reasonable conservation, as shown in Figure 3A (31). When these arginine residues were mutated to alanine (PDGF-DR247,249A), matriptase failed to process PDGF-D into GFD, therefore identifying the cleavage site of matriptase within the hinge region of FL-PDGF-D (Fig. 3B).

The aforementioned results show that the catalytic domain of matriptase activates FL-PDGF-D in a 2-step process, with the HD as an intermediate dimer species as depicted by the diagram shown in Figure 3C. To test whether HD can be generated in HeLa cells, which express matriptase at a high level (32), PDGF-D was expressed in these cells by using the vaccinia expression system. Immunoblot analysis in a nonreducing condition revealed 3 major PDGF-D dimer species, presumably FL-D, HD, and GFD-D (Fig. 3D). To further reveal the nature of these species, these 3 protein bands were isolated by gel extraction, followed by immunoblot analysis under both nonreducing and reducing conditions. FL-D contained 50-kDa FL-M in a reducing condition as expected (Fig. 3D, lane 3). The HD (lane 2) consisted of FL-M and GFD-M, whereas GFD-D (lane 1) contained GFD-M as well as a smaller GFD-M species. This confirmed that HD contains 1 FL-M and 1 GFD-M and also showed further proteolytic cleavage within GFD-D, generating a 15-kDa fragment.

**Increased PDGF-D expression, colocalization of PDGF-D with matriptase, and increased β-PDGFR phosphorylation in human prostate carcinoma**

To examine whether PDGF-D and matriptase is expressed in human prostate carcinoma specimens, resulting in β-PDGFR activation, and whether colocalization of PDGF-D and matriptase can be detected, we obtained tissue sections of both benign prostate and prostate carcinoma specimens from our Pathology Research Core. Sixty-six individuals were included in the immunohistochemical analysis, and the distribution of individuals was tabulated by American Joint Committee on Cancer tumor stage and Gleason score (Supplementary Tables 1 and 2).
The immunohistochemical staining was evaluated by an uropathologist, and a blind assessment of the percentage of cells stained for PDGF-D and matriptase was performed for semiquantitation. Kendall’s tau was used to assess the association of tumor stage and Gleason score with PDGF-D and matriptase staining. PDGF-D staining is strongly associated with both higher tumor stage ($P = 0.006$) and higher Gleason score ($P = 0.01$), whereas matriptase has a weaker association with both tumor stage ($P = 0.08$) and Gleason score ($P = 0.11$) (Table 1).

When the staining patterns of PDGF-D and matriptase were analyzed (Fig. 4A and B), the expression of PDGF-D was detected in the basal cells but not in luminal cells within benign prostatic glands whereas the expression of matriptase was barely detected in benign glands. A mild increase of both markers was observed in some of the glands with high-grade prostatic intraepithelial neoplasia. The neoplastic glands in well to moderately differentiated prostate carcinoma (Gleason score 6) largely showed focal and variable staining for PDGF-D and matriptase. Significantly increased staining of both markers was observed in poorly differentiated prostate carcinoma (Gleason score ≥8). Unlike matriptase, which is an epithelial cell–specific enzyme, PDGFs can be produced by many different cell types, including epithelium and mesenchyme. To localize the cells that produce PDGF-D in these tissue samples, we performed an *in situ* hybridization analysis of PDGF-D mRNA, which was almost exclusively localized in epithelium (Fig. 5). Consistent with the immunostaining patterns of PDGF-D proteins, PDGF-D mRNA was detected in the basal cells but not in secretory cells within benign prostatic glands, and a marked increase in hybridization signal was observed in poorly differentiated prostate carcinoma cells.

A striking feature of PDGF-D and matriptase expression was the conspicuous overexpression of both markers in the neoplastic glands surrounding nerves, referred to as perineural invasion (Fig. 6A). Consistent with our *in vitro* finding that matriptase is an activator of PDGF-D/β-PDGFR signaling, phosphorylated β-PDGFR was readily detected in prostate carcinoma cells surrounding nerves. Of note, β-PDGFR phosphorylation was barely detected in nerve cells that express PDGF-D without matriptase. Marked overexpression of both markers and β-PDGFR phosphorylation were also detected in the mucinous variant of poorly differentiated prostate carcinoma, typically Gleason score of 8 or higher (Fig. 6B). Overall, as the levels of PDGF-D and matriptase increased, phosphorylated β-PDGFR expression was prominent in high-grade prostate carcinoma compared with the benign or low-grade prostate carcinoma (Supplementary Fig. 1).

We examined whether colocalization of PDGF-D and matriptase could be detected by confocal immunofluorescence microscopic analysis. As shown in Figure 6C, colocalization of PDGF-D and matriptase was readily detected both in PCa specimens and in the mucinous variant of poorly differentiated prostate carcinoma, in which increased expression of both markers was detected by immunohistochemical analysis. Taken together, the present study unveiled a novel signaling axis of matriptase/PDGF-D/β-PDGFR during human PCa progression.

### Table 1. Association between tumor stage and Gleason score with PDGF-D and matriptase staining assessed using Kendall’s tau

<table>
<thead>
<tr>
<th></th>
<th>Tumor stage</th>
<th>Gleason score</th>
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<tbody>
<tr>
<td>PDGF-D</td>
<td>$0.27 (0.006)$</td>
<td>$0.25 (0.01)$</td>
</tr>
<tr>
<td>Matriptase</td>
<td>$0.18 (0.08)$</td>
<td>$0.17 (0.11)$</td>
</tr>
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$P$ values are shown within parentheses.
PDGF-D Is a Substrate for Matriptase

The present study identifies PDGF-D as a substrate for matriptase and provides molecular insight into how matriptase regulates the activities of PDGF-D by its dynamic proteolytic processing. This finding is of particular importance not only for understanding molecular actions of PDGF-D but also for unveiling the oncogenic activities of matriptase in human cancer. Matriptase was originally identified from human breast cancer cells (29) and independently from a human cancer. Matriptase was originally identified from the primary site, as in vitro studies show mutual beneficial effects between PCa cells and ganglia/nerve in terms of neurite growth, proliferation, and migration of PCa cells (44, 45). These studies suggest that the cross talk between nerve cells and cancer cells supports cell proliferation and that the axons may provide a pathway for cancer cells to escape from the primary site. A role of PDGF-D in PNI and tumor interactions with the nerve cells remains to be fully investigated.

Recent studies revealed a critical role for β-PDGFR in PCa. A majority of prostate tumor tissues at both primary and metastatic sites in the bone express β-PDGFR, as determined by immunohistochemical analysis (12). Consistent with the potential oncogenic activity of PDGF signaling in PCa, investigators have reported a therapeutic potential of PDGF/PDGFR targeting in clinical trials of hormone refractory PCa (12, 14–16, 46). In animal experiments, imatinib mesylate (also known as ST1571 or Gleevec) was shown to inhibit the phosphorylation of PDGF and significantly suppress experimental human PCa bone metastasis and angiogenesis, especially when administrated in combination with paclitaxel (Taxol) (11) or with paclitaxel and PKI166, an inhibitor of epidermal growth factor receptor (13). However, recent
clinical trials with imatinib mesylate showed only limited success with unanticipated side effects such as diarrhea related to inhibition of c-kit in intestines, hypopigmentation in skin, and cardiotoxicity associated with inhibition of c-abl in cardiac myocytes (47–49). Improvement in β-PDGFR targeting specific to PCa would require the identification of the prostate-specific PDGF ligand and characterization of its molecular actions. Importantly, the present study identified PDGF-D as a ligand for β-PDGFR in PCa. Our study revealed statistically significant correlation between PDGF-D expression with higher Gleason scores or advanced tumor stages, identifying PDGF-D as one of the very few growth factors with potential prognostic values in PCa. Importantly, the present study also revealed increased expression of matriptase, a regulator of PDGF-D, in human prostate carcinoma, resulting in the activation of β-PDGFR signaling. Taken together, our studies unveil a novel signaling axis of matriptase/PDGF-D/β-PDGFR in PCa, providing valuable information for the design of more specific therapeutic approaches for the improvement in the effectiveness with fewer side effects.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We are grateful to Dr. R. Fridman and his laboratory for assistance with the vaccinia virus system and the Wayne State University (WSU) Pathology Research Core Facility for assistance with immunohistochemistry assays. Special thanks to Dr. Alan Dombkowski and Amy Diakiw of the WSU Microarray and Bioinformatics Facility Core at the Institute of Environmental Health Sciences for their work with the microarray analysis.

**Grant Support**

Microarray and Bioinformatics Core was supported in part by NIEHS Center grant P30 ES06639. The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by NIH Center grant P30CA22453 to Karmanos Cancer Institute and the Perinatology Research Branch of the National Institutes of Child Health and Development, WSU.

This work was supported by NIH/NCI RO1 grants CA064139 and CA123362 (H.R.C. Kim), as well as the Ruth L. Kirschstein National Research Service Award T32-C-A009531 (M.K. Conley-LaComb).

Received 02/12/2010; revised 09/09/2010; accepted 09/30/2010; published OnlineFirst 11/23/2010.
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Cancer Res  Published OnlineFirst November 23, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-0511

Supplementary Material  Access the most recent supplemental material at:
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