A Potential Oncogenic Activity of Platelet-Derived Growth Factor D in Prostate Cancer Progression

Carolyn V. Ustach,1 Marcus E. Taube,1 Newton J. Hurst, Jr.,1 Sunita Bhagat,2 R. Daniel Bonfil,1,2 Michael L. Cher,1,2 Lucia Schuger,1 and Hyeong-Reh Choi Kim1

Departments of 1Pathology and 2Urology, Barbara Ann Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, Michigan

ABSTRACT

The platelet-derived growth factor (PDGF) proteins are potent stimulators of cell proliferation/transformation and play a major role in cell-cell communication. For over two decades, PDGFs were thought to exist as three dimeric polypeptides (the homodimers AA and BB and the heterodimer AB). Recently, however, the PDGF C and D chains were discovered in a BLAST search of the expressed sequence tag databases. The PDGF CC and DD dimers have a unique two-domain structure with an NH2-terminal CUB (complement subcomponents C1r/C1s, Uegf, and Bmpl) domain and a COOH-terminal PDGF/vascular endothelial growth factor domain. Whereas secreted PDGF AA, BB, and AB readily activate their cell surface receptors, it was suggested that extracellular proteolytic removal of the CUB domain is required for the PDGF/vascular endothelial growth factor domain of PDGF CC and DD to activate PDGF receptors. In the present study, we examined the processing of latent PDGF D into its active form and the effects of PDGF D expression on prostate cancer progression. We show that LNCaP cells auto-activate latent PDGF DD into the active PDGF domain, which can induce phosphorylation of the β-PDGFR receptor and stimulates LNCaP cell proliferation in an autocrine manner. Additionally, LNCaP-PDGF D-conditioned medium induces migration of the prostate fibroblast cell line 1532-FTX, indicating LNCaP-processed PDGF DD is active in a paracrine manner as well. In a severe combined immunodeficient mouse model, PDGF DD expression accelerates early onset of prostate tumor growth and drastically enhances prostate carcinoma cell interaction with surrounding stromal cells. These demonstrate a potential oncogenic activity of PDGF DD in the development and/or progression of prostate cancer.

INTRODUCTION

Primary prostate carcinomas typically grow slowly and often do not progress to clinically relevant disease (1). The main cause for mortality from prostate cancer is metastasis, a complex process involving cancer cell invasion through stromal components and colonization at the distant organs (2, 3). Although it has become clear that paracrine signaling between the tumor cells and the surrounding mesenchymal cells plays a critical role for tumor progression, the molecular mechanisms of these interactions are much less understood. The platelet-derived growth factor (PDGF) protein family is a potent stimulator of cell proliferation, chemotaxis, and transformation and is known to play a major role in cell-cell communication for normal development and also during pathogenesis (reviewed in Refs. 4 and 5). PDGF isoforms exert their biological functions through activation of two structurally related cell surface receptor tyrosine kinases [α-PDGFR receptor (PDGFR) and β-PDGFR (4, 6)]. Immunohistochemical analysis showed that ~80% of prostate tumor tissues express PDGFRs at both primary and metastasized sites (7). Interestingly, β-PDGFR staining is more prominent in endothelial cells when they are exposed to prostate tumor cells that express PDGF (8), suggesting significance of PDGF signaling for prostate cancer progression in a paracrine manner. Consistently, PDGFR inhibitor STI571 (Gleevec) in combination with paclitaxel was shown to substantially reduce prostate cancer bone metastasis in a mouse model (8).

For more than two decades, PDGFs were thought to exist in the form of three dimeric polypeptides (the homodimers PDGF AA and BB and the heterodimer PDGF AB). The PDGF A chain can activate α-PDGFR only, whereas the PDGF B chain activates both α- and β-PDGFR (4, 6). Recently, however, the PDGF C and D chains were discovered in a BLAST search of the expressed sequence tag databases at the National Center for Biotechnology Information (9–11). The PDGF C and D chains have a unique two-domain structure with an NH2-terminal CUB (complement subcomponents C1r/C1s, Uegf, and Bmpl) domain and a COOH-terminal PDGF/vascular endothelial growth factor domain. Whereas secreted PDGF AA, BB, and AB can readily activate their cell surface receptors, it was suggested that extracellular proteolytic removal of the CUB domain is required for the growth factor domain of PDGF CC and DD dimers to preferentially activate α-PDGFR and β-PDGFR, respectively (9–11).

Whereas the PDGF A- and B-mediated signal transduction pathways leading to specific cellular processes have been extensively studied, little is known about the functions of the new family members, PDGF C and D. Increased expression of PDGF D has been found in ovarian, lung, renal, and glioma-derived cell lines as well as in clinical tumor samples, suggesting a correlation between increased PDGF D expression and human cancer (12, 13). Because β-PDGFR is thought to be critical for prostate cancer progression, and PDGF D is a specific ligand for β-PDGFR, we examined the effects of PDGF D expression in prostate cancer cell growth and its interactions with stromal cells. Whereas the previous studies suggested that the processing of latent PDGF DD into its active form requires proteases in serum (9, 10), we show in this study that human prostate carcinoma LNCaP processes latent PDGF DD into the active form under serum-free conditions, resulting in promotion of LNCaP growth. Additionally, PDGF DD produced by LNCaP cells induces cell motility of the prostate fibroblast cell line TFX-1532, indicating paracrine signaling of PDGF DD for the interactions between carcinoma and mesenchymal cells. We also show that PDGF DD expression greatly enhances prostate carcinoma cell interaction with the surrounding stromal layers in a severe combined immunodeficient (SCID) mouse model, demonstrating a potential oncogenic activity of PDGF D in human prostate cancer progression.

MATERIALS AND METHODS

Production of Recombinant PDGF D (rPDGF D) Protein. A two-step reverse transcription-PCR approach was taken to clone PDGF D into a vaccinia expression vector. Total RNA isolated from the prostate cell line DU145 using Trizol reagent was used in a cDNA synthesis reaction using SuperScript RTII (Invitrogen, Carlsbad, CA). The resultant cDNA was used in a first PCR reaction that yielded a 2107-bp product containing the 1113-bp open reading frame of PDGF D (forward, 5′-ggga-gacgtgctgclctc-3′; reverse, 5′-tttcggtgctacctgta-3′). This PCR product was gel purified and used as a template in a second PCR reaction designed to add the restriction enzyme sites AflIII and

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Requests for reprints: Hyeong-Reh Choi Kim, Department of Pathology, Wayne State University School of Medicine, 540 East Canfield, Detroit, Michigan 48201. Phone: (313) 577-2407/0193; Fax: (313) 577-0057; E-mail: hrckim@med.wayne.edu.

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BeII at the 5' and 3' ends, respectively, of the PDGF D open reading frame, as well as the addition of a 6×His epitope tag at the carboxyl end of the PDGF D protein (forward: 5'-caaagcttgcagacgcggct-c-3'; reverse, 5'-catgctta-caugagctcttct-cgtggt-3'; the 6×His tag is underlined). The resultant 1150-bp product was digested with A/IIf and BeII, and inserted into the Neo/BamHI site of the vaccinia virus expression vector pTF7-ECM1 (a kind gift from Dr. R. Fridman, Wayne State University). pTF7-ECM1 contains the T7 promoter upstream of the Neo/BamHI cloning site; therefore, transcription of the inserted gene requires the presence of the T7 polymerase, supplied by infecting the cells with recombinant vaccinia virus. Fidelity of the in-frame sequence encoding the PDGF D:His fusion protein was confirmed by DNA sequencing (Eli Lilly Pharmaceuticals, Inc., San Francisco, CA). This plasmid is referred to as pTF7-PDGFD:His and was used to produce recombinant full-length PDGF D following established vaccinia virus protocols (14). In short, BS-C-1 (green monkey kidney) cells were first infected with the recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase. After 30 min of infection, the cells were washed with PBS and then transfected with the plasmid pTF7-PDGFD:His, using Effectene reagent (Qiagen). Expression of PDGF D inserted into the pTF7 plasmid is reliant on infection of the cells by vTF7-3, which expresses the T7 RNA polymerase gene. Cell-host machinery then transcribes the gene of interest. Forty-eight h after co-infection/transfection with vaccinia virus and PDGF D, the serum-free conditioned medium (CM) was collected and cleared of cellular debris by a 5-min centrifugation at 2000 × g. The resultant CM containing recombinant full-length PDGF D was used in subsequent PDGF D processing experiments.

Construction of a Mammalian PDGF D Expression Vector. To insert PDGF D into the mammalian expression vector pcDNA3, pTF7-PDGFD:His (described in the preceding paragraph) was used as the template in a PCR reaction with the primers (forward, 5'-ctgcttgaatagctggccgct-c-3'; and reverse, 5'-ctgcttgagctgagctaggccgct-c-3') to add an A/IIf site at the 5' end and to restore the BS/BamHI site at the 3' end of the PDGF D:His open reading frame. The subsequent 1150-bp PCR product was then digested with A/IIf and BeII and inserted into the A/IIf/BamHI site of the mammalian expression vector pcDNA3.1(+) (Invitrogen). This plasmid is referred to as pcDNA3-PDGF D:His and was used to create the stably transfected cell line LNCaP-PDGF D.

Cell Culture. Human prostate carcinoma LNCaP cells and resultant transfected lines were cultured in a humidified 5% CO2 incubator with RPMI 1640 supplemented with 5% fetal bovine serum, 2 mm glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies Inc., Carlsbad, CA). Medium and incubator conditions were the same for the human prostate cancer cell line LNCaP-PDGF D. RPMI 1640 with 10% fetal bovine serum, pooled together, and are referred to as RPMI 1640, and viable cells were counted by trypan blue exclusion assay. One million cells were s.c. injected into 5-week-old male homozygous C.B.-17 scid/scid mice (Taconic Farms, Germantown, NY). For the 2-week experiment, each animal received one injection of 1 × 106 cells in the left flank. Tumor size was too small to measure reliably by caliper; therefore, animals were sacrificed at the end of 2 weeks to dissect out the developing tumors. For the 5-week experiment, six animals received two s.c. injections of 1 × 106 cells/injection, one injection in each flank, and tumor growth was assessed weekly by two-dimensional measurements (mm) using a caliper. Tumor volume was estimated by the formula \( V = \frac{a \times b^2}{2} \), where \( a \) is the longest dimension, and \( b \) is the width. Statistical analysis of tumor volume (average and SE) was performed using the program GraphPad Instat Version 3 for Windows (GraphPad Software, San Diego, CA). Mice were maintained according to the guidelines for the care and use of experimental animals established by the NIH.

Wound Migration Assay. Cells were grown in complete medium in a 24-well plate to 90% confluence in a 24-well plate and to 90% confluence and pretreated with mitomycin C (25 μg/ml) for 30 min before an injury line was made using a 2-mm-wide plastic pipette tip. After rinsing with PBS, cells were allowed to migrate in appropriate medium, and photographs were taken (×40) at the indicated time points to assess cell motility.

Cell Migration Assay. Twenty-four-well Transwell units with polycarbonate filters (Corning Costar, Cambridge, MA) coated with type I collagen (Sigma, St. Louis, MO) were used for all cell migration assays. To determine 1532-FTX cell migration in the presence of CM, the lower compartments of the wells were filled with 600 μl of appropriate medium, and 5 × 104 cells were placed in the upper part of the Transwell and incubated for 12 h. To determine LNCaP-PDGF D migration in the presence of 1532-FTX cells, 1532-FTX cells were grown to 90% confluence in a 24-well plate, and then medium was changed to serum-free medium. LNCaP-neo or LNCaP-PDGF D cells (5 × 104) were then placed in Transwell units and placed in the wells containing 1532-FTX cells in serum-free medium at the bottom of the well. Cells were co-cultured for 48 h. Filters from all assays were prepared in the following manner: cells were fixed with methanol and stained with hematoxylin for 10 min, followed briefly by eosin staining. Cells on the upper surface of the filter were removed mechanically by wiping with a cotton swab. The total number of cells migrating to the lower side of the filter was determined using microscopy at ×400.

Histology. Mice were sacrificed 5 weeks after injection of cells by cervical dislocation under anesthesia using isoflurane. The tumors and surrounding mouse tissues were dissected and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E for histological analysis.
RESULTS

Human Prostate Carcinoma LNCaP Processes PDGF D into Active Growth Factor Domains. To examine the effects of PDGF D expression on human prostate carcinoma cell growth and tumorigenicity, LNCaP-PDGFD and LNCaP-neo cell lines were established as described in “Materials and Methods.” PDGF D overexpression in LNCaP-PDGFD was confirmed by reverse transcription-PCR analysis (Fig. 1A) as well as immunoblot analysis using anti-PDGF D/GD antibody (Fig. 1B). It was shown previously that when a PDGF D expression vector was transfected into human embryonic kidney (HEK 293) cells, only the full-length PDGF D protein was secreted from HEK 293 cells under serum-free conditions; HEK 293-secreted pro-PDGF D was found to be processed into an active growth factor dimer by unknown protease(s) in serum (10). Surprisingly, however, immunoblot analysis of secreted proteins collected from LNCaP-PDGFD cells in the absence of serum revealed that both the pro-form (Mr 50,000) and the processed forms (Mr 20,000 and 15,000), with sizes similar to what were reported to be growth factor monomers (9, 10), were detected under reducing condition using anti-PDGF D/GD antibody (Fig. 1B).

The above results suggest that the pro-PDGF D protein may be processed into the growth factor domain by protease(s) expressed in human prostate carcinoma LNCaP cells independent of serum. To examine PDGF D processing by proteinases produced by LNCaP cells, rPDGF D protein was produced using a vaccinia mammalian cell expression system as described in “Materials and Methods.” When BS-C-1 cells were cultured in serum-free medium after coinfection/transfection with vaccinia virus and pTF7-PDGF D-HIS (see “Materials and Methods”), only pro-PDGF D protein was detected, migrating as a dimer with a Mr of ~90,000 under nonreducing conditions and as a monomer with a Mr of ~50,000 under reducing conditions (Fig. 2A, left panel). In agreement with previous reports (9, 10), the pro-PDGF D protein was processed into the growth domain in the presence of serum, migrating as a dimer with Mr ~35,000 and as monomer species with Mr 20,000 and 15,000 (Fig. 2A, right panel). Using rPDGF D, we examined whether pro-PDGF D can be processed by secreted proteinase(s) or by cell surface/pericellular proteinase(s) produced from LNCaP cells. Whereas CM collected from LNCaP cells contained little proteolytic enzyme capable of processing rPDGF D (Fig. 2B, right panel), incubation of rPDGF D with LNCaP-neo cells resulted in cleavage of rPDGF D, suggesting that cell surface or pericellular proteinase(s) may be responsible for processing pro-PDGF D into the growth factor domain (Fig. 2B, left panel). It was noticed that rPDGF D was processed into several species with Mr ~25,000 by proteinase(s) produced by LNCaP-neo cells, whereas two major monomer species with Mr 20,000 and 15,000 are usually detected in CM collected from LNCaP-PDGFD cells (Fig. 1B). Next, we examined whether rPDGF D is more effectively processed into Mr 20,000 and 15,000 monomers in the presence of LNCaP-PDGFD cells.
immunoblotted with an Ab that recognizes both active and inactive forms of ERK1/2. pERK1/2 using an Ab specific to active extracellular signal-regulated protein kinase (ERK) 1/2. CM (Lane 4) were resolved on reducing SDS-PAGE, and the immunoblot was analyzed.

vitro mitogenic activity of PDGF D through an autocrine mechanism, B, immunoprecipitation. C, LNCaP-PDGF D CM (Lane 4), LNCaP-neo CM (Lane 3), or LNCaP-neo (Lane 1) supplemented with 25 ng/ml PDGF BB served as a positive control (Lane 2). 50 μg of lysate from NIH3T3 cells treated with 25 ng/ml PDGF BB (Lane 1), serum-free medium (Lane 2), LNCaP-PDGF D CM (Lane 3), or LNCaP-neo CM (Lane 4) were resolved on reducing SDS-PAGE, and the immunoblot was analyzed using an Ab specific to active extracellular signal-regulated protein kinase (ERK) 1/2 (pERK1/2). To determine basal levels of ERK1/2, the same blot was stripped and immunoblotted with an Ab that recognizes both active and inactive forms of ERK1/2.

cells compared with LNCaP neo cells. As shown in 2C, whereas rPDGF D was processed mostly to a M, 25,000 fragment after 4 h of incubation with LNCaP-neo cells, it was more effectively processed into growth factor domains (M, 25,000, 20,000, and 15,000) in the presence of LNCaP-PDGF D cells. This suggests that protease expression or its activation, responsible for PDGF D processing, was up-regulated by constitutive activation of PDGF D signaling in LNCaP-PDGF D cells.

To test whether the processed forms of PDGF D are biologically active, serum-starved NIH3T3 cells were treated with CM collected from LNCaP-neo and LNCaP-PDGF D. The β-PDGFR protein was immunoprecipitated with an anti β-PDGFR Ab, and the active form was detected by immunoblot analysis using an anti-phosphotyrosine Ab. As a positive control, serum-starved NIH3T3 cells were treated with recombinant PDGF BB protein at a concentration of 50 ng/ml. As shown in 3A, β-PDGFR was readily activated by treatment for 10 or 60 min with LNCaP-PDGF D CM as well as by recombinant PDGF BB protein treatment for 10 min, whereas no significant activation was detected with LNCaP CM. To further confirm β-PDGFR activation, we also examined activation of its downstream signaling molecule, ERKs. As shown in 3B, ERKs were phosphorylated on LNCaP-PDGF D CM treatment as effectively as PDGF BB treatment, whereas LNCaP-neo CM had little effect on ERK phosphorylation. These results demonstrate that the growth factor domain of PDGF D produced by LNCaP-PDGF D cells can activate its cognate receptor, β-PDGFR, transducing its downstream signaling pathway.

PDGF D Enhances LNCaP Cell Proliferation. To assess the in vitro mitogenic activity of PDGF D through an autocrine mechanism, we examined the growth rates of LNCaP-neo and LNCaP-PDGF D under normal growth conditions. During the first 24 h of cultures, neither LNCaP-neo nor LNCaP-PDGF D significantly increased their cell numbers. However, drastic differences in cell numbers between LNCaP-neo and LNCaP-PDGF D were observed after the medium was conditioned by these cells for 48 h. Whereas LNCaP-neo cells barely doubled the cell number, LNCaP-PDGF D exhibited a 3–4-fold increase in cell numbers after 48 h in normal culture medium (Fig. 4), showing that PDGF D expression in prostate carcinoma cells has growth-promoting activity through an autocrine mechanism.

PDGF D Accelerates Early Onset of Prostate Tumor Growth and Stimulates Prostate Carcinoma Cell Interactions with Stromal Cells in a Mouse Model. The above-mentioned studies showed that tumor cell-derived PDGF D enhances tumor cell growth in an autocrine manner. These effects may be transient in vitro or may result in profound differences in vivo for tumorigenicity and/or their interactions with stromal cells that express PDGFRs. To examine the in vivo effects of PDGF D expression in human prostate carcinoma, LNCaP-neo and LNCaP-PDGF D cells were injected s.c. in the flank of male SCID mice (1 injection/animal). When the formation of tumor nodule was examined in mice sacrificed 2 weeks after injection, only one of five injections developed from LNCaP-neo cells, whereas five of five animals developed lesions from LNCaP-PDGF D cells (20% versus 100%; Fig. 5A). To further examine the onset of tumor nodules and their growth, LNCaP-neo and LNCaP-PDGF D cells were s.c. injected into both the right and left flanks (2 injections/animal) of an additional six animals, and tumor incidence and size were monitored at weekly intervals. The differences in tumor incidence between LNCaP-neo and LNCaP-PDGF D became less significant after 5 weeks (80% versus 100%; Fig. 5A), and average tumor volume was similar between these two cell lines (Fig. 5B). These results indicate that whereas the onset of tumor nodules was shortened by 10–14 days in LNCaP-PDGF D tumors compared with LNCaP-neo tumors, overall tumor incidence and tumor volume were similar between these two cell lines.

To examine the effects of tumor cell-derived PDGF D expression on host stromal cell responses in vivo, LNCaP-neo and LNCaP-PDGF D tumor tissues were harvested 5 weeks after injection, sectioned, and stained with H&E. Histological examination of LNCaP-neo tumors revealed a solid tumor mass that is well capsulated and separated from mouse stromal tissue (Fig. 6A). In contrast, LNCaP-PDGF D tumors showed a close interaction with the mouse stromal cells (Fig. 6B), suggesting a role for PDGF D in close reciprocal interplay between tumor cells and local mesenchymal cells. Among two different sections for each of eight individual LNCaP-neo and LNCaP-PDGF D tumors, we found differences in tumor incidence and/or their interactions with stromal cells that express PDGFRs. To examine the interactions between LNCaP-neo and LNCaP-PDGF D, we used a mouse model.

Fig. 3. LNCaP-processed platelet-derived growth factor (PDGF) D is biologically active. A, serum-starved NIH3T3 cells were stimulated with 5 ml of conditioned medium (CM) from either LNCaP-PDGF D (Lanes 3 and 6) or LNCaP-neo (Lanes 4 and 7) for 10 or 60 min. Serum-starved NIH3T3 cells stimulated with 5 ml of serum-free medium supplemented with 25 ng/ml PDGF BB served as a positive control (Lane 1), and cells stimulated with serum-free medium (SF) served as a negative control (Lanes 2 and 5). β-PDGFR receptor was immunoprecipitated from 250 μg of lysate, as described in Materials and Methods. Resultant immunoprecipitates were resolved by reducing SDS-PAGE and analyzed by immunoblot with the indicated antibodies (Abs). The blot was stained with H&E. Histological examination of LNCaP-neo tumors stained with H&E. Histological examination of LNCaP-neo tumors showed a close interaction with the mouse stromal cells (Fig. 6A), showing that PDGF D expression in prostate carcinoma cells enhances tumor cell growth in an autocrine manner. These effects may be transient in vitro or may result in profound differences in vivo for tumorigenicity and/or their interactions with stromal cells that express PDGFRs. To examine the in vivo effects of PDGF D expression in human prostate carcinoma, LNCaP-neo and LNCaP-PDGF D cells were injected s.c. in the flank of male SCID mice (1 injection/animal). When the formation of tumor nodule was examined in mice sacrificed 2 weeks after injection, only one of five injections developed from LNCaP-neo cells, whereas five of five animals developed lesions from LNCaP-PDGF D cells (20% versus 100%; Fig. 5A). To further examine the onset of tumor nodules and their growth, LNCaP-neo and LNCaP-PDGF D cells were s.c. injected into both the right and left flanks (2 injections/animal) of an additional six animals, and tumor incidence and size were monitored at weekly intervals. The differences in tumor incidence between LNCaP-neo and LNCaP-PDGF D became less significant after 5 weeks (80% versus 100%; Fig. 5A), and average tumor volume was similar between these two cell lines (Fig. 5B). These results indicate that whereas the onset of tumor nodules was shortened by 10–14 days in LNCaP-PDGF D tumors compared with LNCaP-neo tumors, overall tumor incidence and tumor volume were similar between these two cell lines.

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xenografts examined, all of the LNCaP-PDGF D sections, but none of the LNCaP-neo sections, showed drastically increased carcinoma interactions with the stromal cells, an event known to be critical for cancer progression leading to metastasis. It should be mentioned that the average weight of xenografts formed by LNCaP-neo and LNCaP-PDGF D was comparable at the time of histological examination (1.5 versus 1.3 g, respectively), suggesting that PDGF D-mediated stromal-epithelial interactions are not directly associated with its growth-promoting activity.

PDGF D Processed by LNCaP Enhances Cell Motility of Prostate Fibroblasts in a Paracrine Manner and Induces LNCaP Migration in a Secondary Paracrine Manner. We next investigated whether PDGF D stimulates prostate fibroblast cell migration in a paracrine manner and/or promotes migrative phenotype of human prostate cancer cells in an autocrine manner. As shown in Fig. 7A, cell motility of human prostate fibroblasts 1532-FTX was greatly induced in the presence of CM collected from LNCaP-PDGF D compared with treatment with CM collected from LNCaP-neo. To ensure that LNCaP-PDGF D CM-induced 1532-FTX migration does not result from increased cell proliferation rates, the wound migration assay was performed using cells pretreated with mitomycin C, a cell cycle blocker at the S phase (15). We then quantitated cell motility by a migration assay using a Transwell chamber. Whereas <2-fold induction of 1532-FTX cell migration was observed in the presence of LNCaP-neo CM compared with the serum-free medium condition, ~10-fold induction was detected in the presence of LNCaP-PDGF D CM (Fig. 7B). These results suggest that PDGF D-mediated stromal-prostate carcinoma interactions involve migration of surrounding mesenchymal cells in a paracrine manner.

When cell motility of LNCaP-neo and LNCaP-PDGF D was examined by a Transwell chamber assay (Fig. 8) as well as wound healing assay (data not shown), no significant difference was detected, suggesting that PDGF D expression has little effect on human prostate epithelial cell motility in an autocrine manner. Next, we asked whether prostate epithelial cell-produced PDGF D in the presence of prostate fibroblasts induces epithelial cell migration. To this end, cell migration of LNCaP-neo and LNCaP-PDGF D was examined by a Transwell chamber assay in the presence of prostate 1532-FTX fibroblasts on the bottom of the wells. Importantly, migration of LNCaP-PDGF D was induced significantly compared with migration of LNCaP-neo in the presence of 1532-FTX cells (Fig. 8). Taken together, these results suggest that prostate carcinoma-produced PDGF D induces PDGFR-mediated signaling transduction pathways in stromal cells, resulting in secretion of chemotactic molecule(s), which, in turn, induce prostate cell motility in a secondary paracrine manner.

DISCUSSION

A causative role for PDGF in tumorigenesis was suggested when v-sis (the oncogene of simian sarcoma virus) was found to be 92% homologous to PDGF-B (6, 16, 17). Consistently, studies during the past two decades have clearly indicated the significance of PDGF in human tumors including glioma (18–20), dermatofibrosarcoma (21, 22), neurofibroma (23), myelomonocytic leukemia (24), and osteo-

Fig. 5. Onset of LNCaP-PDGF D tumor formation is accelerated compared with LNCaP-neo tumors. A, 1 × 10⁶ cells were injected s.c. in the flank of male severe combined immunodeficient mice, and tumor incidence was examined. To determine the 2 week incidence point, one injection was given per mouse, with a total of 5 mice/group (total, 5 injections/cell line). Because tumors were not palpable at 2 weeks, mice were sacrificed, and the skin was dissected away to note the appearance of small nodules. To determine tumor incidence over a 5-week period, a second set of mice were given 2 injections/mouse, 6 mice/group (total, 12 injections/cell line). Palpable tumors were examined at week 4 and 5. B, tumor growth is represented by average tumor volume. Error bars represent ±SE.

Fig. 6. Increased tumor-stromal interactions observed in LNCap-PDGF D tumors compared with LNCaP-neo tumors. Tumors were harvested 5 weeks after injection, fixed, sectioned, and stained with H&E. A, LNCaP-neo tumor; B, LNCaP-PDGF D tumor. Asterisk indicates tumor-stromal interactions.
blastoma and osteosarcoma (25). 

In vitro, overexpression of the v-sis oncogene product (p28v-sis) or PDGF B in cells that express their receptors enhances transformation, indicating an autocrine mechanism in tumorigenesis (26, 27). In addition to the autocrine mechanism, recent studies revealed a critical role for paracrine PDGF signaling in carcinogenesis involving epithelial-stromal interactions. Using nude mice, it was demonstrated that PDGFR activation of stromal cells results in tumorigenic conversion of immortal human keratinocytes (28). Mounting evidence suggests that aberrant interaction between stroma and epithelium is critical for the neoplastic progression of breast epithelium (29, 30), and PDGF was suggested to be among the critical mediators (31–34). Expression of PDGF B-protein and mRNA was restricted to the breast epithelium and tumor cells, whereas membranous PDGFR immunostaining was detected in stromal cell populations in all of the breast tissues examined (32). PDGFR staining was particularly localized in the periepithelial stroma of breast carcinoma, suggesting a paracrine stimulation of adjacent stromal tissue by breast tumor cells (32). Similar to breast cancer, paracrine mechanisms were also observed in colorectal cancer and small cell lung carcinoma (35, 36).

The present study reveals the potential oncogenic activity of PDGF D-mediated β-PDGFR in human prostate cancer in both autocrine and paracrine manners. In agreement with our hypothesis, previous studies showed that approximately 80% of prostate tumor tissues express PDGFRs at both primary and metastasized sites (7), and inhibition of PDGFR signaling using Gleevec significantly reduces prostate cancer bone metastasis in a mouse model (8). Interestingly, whereas increased expression levels of α-PDGFR and its cognate ligand, PDGF A, were detected in prostatic intraepithelial neoplasia and adenocarcinoma, only β-PDGFR (and not its ligand, PDGF B) was expressed in advancing stages of prostate cancer (37, 38). These prostate cancer cells were shown to exhibit phenotypes typical to β-PDGFR activation, suggesting that an alternative ligand, besides PDGF B, may play a critical role for β-PDGFR signaling during the progression of prostate cancer. Recent discovery of PDGF D as a specific activator of β-PDGFR and our present study suggest the involvement of PDGF D in human prostate cancer development and/or progression.

Many investigators have reported a more potent transforming signal by β-PDGFR than the signal transduced by α-PDGFR (26, 39, 40). Although both PDGF AA and BB are strong mitogens, only PDGF BB can induce phenotypic transformation in murine fibroblasts. A fundamental question in cell signaling is “how do two structurally related cell surface receptor tyrosine kinases (α- and β-PDGFRs) mediate differential cellular processes often using overlapping signaling pathways?” To address this issue, we previously established NIH3T3 clones in which α-PDGFR signaling is inhibited by a dominant-negative α-PDGFR or an antisense construct of α-PDGFR (41). We showed that inhibition of α-PDGFR signaling enhanced PDGF BB-mediated phenotypic transformation, suggesting that α-PDGFR antagonizes β-PDGFR-induced transformation. Whereas both α- and β-receptors effectively activate ERKs, α-PDGFR, but not β-PDGFR, activates stress-activated protein kinase-1/e-Jun NH2-terminal kinase-1 (JNK-1). Inhibition of JNK-1 activity using a dominant-negative JNK-1 mutant markedly enhanced PDGF BB-mediated anchorage-independent cell growth, demonstrating an

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**Fig. 7.** Platelet-derived growth factor (PDGF) D induces cell motility of human prostate fibroblasts in a paracrine manner. A, wound healing assay. TFX-1532 cells were grown to 100% confluence and then pretreated with mitomycin C for 30 min to prevent cell proliferation. An injury line was made, and cells were cultured in the presence of either LNCaP-neo conditioned medium or LNCaP-PDGF D conditioned medium. Pictures were taken at indicated time points. B, 1532-FTX cell migration assay using a Transwell. Migrating cells were counted after 12 h of culture. Data points represent average total number of cells migrating to bottom of filter; experiments were done in triplicate. *, P < 0.05 as determined by Student’s t test.

**Fig. 8.** Platelet-derived growth factor D induces LNCaP cell motility in the presence of human prostate fibroblasts in a secondary paracrine manner. LNCaP-neo or LNCaP-PDGF D cells were placed on top of a Transwell chamber filter and cultured for 48 h in the absence or presence of 1532-FTX fibroblasts in serum-free medium. Data points represent average total number of cells migrating to bottom of filter; experiments were done in triplicate. *, P < 0.05 as determined by Student’s t test.
agonistic role for α-PDGFR-activated JNK-1 in PDGF-induced transformation. These results revealed a striking feature of PDGF signaling: the specificity and the strength of the PDGF growth signal is modulated by α-PDGFR-mediated simultaneous activation of growth-stimulatory and -inhibitory signals. With the discovery of new PDGF ligands, it has become clear that PDGF signaling is finely modulated not only by PDGF A activation of α-PDGFR and PDGF B activation of both α- and β-PDGFR, but also by PDGF C activation of α-PDGFR and PDGF D activation of β-PDGFR. Recently, it was shown that PDGF DD dimer activates ββ-PDGFR, not αα-PDGFR, but it can activate α-PDGFR through its dimerization with ββ-PDGFR \([αβ-PDGFR (9, 10)]\). Thus, for better understanding of roles of PDGF signaling during human cancer progression, it would be of particular importance to unveil signal transduction pathways and oncogenic activities induced by PDGF DD in comparison with better characterized pathways induced by PDGF BB, which activates all three forms of PDGFRs (αα, αβ, and ββ).

The unique structure of PDGF D, which requires proteolytic cleavage of the CUB domain to allow the growth factor domain to activate β-PDGFR, implements another level of regulation in PDGF signaling. The present study demonstrates that human prostate carcinoma LNCaP is capable of processing full-length PDGF D into a biologically active PDGF ligand for β-PDGFR activation and that this processing most likely occurs at or near the cell surface. This localized activation leads us to speculate a possible function of the CUB domain of the full-length PDGF DD. The CUB domain, composed of approximately 110 amino acids, shares sequence homology with the CUB domains of the complement subcomponents C1r/C1s, urokinase epidermal growth factor-like protein (Uegf), and bone morphogenetic protein 1 (Bmp1, Ref. 42). The CUB domain is found in several extracellular proteins and is thought to mediate protein-protein or protein-carbohydrate interactions (43–45). Thus, the CUB domain may have unique regulatory functions toward PDGF D activity in addition to its activity in inhibiting PDGF D binding to the PDGFR.

The CUB domain interaction with unidentified proteins may be critical for sequestration of the full-length PDGF D protein in extracellular and/or pericellular matrix, thereby regulating PDGF D protein localization. Conversely, the CUB domain could facilitate activation of PDGF DD by interacting with a specific protein(s) near the cell surface, localizing PDGF DD in appropriate association with the protease(s); only on this association would the protease be able to activate PDGF DD. It is also possible that the CUB domain may not serve as a mere “anchor” but that its interaction with co-receptor-like proteins may alter PDGFR signaling pathways. Considering its sequence similarities with epidermal growth factor-like protein and bone morphogenetic protein 1, it may be also worthy of investigation to determine whether the CUB domain alone after proteolytic cleavage from the full-length PDGF D has biological activity. In relation to prostate cancer, the CUB domain’s homology with bone morphogenetic protein (a protein known to play role in bone growth) may have implications in prostate cancer metastasis to the bone. This is worthy of further investigation using in vivo models.

Recent reports showed that PDGF D induces phenotypic transformation of NIH3T3 cells in vitro and also promotes tumor formation in mice (12, 46). In this study, we show that although PDGF D promotes human prostate carcinoma cell growth in vitro, it accelerates tumor formation only at the early stages, with little effect on the overall tumor incidence or the size of the tumors in vivo. However, PDGF D drastically increased the ability of human prostate carcinoma cells to interact with host stromal components in mice, an event known to be critical for prostate cancer progression. Our in vitro studies suggest that PDGF D is sufficient to induce migration of fibroblasts but that PDGF D alone is not sufficient to induce epithelial migration. Rather, PDGF D induces an as yet unidentified signal from the fibroblasts, which then induces the epithelial cell migration in a secondary paracrine manner. Taking our results together with those of recent studies reporting the significance of PDGF signaling in the progression of prostate cancer as well as β-PDGFR signaling in metastasis, we propose that prostate carcinoma cells auto-activate PDGF D and use its signaling in both autocrine and paracrine manners, leading to a more malignant phenotype.

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A Potential Oncogenic Activity of Platelet-Derived Growth Factor D in Prostate Cancer Progression

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