PDGF-D/PDGF- $\beta\beta$ Receptor-Regulated Chemotaxis of Malignant Mesothelioma Cells

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Key Words

Malignant mesothelioma cell • Chemotaxis • PDGF- $\beta\beta$ receptor • ROCK • ERK

Abstract

Background/Aims: Our earlier study suggested that platelet-derived growth factor (PDGF)- $\beta\beta$ receptor regulates chemotaxis of human malignant mesothelioma cells such as MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells, but not non-malignant Met5A cells. The present study was designed to gain further insight into the PDGF- $\beta\beta$ receptor signals underlying the chemotaxis. **Methods:** PDGF-D secreted from cells, activation of Akt and ERK, and cell migration were monitored for cells with and without knocking-down PDGF- $\beta\beta$ receptor. **Results:** FBS significantly stimulated PDGF-D secretion from malignant mesothelioma cells, but not Met5A cells. PDGF-D activated Akt and ERK in both the non-malignant and malignant cells. PDGF-D significantly facilitated migration of malignant mesothelioma cells, but not Met5A cells, with the extent varying among the cell types. The facilitatory action of PDGF-D was clearly prevented by knocking-down PDGF- $\beta\beta$ receptor or inhibitors of PI3 kinase, PDK1, Akt, Rac1,

ROCK, and MEK. **Conclusion:** The results of the present study indicate that PDGF-D promotes malignant mesothelioma cell chemotaxis through PDGF- $\beta\beta$ receptor signaling pathways along a PI3 kinase/PDK1/Akt/Rac1/ROCK axis and relevant to ERK activation.

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Introduction

Malignant mesothelioma, originated from mesothelial cells, is a locally aggressive and highly lethal tumor, that is caused by exposure to asbestos [1]. Malignant mesotheliomas are characterized by rapid and diffuse invasion into the peritoneum through the diaphragm or the pleura through the mediastinum. Mesothelioma cells express a variety of growth factors including platelet-derived growth factor (PDGF), that act in an autocrine manner [2, 3]. PDGFs such as PDGF-A, -B, -C and -D regulate cell growth and chemotaxis both in cancer cells and normal cells [4]. Higher expression of PDGF-A and

-B is found with lung and pleural tumors [5]. PDGF-A and -B are extracellularly secreted from malignant mesothelioma cells [6-8]. PDGF-A plays a role in mesothelial tumor initiation at early stages [9] and PDGF-B acts as a chemoattractant for malignant mesothelioma [10, 11].

PDGF-A and -B are secreted as active dimers composed of single-domain protein chains (PDGF-AA and -BB), but otherwise, PDGF-C and -D, containing an N-terminal CUB and a conserved C-terminal growth factor domain, are secreted as a latent dimeric factor and undergo proteolytic processing at the hinge region between the CUB domain and the growth factor domain, to produce active form of PDGF-CC and -DD [12-14]. PDGF receptors consist of the PDGF- α and/or - β subunit such as $\alpha\alpha$ homodimer, $\alpha\beta$ heterodimer, and $\beta\beta$ homodimer [12]. PDGF- $\alpha\alpha$ and - $\alpha\beta$ receptors are activated by PDGF-AA, -BB, and -CC, and PDGF- $\beta\beta$ is activated by PDGF-DD [12-14].

Evidence has pointed to significance of PDGF-D in tumor development and progression [15]. Urokinase plasminogen activator (uPA), that forms the active dimer PDGF-DD through extracellular proteolytic processing, contributes to progression of prostate cancer [16, 17]. Conversely, urinary trypsin inhibitor (UTI), a potent inhibitor of uPA, is capable of preventing metastasis in a variety of cancer cells [18-23]. In our earlier study, UTI inhibited fetal bovine serum (FBS)-induced migration of human malignant mesothelioma cells [24]. This suggests that PDGF-D, undergoing uPA-mediated proteolytic processing, promotes malignant mesothelioma cell chemotaxis through a PDGF- $\beta\beta$ receptor signaling pathway.

The present study was conducted to obtain direct evidence for PDGF-D/PDGF- $\beta\beta$ receptor-regulated chemotaxis of malignant mesothelioma cells. We show here that PDGF-D promotes malignant mesothelioma cell chemotaxis by activating phosphoinositide 3-kinase (PI3 kinase), phosphoinositide-dependent kinase-1 (PDK1), Akt, Rac1, and Rho-associated coiled-coil forming protein kinase (ROCK) or extracellular signal-regulated kinase (ERK) as mediated via PDGF- $\beta\beta$ receptors.

Materials and Methods

Cell culture

Human malignant mesothelioma cell lines such as MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells and the human non-malignant mesothelial cell line, Met5A cells, were

purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 0.003% L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Monitoring of PDGF-D secretion

Cells were untreated and treated with 1% (v/v) FBS for 24 h, and extracellular culture medium was collected. The medium was incubated with an anti-PDGF-D antibody (1 μ g) (R&D systems, Minneapolis, MN, USA) at 4 °C for 24 h, and subsequently, 20 μ l of protein G agarose (GE healthcare, Piscataway, NJ, USA) was added followed by further incubation at 4 °C for 24 h by continuously shaking. After centrifugation at 3,000 rpm for 1 min, the pellets were washed twice with Tris Buffered Saline with Tween 20 (TBST)[150 mM NaCl, 0.1% (v/v) Tween20, and 20 mM Tris, pH 7.5] and dissolved in 40 μ l of a sodium dodecyl sulfate (SDS)-polyacrylamide sample buffer [0.2 mM Tris-HCl, 0.04% (w/v) SDS, 20% (v/v) glycerol, pH 6.8]. After boiling at 100 °C for 5 min, proteins were separated by 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. Blotting membranes were blocked with 5% (w/v) bovine serum albumin (BSA) and reacted with an anti-PDGF-D antibody (1:1000)(R&D systems), followed by a horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody (1:5000)(MP Biomedicals Inc., Ohio, USA). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) kit (GE Healthcare) and visualized with an Image Gauge software (FUJIFILM, Tokyo, Japan). The amount of PDGF-D secretion was normalized by regarding immunoreactive signals before FBS treatment as 1.

Knocking-down PDGF- $\beta\beta$ receptor

Small interfering RNAs (siRNAs) silencing the PDGF receptor β subunit-targeted gene (PDGF- β R siRNA): sense, 5'-GGA AUG AGG UGG UCA ACU UTT-3' and anti-sense, 5'-AAG UUG ACC ACC UCA UUC CTT-3'; sense, 5'-GCU CAU GGC CUG AGC CAU UTT-3' and anti-sense, 5'-A AUG GCU CAG GCC AUG AGC TT-3'; and sense, 5'-GAG AGG ACC UGC CGA GCA ATT-3' and anti-sense, 5'-UUG CUC GGC AGG UCC UCU CTT-3', and negative control siRNA (NC siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNAs were transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA).

Chemotaxis assay

Cell migration was monitored with an EZ-TAXIScan (Effector Cell Institute, Piscataway, NJ, USA) equipped with a 6-channel chamber by the method as previously described [24]. Briefly, cells (5 x 10⁶ cells/ml), transfected with the NC siRNA or the PDGF- β R siRNA, were seeded into a hole (1 mm³ in volume) in a KK-chamber filled with RPMI-HEPES medium at 37 °C, aspirated from the opposite side, to come out from the hole, and lined up on the plate to adjust the starting grid. Subsequently, PDGF (4 ng) together with and without a variety

of inhibitors was applied into another hole (1 mm³ in volume) located on the opposite side of the chamber. Cell mobilizations were detected at 3-min intervals through a high performance lens connected to a CCD camera with a coaxial episcopic illumination system, and analyzed with a developed using a C⁺⁺ Builder 6 software (Borland, CA, USA). The migratory pathway was recorded by clicking each cell on a display.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were purified from cells, transfected with and without the NC siRNA or the PDGF- β R siRNA, by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase free-DNase I (2 unit) at 37 °C for 30 min to remove genomic DNAs, and 10 μ g of RNAs were resuspended in water. Then, oligo dT primers, dNTPs, 5 x First Strand buffer, and SuperScript III RNase H-Reverse Transcriptase were added to the RNA solution and incubated at 65 °C for 5 min followed by 56 °C for 60 min, 58 °C for 60 min, 85 °C for 5 min to synthesize the first strand cDNA. Subsequently, 1 μ l of the reaction solution was diluted with water and mixed with 10 x PCR reaction buffer, dNTPs, MgCl₂, oligonucleotide, dimethylsulfoxide [final concentration, 5% (v/v)] and 1 unit of Taq polymerase (Fermentas, St. Leon-Roth, Germany) (final volume, 20 μ l). RT-PCR was carried out with a GeneAmp PCR system model 9600 DNA thermal cycler (Applied Biosystems, Indianapolis, IN, USA) programmed as follows: the first one step, 94 °C for 4 min and the ensuing 30 cycles, 94 °C for 1 s, 62 °C for 15 s, and 72 °C for 30 s. The primers used here were as follows: sense, 5'-CGA GTG GAC ATA CCC CCG CAA-3' and anti-sense, 5'-CAG CAC TCG GAC AGG GAC ATT GAT-3' for the PDGF receptor β subunit and sense, 5'-GAC TTC AAC AGC GAC ACC CAC TCC-3' and anti-sense, 5'-AGG TCC ACC ACC CTG TTG CTG TAG-3' for GAPDH. PCR products were stained with ethidium bromide and visualized by 2% (w/v) agarose gel electrophoresis.

Monitoring activation of Akt and ERK

Cells, transfected with the NC siRNA or the PDGF- β R siRNA, were treated with PDGF-D for 10 min, and then lysed in a lysate solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) SDS, pH 7.5] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail, and then centrifuged at 3,000 rpm for 5 min at 4 °C. Proteins were separated by SDS-PAGE using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to PVDF membranes. Blotting membranes were blocked with TBST containing 5% (w/v) bovine serum albumin and subsequently reacted with an anti-pAkt (Thr308) antibody (Cell Signaling, Beverly, MA, USA), an anti-Akt antibody (Cell Signaling), an anti-pERK antibody (Santa Cruz), an anti-ERK antibody (Santa Cruz), or an anti- β -actin antibody (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare) and visualized using a chemiluminescence

detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

Results

FBS stimulates PDGF-D secretion from malignant mesothelioma cells

We have earlier found that the uPA inhibitor UTI inhibited FBS-induced migration of human malignant mesothelioma cells [24]. This, in the light of the fact that an active form of PDGF-D is produced through uPA-mediated proteolytic processing, raises the possibility that FBS stimulates PDGF-D secretion to activate PDGF- $\beta\beta$ receptors, responsible for malignant mesothelioma cell chemotaxis. To obtain evidence for this, we initially monitored PDGF-D secretion from malignant mesothelioma cells.

Human non-malignant Met5A mesothelial cells and malignant mesothelioma cells such as MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells were cultured in the presence and absence of 1% (v/v) FBS for 24 h. Then, extracellular culture medium was collected and immunoprecipitated with an anti-PDGF-D antibody. Immunoprecipitants were subjected to SDS-PAGE followed by Western blotting using an anti-PDGF-D antibody, to detect PDGF-D extracellularly secreted. For MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells, FBS significantly increased the amount of extracellular PDGF-D to a variety of extents ranging from 1.5 to 3 folds of basal levels, depending upon cell types, while no significant increase was obtained with non-malignant Met5A cells (Fig. 1). This indicates that FBS stimulates PDGF-D secretion from malignant mesothelioma cells, but not non-malignant cells.

PDGF-D promotes malignant mesothelioma cell chemotaxis in a PDGF- $\beta\beta$ receptor-dependent manner

PDGF receptors include PDGF- $\alpha\alpha$, - $\alpha\beta$, and $\beta\beta$ receptors, and PDGF-D is an agonist of PDGF- $\beta\beta$ receptor. Then, we thought that PDGF-D might regulate malignant mesothelioma cell chemotaxis by targeting PDGF- $\beta\beta$ receptor. To address this question, we constructed the siRNA silencing the PDGF receptor β

subunit-targeted gene. Expression of the PDGF- β subunit mRNA was clearly suppressed for cells transfected with the PDGF- β R siRNA as compared with the expression for cells transfected with the NC siRNA (Fig. 3A). This confirms knocking-down of PDGF- β R receptor for cells transfected with the PDGF- β R siRNA.

We next monitored cell migration using an EZ-TAXIScan (Fig. 2). For malignant mesothelioma cells such as MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells transfected with the NC siRNA, PDGF-D facilitated migration, with the different potency among cell types (Fig. 3B). The facilitatory action of PDGF-D was significantly prevented by knocking-down PDGF- β R receptor (Fig. 3B). In contrast, PDGF-D did not facilitate migration of non-malignant Met5A cells transfected with the NC siRNA, and the migration was not affected by knocking-down PDGF- β R receptor (Fig. 3B). It is indicated from these results that PDGF-D promotes migration of malignant mesothelioma cells, but not non-malignant cells, by activating PDGF- β R receptor.

PDGF-D promotes malignant mesothelioma cell migration by activating PI3 kinase, PDK1, Akt, Rac1, and ROCK or ERK as mediated via PDGF- β R receptors

PDGF- β R receptor as well as other growth factor receptors would be linked to two major signaling pathways: one is a pathway along a PI3 kinase/PDK1/Akt/Rac1 (Cdc42)/ROCK axis and another is a pathway along a Ras/Raf/MAP kinase kinase kinase (MEKK)/MAP kinase kinase (MEK)/MAP kinase (MAPK) axis (Fig. 4). To ascertain the implication of PDGF- β R receptor in those signaling pathways, we examined activation of Akt and ERK, a MAPK, in cells with and without knocking-down of PDGF- β R receptor. Akt is phosphorylated and activated by PDK1. ERK is phosphorylated and activated by MEK. In the Western blot analysis, PDGF-D increased phosphorylated Akt for all the non-malignant and malignant mesothelioma cells used here, and the effect was significantly attenuated by knocking-down PDGF- β R receptor (Fig. 5A-E), indicating that PDGF-D actually activates Akt in non-malignant and malignant mesothelioma cells via PDGF- β R receptors. This also suggests that Akt is activated as a downstream effector of PDK1 in a PDGF- β R receptor/PI3 kinase/PDK1/Akt/Rac1/ROCK pathway.

For ERK, immunoreactive signals against an anti-ERK-antibody were detected at 42 and 44 kDa, which correspond to ERK2 and ERK1, respectively (Fig. 5A-E). PDGF-D increased phosphorylated ERK at 44 kDa

Fig. 1. PDGF-D secretion from non-malignant and malignant mesothelioma cells. Culture medium for Met5A, MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells was collected after 24-h non-treatment and treatment with 1% (v/v) FBS and immunoprecipitated with an anti-PDGF-D antibody followed by Western blotting using an anti-PDGF-D antibody. The ratio of the PDGF-D immunoreactive signal intensity for FBS-treated samples against the intensity for FBS-untreated samples was calculated. In the graph, each value represents the mean ($\pm$ SEM) relative intensity ($n=3$ independent experiments). P values, unpaired t -test.

Fig. 2. Chemotaxis monitoring. Cells were seeded in a KK-chamber. PDGF-D (4 ng) was applied into a hole located on the opposite side of the chamber, and cell mobilizations were monitored for 20 min. Cells shown here are NCIH-2052 cells. Note that cells move towards PDGF-D at 20 min.

Fig. 3. PDGF-D-induced facilitation of malignant mesothelioma cell chemotaxis as mediated via PDGF-β receptors. (A) Cells as indicated were transfected with the NC siRNA [PDGF-βR siRNA (-)] or the PDGF-βR siRNA [PDGF-βR siRNA (+)], and RT-PCR was carried out (n=3 independent experiments). Note that no signal band for the PDGF receptor β subunit mRNA is visible in cells transfected with the PDGF-βR siRNA. (B) Chemotaxis assay was carried out in cells transfected with the NC siRNA or the PDGF-βR siRNA by applying PDGF-D (4 ng). In the graph, each value represents the mean (± SEM) velocity (n=15 independent experiments). *P* values, unpaired *t*-test.

for all the non-malignant and malignant mesothelioma cells, and the effect was significantly inhibited by knocking-down PDGF-β receptors (Fig. 5A-E), indicating that PDGF-D activates ERK1 in mesothelioma cells via PDGF-β receptors. This also suggests that ERK1 is

Fig. 4. Schematic diagram for PDGF-D/PDGF-β receptor signaling pathways. MMC, malignant mesothelioma cell.

activated as a downstream effector of MEK in a PDGF-β receptor/Ras/Raf/MEKK/MEK/MAPK pathway.

Our final attempt was to see whether malignant mesothelioma cell chemotaxis is regulated through those pathways. PDGF-D promoted migration of all the malignant mesothelioma cells used here, with the different potency among cell types, and the effect was abolished by the PI3 kinase inhibitor wortmannin, although PDGF-D had no effect on Met5A cell migration in the presence and absence of wortmannin (Fig. 6A). This indicates the participation of PI3 kinase, a downstream effector of PDGF-β receptor, in PDGF-D-engaged regulation of malignant mesothelioma cell migration.

PDGF-D-induced facilitation of malignant mesothelioma cell migration was clearly prevented by the PDK1 inhibitor BX912, while Met5A cell migration was not affected by PDGF-D in the presence and absence of BX912 (Fig. 6B). This indicates the participation of PDK1, a downstream effector of PI3 kinase, in PDGF-D-engaged regulation of malignant mesothelioma cell chemotaxis.

PDGF-D-induced facilitation of malignant mesothelioma cell migration was significantly inhibited by

Fig. 5. PDGF-D-induced activation of Akt and ERK in mesothelioma cells. Western blotting was carried out in Met5A (A), MSTO-211H (B), NCIH-H2052 (C), NCIH-H2452 (D), and NCIH-28 cells (E), transfected with the NC siRNA (NC) or the PDGF- β R siRNA (PDGF- β R KD) before and after 10-min treatment with PDGF-D (40 ng/ml). In the blotting membranes shown, arrows indicate 44 kDa of ERK, corresponding to ERK1. In the graphs, expression levels for each protein were normalized by β -actin. Each value represents the mean ($\pm$ SEM) ratio of phosphorylated Akt (p-Akt) against total amount of Akt or the phosphorylated ERK (p-ERK) against total amount of ERK (n=4 independent experiments). Note that each value before treatment with PDGF-D is regarded as 1. *P* values, Dunnett's test.

the Akt inhibitor MK2206, while Met5A cell migration was not affected by PDGF-D in the presence and absence of MK2206 (Fig. 6C). This indicates the participation of

Akt, a downstream effector of PDK1, in PDGF-D-engaged regulation of malignant mesothelioma cell chemotaxis.

Fig. 6. PDGF-D-induced facilitation of malignant mesothelioma cell chemotaxis through a PDGF- β receptor signaling pathway along a PI3 kinase/PDK1/Akt/Rac1/ROCK axis or by activating ERK1. Chemotaxis assay was carried out in cells as indicated by applying PDGF-D (4 ng) in the presence and absence of wortmanin (50 nmol)(A), BX912 (10 nmol)(B), MK2206 (5 nmol)(C), NSC23766 (100 nmol)(D), Y27632 (10 nmol)(E) or PD98059 (1 pmol) (F). In the graph, each value represents the mean ($\pm$ SEM) velocity ($n=15$ independent experiments). P values, unpaired t -test.

PDGF-D-induced facilitation of malignant mesothelioma cell migration was significantly attenuated by the Rac1 inhibitor NSC23766, while Met5A cell migration was not affected by PDGF-D in the presence and absence of NSC23766 (Fig. 6D). This indicates the

participation of Rac1, a downstream effector of Akt, in PDGF-D-engaged regulation of malignant mesothelioma cell chemotaxis.

PDGF-D-induced facilitation of malignant mesothelioma cell migration was significantly suppressed

by the ROCK inhibitor Y27632, while Met5A cell migration was not affected by PDGF-D in the presence and absence of Y27632 (Fig. 6E). This indicates the participation of ROCK, a downstream effector of Rac1, in PDGF-D-engaged regulation of malignant mesothelioma cell chemotaxis.

PDGF-D-induced facilitation of malignant mesothelioma cell migration, alternatively, was significantly suppressed by the MEK1 inhibitor PD98059, while Met5A cell migration was not affected by PDGF-D in the presence and absence of PD98059 (Fig. 6F). This indicates that ERK1 still participates in PDGF-D-engaged regulation of malignant mesothelioma cell chemotaxis.

Overall, these results lead to a conclusion that PDGF-D promotes malignant mesothelioma cell chemotaxis as mediated via PDGF- $\beta\beta$ receptors linked to activation of PI3 kinase/PDK1/Akt/ Rac1/ROCK and ERK1.

Discussion

Chemotaxis, cell motility directed towards higher concentrations of chemoattractants, plays a critical role in invasion and metastasis of cancers [25]. The motility is regulated according to gradients of chemoattractants such as chemokines and growth factors, that activate chemokine receptors and receptor tyrosine kinases, respectively. Malignant mesothelioma cells are recognized to progressively invade into the peritoneum or the pleura. Several avenues of studies have suggested the implication of PDGF, a growth factor, in metastasis of a variety of cancers, but little is known about the effect on malignant mesothelioma cells.

In our earlier study, FBS facilitated malignant mesothelioma cell migration and the effect was inhibited by the uPA inhibitor UTI [24]. This suggests that FBS stimulates secretion of PDGF-D, that is converted to active dimer of PDGF-DD through uPA-mediated proteolytic processing, thereby activating PDGF- $\beta\beta$ receptors bearing facilitation of malignant mesothelioma cell chemotaxis. In the present study, FBS significantly increased the amount of extracellular PDGF-D for malignant mesothelioma cells such MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells. FBS also increased the amount of extracellular PDGF-D for non-malignant Met5A cells, but not significantly. This provides evidence for FBS-induced PDGF-D secretion from mesothelioma cells, with the extent being greater for malignant mesothelioma cells than that for non-malignant

mesothelioma cells. This also suggests that malignant mesothelioma cells favor an autocrine activation of PDGF- $\beta\beta$ receptors.

PDGF- $\beta\beta$ receptor is a receptor tyrosine kinase. For a PDGF- $\beta\beta$ receptor signaling pathway, activation of PDGF- $\beta\beta$ receptor by PDGF-D, i.e., activation of tyrosine kinase, phosphorylates insulin substrate protein (IRS)-1/-2. Phosphorylated IRS-1/-2, in turn, recruits and activates PI3 kinase, to produce phosphatidylinositol (3,4,5)-trisphosphate [PI (3,4,5) P_3] from phosphatidylinositol (4,5)-biphosphate [PI (4,5) P_2]. PI (3,4,5) P_3 binds to and activates PDK1, which phosphorylates and activates Akt. Activated Akt activates the small G-protein Rac1/Cdc42 followed by activation of the effector ROCK. For another PDGF- $\beta\beta$ receptor signaling pathway, activation of PDGF- $\beta\beta$ receptor by PDGF-D phosphorylates Shc2, to recruit and associate Grb2 and SOS, a guanine nucleotide exchange factor for Ras, thereby activating Ras. Activated Ras activates Raf, a serine/threonine protein kinase. Activated Raf phosphorylates and activates MEKK, a serine/threonine protein kinase, followed by phosphorylation and activation of MEK, a serine/threonine protein kinase, to phosphorylate and activate MAPK, a serine/threonine protein kinase. In the present study, PDGF-D increased phosphorylated Akt, i.e., the active form of Akt, and phosphorylated ERK1, the active form of ERK1, in all the non-malignant and malignant mesothelioma cells used here, and those effects were inhibited by knocking-down PDGF- $\beta\beta$ receptor. This suggests that PDGF- $\beta\beta$ receptor is capable of activating ROCK along a PI3 kinase/PDK1/Akt/Rac1 (Cdc42) axis and activating MAPK along a Ras/Raf/MEKK/MEK axis.

In the chemotactic assay, PDGF-D facilitated migration of all the malignant mesothelioma cells used here, MSTO-211H, NCIH-2052, NCIH-2452, and NCIH-28 cells, with the potency varying among cell types, while the facilitatory effect was not obtained with non-malignant Met5A cells. This confirms that PDGF-D serves as a chemoattractant for malignant mesothelioma cell chemotaxis. If this is true, then PDGF-D might facilitate malignant mesothelioma cell chemotaxis by activating PDGF- $\beta\beta$ receptors. PDGF-D-induced facilitation of malignant mesothelioma cell migration was abolished by knocking-down PDGF- $\beta\beta$ receptor, providing direct evidence for the implication of PDGF- $\beta\beta$ receptor in the facilitatory action of PDGF-D on malignant mesothelioma cell chemotaxis.

PDGF-D-induced facilitation of malignant mesothelioma cell migration was clearly prevented by the

PI3 kinase inhibitor wortmannin, the PDK1 inhibitor BX912, the Akt inhibitor MK2206, the Rac1 inhibitor NSC23766, or the ROCK inhibitor Y27632. This indicates that PDGF-D promotes malignant mesothelioma cell chemotaxis through a PDGF- $\beta\beta$ receptor signaling pathway along a PI3 kinase/PDK1/Akt/Rac1/ROCK axis. PDGF-D-induced facilitation of malignant mesothelioma cell migration, on the other hand, was still inhibited by the MEK1 inhibitor PD98059, suggesting that PDGF-D promotes malignant mesothelioma cell chemotaxis through another PDGF- $\beta\beta$ receptor signaling pathway along a Ras/Raf/MEKK/MEK/MAPK axis.

Several avenues of studies have provided direct and indirect evidence for PDGF-D/PDGF- $\beta\beta$ receptor as a mediator for development, progression, and metastasis in a variety of cancer cells [15-23]. Imatinib mesylate, an inhibitor of receptors tyrosine kinases including PDGF- $\beta\beta$ receptor, has been attempted for treatment of malignant

mesothelioma as well as other cancers. Unfortunately, clinical trials with imatinib mesylate show limited efficacy in malignant mesothelioma, while the drug induces cytotoxicity and apoptosis selectively on PDGF- $\beta\beta$ receptor-positive mesothelioma cells [26-28]. The results presented here point to the critical role of PDGF-D/PDGF- $\beta\beta$ receptor in malignant mesothelioma cell chemotaxis. This may represent the mechanism underlying rapid and aggressive invasion of malignant mesothelioma cells into the peritoneum or the pleura. PDGF-D/PDGF- $\beta\beta$ signaling cascades, accordingly, could be a promising target of malignant mesothelioma therapy, even though imatinib mesylate currently exhibits less beneficial effect in clinical trials.

In conclusion, the results of the present study show that PDGF-D activates PDGF- $\beta\beta$ receptors in an autocrine manner, involving activation of PI3 kinase/PDK1/Akt/Rac1/ROCK and MAPK, to promote malignant mesothelioma cell chemotaxis.

References

- Nakano T: Malignant mesothelioma-diagnosis and treatment strategies. *Gan To Kagaku Ryoho* 2006;33:1215-1220.
- Garlepp MJ, Leong CC: Biological and immunological aspects of malignant mesothelioma. *Eur Respir J* 1995;8:643-650.
- Upham JW, Garlepp MJ, Musk AW, Robinson BW: Malignant mesothelioma: new insights into tumour biology and immunology as a basis for new treatment approaches. *Thorax* 1995;50:887-893.
- Halper J: Growth factors as active participants in carcinogenesis: a perspective. *Vet Pathol* 2010;47:77-97.
- Filiberti R, Marroni P, Neri M, Ardizzoni A, Betta PG, Cafferata MA, Canessa PA, Puntoni R, Ivaldi GP, Paganuzzi M: Serum PDGF-AB in pleural mesothelioma. *Tumour Biol* 2005;26:221-226.
- Versnel MA, Hagemeijer A, Bouts MJ, van der Kwast TH, Hoogsteden HC: Expression of *c-sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* 1988;2:601-605.
- Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC: Comparison of production of transforming growth factor- β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. *Cancer Res* 1987;47:6180-6184.
- Van der Meeren A, Seddon MB, Betsholtz CA, Lechner JF, Gerwin BI: Tumorigenic conversion of human mesothelial cells as a consequence of platelet-derived growth factor-A chain overexpression. *Am J Respir Cell Mol Biol* 1993;8:214-221.
- Metheny-Barlow LJ, Flynn B, van Gijssel HE, Marrogi A, Gerwin BI: Paradoxical effects of platelet-derived growth factor-A overexpression in malignant mesothelioma. Antiproliferative effects in vitro and tumorigenic stimulation in vivo. *Am J Respir Cell Mol Biol* 2001;24:694-702.
- Klominek J, Baskin B, Hauzenberger D: Platelet-derived growth factor (PDGF) BB acts as a chemoattractant for human malignant mesothelioma cells via PDGF receptor β -integrin $\alpha 3\beta 1$ interaction. *Clin Exp Metastasis* 1998;16:529-539.
- Zhong J, Gencay MM, Bubendorf L, Burgess JK, Parson H, Robinson BW, Tamm M, Black JL, Roth M: ERK1/2 and p38 MAP kinase control MMP-2, MT1-MMP, and TIMP action and affect cell migration: a comparison between mesothelioma and mesothelial cells. *J Cell Physiol* 2006;207:540-552.
- Alvarez RH, Kantarjian HM, Cortes JE: Biology of platelet-derived growth factor and its involvement in disease. *Mayo Clin Proc* 2006;81:1241-1257.
- Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U: PDGF-D is a specific, protease-activated ligand for the PDGF β -receptor. *Nat Cell Biol* 2001;3:512-516.

- 14 Fredriksson L, Ehnman M, Fieber C, Eriksson U: Structural requirements for activation of latent platelet-derived growth factor CC by tissue plasminogen activator. *J Biol Chem* 2005;280:26856-26862.
- 15 Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, Sarkar FH: Emerging roles of PDGF-D signaling pathway in tumor development and progression. *Biochim Biophys Acta* 2010;1806:122-130.
- 16 Ustach CV, Taube ME, Hurst NJ Jr, Bhagat S, Bonfil RD, Cher ML, Schuger L, Kim HR: A potential oncogenic activity of platelet-derived growth factor D in prostate cancer progression. *Cancer Res* 2004;64:1722-1729.
- 17 Ustach CV, Kim HR: Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. *Mol Cell Biol* 2005;25:6279-6288.
- 18 Kobayashi H: Suppression of urokinase expression and tumor metastasis by bikunin overexpression. *Hum Cell* 2001;14:233-236.
- 19 Kobayashi H, Gotoh J, Kanayama N, Hirashima Y, Terao T, Sugino D: Inhibition of tumor cell invasion through matrigel by a peptide derived from the domain II region in urinary trypsin inhibition. *Cancer Res* 1995;55:1847-1852.
- 20 Kobayashi H, Shinohara H, Gotoh J, Fujie M, Fujishiro S, Terao T: Anti-metastatic therapy by urinary trypsin inhibitor in combination with an anti-cancer agent. *Br J Cancer* 1995;72:1131-1137.
- 21 Kobayashi H, Shinohara H, Takeuchi K, Itoh M, Fujie M, Saitoh M, Terao T: Inhibition of the soluble and the tumor cell receptor-bound plasmin by urinary trypsin inhibitor and subsequent effects on tumor cell invasion and metastasis. *Cancer Res* 1994;54:844-849.
- 22 Kobayashi H, Suzuki M, Sun GW, Hirashima Y, Terao T: Suppression of urokinase-type plasminogen activator expression from human ovarian cancer cells by urinary trypsin inhibitor. *Biochim Biophys Acta* 2000;1481:310-316.
- 23 Yoshioka I, Tsuchiya Y, Aozuka Y, Onishi Y, Sakurai H, Koizumi K, Tsukada K, Saiki I: Urinary trypsin inhibitor suppresses surgical stress-facilitated lung metastasis of murine colon 26-L5 carcinoma cells. *Anticancer Res* 2005;25:815-820.
- 24 Yaguchi T, Muramoto M, Nakano T, Nishizaki T: Urinary trypsin inhibitor suppresses migration of malignant mesothelioma. *Cancer Lett* 2010;288:214-218.
- 25 Condeelis J, Singer RH, Segall JE: The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annu Rev Cell Dev Biol* 2005;21:695-718.
- 26 Bertino P, Porta C, Barbone D, Germano S, Busacca S, Pinato S, Tassi G, Favoni R, Gaudino G, Mutti L: Preliminary data suggestive of a novel translational approach to mesothelioma treatment: imatinib mesylate with gemcitabine or pemetrexed. *Thorax* 2007;62:690-695.
- 27 Barbieri F, Würth R, Favoni RE, Pattarozzi A, Gatti M, Ratto A, Ferrari A, Bajetto A, Florio T: Receptor tyrosine kinase inhibitors and cytotoxic drugs affect pleural mesothelioma cell proliferation: insight into EGFR and ERK1/2 as antitumor targets. *Biochem Pharmacol* 2011;82:1467-1477.
- 28 Zucali PA, Ceresoli GL, De Vincenzo F, Simonelli M, Lorenzi E, Gianoncelli L, Santoro A: Advances in the biology of malignant pleural mesothelioma. *Cancer Treat Rev* 2011;37:543-558.