Mesothelioma Cell Proliferation through Autocrine Activation of PDGF-ββ Receptor

Miki Honda1,2*, Takeshi Kanno1*, Yumiko Fujita1,2, Akinobu Gotoh3, Takashi Nakano2 and Tomoyuki Nishizaki1

1Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, Nishinomiya, 2Department of Thoracic Oncology, Hyogo College of Medicine, Nishinomiya, 3Laboratory of Cell and Gene Therapy, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Nishinomiya, *M. Honda and T. Kanno contributed equally to this work

Key Words
Malignant mesothelioma cell • Proliferation • PDGF-D • PDGF-ββ receptor

Abstract
Background/Aims: Growth factors play a critical role in proliferation for a variety of cancer cells. The present study was conducted to understand the signaling cascades underlying PDGF-D/PDGF-ββ receptor-mediated proliferation of mesothelioma cells.

Methods: Cell growth and cell cycle were analyzed in human non-malignant Met5A cells and malignant mesothelioma cells such as MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells. Results: Growth of all the cells used here was not affected by PDGF-D, regardless of concentrations (1-30 ng/ml) or treatment time (48-72 h). Spontaneous growth of those cells was significantly inhibited by knocking-down PDGF-D or PDGF-ββ receptor, without affecting cell cycling. The cell growth was significantly inhibited by the Akt inhibitor MK2206 and the ROCK inhibitor Y27632 for all the cell types, by the PDK1 inhibitor BX912 for NCI-H28 cells alone, and by the Rac1 inhibitor NSC23766 for NCI-H2052 cells alone, while the PI3 kinase inhibitor wortmannin had no effect. The cell growth, alternatively, was significantly attenuated by MAP kinase inhibitor PD98059 or the ERK1/2 inhibitor FR180204 for all the cell types. Conclusion: The results of the present study show that PDGF-D promotes mesothelioma cell proliferation by targeting ROCK or MAP kinase through autocrine activation of PDGF-ββ receptor.

Introduction
Malignant mesothelioma is an aggressive and highly lethal tumor, which is caused by occupational exposure to asbestos fibres, particularly of the amphibole type [1]. Invasion into the mesothelium of the pleural cavity and/or the peritoneal cavity is commonly found with this tumor [1]. As is the case with other type of carcinoma cells, growth factors including platelet-derived growth factor (PDGF) serve as a pivotal mediator for proliferation of malignant mesothelioma cells too [2]. Lines of evidence have shown that PDGF, extracellularly secreted, stimulates malignant mesothelioma cell proliferation [3-8].
Of the PDGF family such as PDGF-A, -B, -C and -D, PDGF-A and -B are secreted as active dimers composed of single-domain protein chains (PDGF-AA and -BB). In contrast, PDGF-C and -D, which contain 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 the active form of PDGF-CC and -DD [9-11]. The serine protease tissue plasminogen activator (tPA) is a potent activator of latent dimeric PDGF-CC and the closely related protease urinary plasminogen activator (uPA), that is inhibited by plasminogen activator inhibitor-1 (PAI-1), activates latent PDGF-DD [12-14] (Fig. 1).

Fig. 1. Schematic regulatory pathway for production of the active form of PDGF-D.

The present study investigated PDGF-D/PDGF-β receptor-mediated proliferation of human non-malignant Met5A cells and malignant mesothelioma cells such as MSTO-211H, NC1-H28, NC1-H2052, and NC1-H2452 cells. We show here that mesothelioma cell proliferation is regulated via a PDGF-D/PDGF-β receptor pathway.

Materials and Methods

Cell culture
Met5A, MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.003% (w/v) 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.

Cell viability
Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [16]. MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA, USA).

Construction and transfection of small interfering RNA (siRNA)
The siRNAs silencing the PDGF-D-targeted gene (PDGF-D siRNA) or the PDGF receptor β subunit-targeted gene (PDGF-βR siRNA) were obtained from Cosmo Bio (Kyoto, Japan). The sequences of siRNAs used here were as follows: sense, 5’-CCA UCA AAG CUU UGC GCA ATT-3’ and anti-sense, 5’-UUG CGC AAA GCU UUG AUG GTT-3’; sense, 5’-GGA AGU UCC AAG GAU ACC UUG AGG AAC UUC UCC CTT-3’ and anti-sense, 5’-AAU GGC UCA GGC CAU GAG CTT-3’ for PDGF-D; sense, 5’-GGA AUG AUG UUG UCA AUG ACC GCC ATT-3’ and anti-sense, 5’-CUU CGG UCA UUG UAU GAC CTT-3’ for PDGF-βR; sense, 5’-GCC GAU GCC CUG AGC GAG CAU UTT-3’ and anti-sense, 5’-GAU GCC UGC AGA CAA GAA GTG AAG CTT-3’ for PDGF-D; sense, 5’-GGA AUG AGU UCC AUG UGC UAC CTT-3’ and anti-sense, 5’-CUU CGG UCA UUG UAU GAC CTT-3’; sense, 5’-GCU GAU GCC CUG AGC GAG CAU UTT-3’ and anti-sense, 5’-AAU GCC UGC AGA CAA GAA GTG AAG CTT-3’; and sense, 5’-GAG AGC UGC CGA GCA ATT-3’ and anti-sense, 5’-UGG CUC GGC AGG ACC UCC UCC CTT-3’ for PDGF-βR, respectively.
receptor. Negative control siRNA (NC siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PDGF-D siRNA, the PDGF-Rβ siRNA, and the NC siRNA were reverse-transfected into cells using a Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were purified from cells, transfected with the NC siRNA, the PDGF-D siRNA or the PDGF-Rβ siRNA, by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit (Nacalai, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37 °C for 30 min to remove genomic DNAs, and 10 μg of RNAs were resuspended in water. Then, oligo dT primers, dNTPs, 5x 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 μl of the reaction solution was diluted with water and mixed with 10x 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 μl). 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: 5’-TAA TGT ATC TTG CTT TTG TGT CGT CCT TTT-3’ and 5’-CCT TTA AAA CAG ACA CAA AGG GTG CAT TCC CAG GTT CTC TA-3’ for GAPDH. PCR products were stained with ethidium bromide and visualized by 2% (w/v) agarose gel electrophoresis.

Real-time RT-PCR

Total RNAs of cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37 °C for 30 min to remove genomic DNAs, and 10 μg of RNAs was resuspended in water. Then, random primers, dNTPs, 10x RT buffer, and Multiscribe Reverse Transcriptase were added to an RNA solution and incubated at 25 °C for 10 min followed by 37 °C for 120 min to synthesize the first-strand cDNA. Real-time RT-PCR was performed using a SYBR Green Realtime PCR Master Mix (Takara Bio, Otsu, Japan) and the Applied Biosystems 7900 real-time PCR detection system. Thermal cycling conditions were as follows: first step, 94 °C for 4 min; the ensuing 40 cycles, 94 °C for 1 s, 62 °C for 15 s, and 72 °C for 30 s. The expressed level of each mRNA was normalized by that of GAPDH mRNA. Primers used for real-time RT-PCR are 5’-GCC CTG CCT GCC CTG GAA C-3’ and 5’-AGC ATG TAG TCC TCC TTC TTT GGG TAA TC-3’ for uPA; 5’-CCC ACC GCC GCC TCT TCC-3’ and 5’-CAT GTC GGT CAT TCC CAG GTT CTC TA-3’ for PAI-1; and 5’-GAC TTC AAC AGC GAC ACC CAC TCC-3’ and 5’-AGG TCC ACC ACC CTG TTG CTG TAG-3’ for GAPDH.

Cell cycle analysis

Cells were harvested by a trypsinization 48 h after transfection with the NC siRNA or the PDGF-Rβ siRNA, fixed with 70% (v/v) ethanol at 4 °C overnight. Fixed cells were incubated in phosphate-buffered saline containing 1.5 μg/ml RNase A for 1 h at 37 °C, followed by staining with 5 μl of propidium iodide (PI) for 20 min on ice. Then, cells were collected on a nylon mesh filter (pore size, 40 μm), and cell cycles were assayed using a flow cytometer (FACS Calibur, Becton Dickinson, USA) at an excitation of 488 nm and an emission of 585 nm, and analyzed using a Mod Fit LT software (Verity Software House Inc., Topsham, USA).

Statistical analysis

Statistical analysis was carried out using Dunnett’s test.

Results

Mesothelioma cell growth in a PDGF-D- and PDGF-β receptor-dependent manner

Growth of non-malignant Met5A cells and malignant MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells was not affected by 48-h treatment with exogenous PDGF-D at concentrations ranging from 1 to 30 ng/ml (Fig. 2A) or treatment with exogenous PDGF-D at 10 ng/ml for 48-72 h (Fig. 2B).

To examine whether endogenous PDGF-D regulates mesothelioma cell growth, the PDGF-D siRNA was constructed and transfected into cells. Expression of the PDGF-D mRNA for cells transfected with the PDGF-D siRNA was clearly suppressed as compared with the NC siRNA (Fig. 3A), confirming PDGF-D knock-down. For all the cell types used here, growth of cells transfected with the PDGF-D siRNA was significantly inhibited as compared with cells transfected with the NC siRNA (Fig. 3A). This suggests regulation of mesothelioma cell proliferation by endogenous PDGF-D.

If this is true, then PDGF-β receptor, that is activated by the active form of PDGF-D, should be implicated in the regulation of mesothelioma cell proliferation. To address this point, the PDGF-β siRNA was constructed and transfected into cells. Expression of the PDGF receptor-β subunit mRNA for cells transfected with the PDGF-β siRNA was abrogated (Fig. 3B), confirming PDGF-β receptor knock-down. Expectedly, spontaneous cell growth for all the cell types

Mesothelioma Cell Proliferation via PDGF-β Receptor
was significantly attenuated by knocking-down PDGF-ββ receptor (Fig. 3B). In contrast, each phase of cell cycling for all the cell types was not influenced by knocking-down PDGF-ββ receptor (Fig. 4). Taken together, these results indicate that endogenous PDGF-D promotes mesothelioma cell proliferation by activating PDGF-ββ receptor, without affecting cell cycle.

**Mesothelioma cell proliferation under the control of ROCK and MAP kinase**

For all the cell types examined here, spontaneous cell growth was significantly inhibited by MK2206 (5 μM), an inhibitor of Akt (Fig. 5C), or Y27632 (10 μM), an inhibitor of ROCK, with the highest potential (Fig. 5E). BX912 (100 nM), an inhibitor of PDK1, and NSC23766 (1 μM), an inhibitor of Rac1, significantly attenuated the
Fig. 4. The effect of PDGF-ββ receptor knock-down on cell cycling. Met5A (A), MSTO-211H (MSTO)(B), NCI-H28(H28)(C), NCI-H2052 (H2052)(D), and NCI-H2452 cells (H2452)(E) were transfected with the NC siRNA or the PDGF-βR siRNA, and 96 h later cell cycle analysis was carried out. Typical profiles are shown in the left panel. In the graphs, each column represents the mean (± SEM) percentage for phases of cell cycling (n=4 independent experiments).

cell growth for NCI-H28 cells alone (Fig. 5B) and for NCI-H2052 cells alone (Fig. 5D), respectively, but wortmannin (10 μM), an inhibitor of PI3 kinase, had no effect (Fig. 5A). This indicates that spontaneous mesothelioma cell proliferation is regulated by ROCK, a signaling cascade downstream PDGF-ββ receptor. This also suggests that ROCK is unlikely activated via a well-recognized pathway along a PI3 kinase/PDK1/
Fig. 5. The effect of inhibitors for PI3 kinase, PDK1, Akt, Rac1, and ROCK on mesothelioma cell proliferation. Cells as indicated were incubated in the absence (Control) and presence of wortmannin (10 μM), BX912 (100 nM), MK2206 (5 μM), NSC23766 (1 μM), or Y27632 (10 μM) for 48 h, and then, MTT assay was carried out. In the graph, each column represents the mean (± SEM) percentage of control (MTT intensities in the absence of inhibitors)(n=6 independent experiments). *P<0.01; **P<0.001; ***P<0.0001 as compared with control, Dunnett’s test.

Fig. 6. The effect of inhibitors for MEK and ERK1/2 on mesothelioma cell proliferation. Cells as indicated were incubated in the absence (Control) and presence of PD98059 (50 μM), or FR180204 (10 μM) for 48 h, and then, MTT assay was carried out. In the graph, each column represents the mean (± SEM) percentage of control (MTT intensities in the absence of inhibitors)(n=6 independent experiments). *P<0.01; **P<0.001; ***P<0.0001 as compared with control, Dunnett’s test.

Fig. 7. Putative PDGF-D/PDGFB receptor signaling pathways for mesothelioma cell proliferation. MEKK, MEK kinase; MAPK, MAP kinase.

Akt/Rac1 (Cdc42)/ROCK axis linked to PDGF-ββ receptor (Fig. 7).

The cell growth, on the other hand, was significantly prevented by PD98059 (50 μM), an inhibitor of MEK (Fig. 6A), or FR180204 (10 μM), an inhibitor of the MAP kinase ERK1/2, for all the cell types (Fig. 6B). This indicates that MEK/ERK also participates in spontaneous mesothelioma cell proliferation, although it has not been examined yet whether those kinases are activated via a pathway along a Ras/Raf/MEKK/MEK/MAP kinase downstream PDGF-ββ receptor (Fig. 7).

Higher expression of the uPA mRNA in malignant mesothelioma cells

uPA produces the active form of PDGF-D to activate PDGF-ββ receptor through proteolytic processing, but PAI-1 inhibits the uPA action (Fig. 1). Expression of the uPA mRNA in malignant mesothelioma cells such as
Fig. 8. Expression of mRNAs for uPA (A) and PAI-1 (B). Real-time RT-PCR was carried out in Met5A, MSTO-211H (MSTO), NCI-H28 (H28), NCI-H2052 (H2052), and NCI-H2452 cells (H2452). In the graphs, each column represents the mean (± SEM) ratio against mRNA intensities for Met5A cells (n=4 independent experiments).

MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells was much higher than the expression in non-malignant Met5A cells (Fig. 8A). In contrast, there was no big difference in the expression of the PAI-1 mRNA between malignant and non-malignant mesothelioma cells (Fig. 8B). In our earlier study, expression of PDGF-β receptor in malignant mesothelioma cells was significantly higher than the expression in Met5A cells, although no difference in PDGF-D expression was found between them [15]. Collectively, these results suggest that the active form of PDGF-D, to activate PDGF-β receptor, is produced more in malignant mesothelioma cells than in non-malignant Met5A cells.

Discussion

In the present study, exogenous PDGF-D had no effect on proliferation for Met5A non-malignant mesothelioma cells and malignant mesothelioma cells such as MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells, regardless of its concentrations and its treatment time. Amazingly, spontaneous cell growth was inhibited by knocking-down PDGF-D or PDGF-β receptor for all the cell types examined here. In contrast, cell cycling for all the cell types was not altered by knocking-down PDGF-β receptor. It is indicated from these results that endogenous PDGF-D, extracellularly secreted, promotes mesothelioma cell proliferation by activating PDGF-β receptor, without affecting cell cycling. This also suggests that PDGF-ββ receptor in mesothelioma cells is sufficiently activated to the maximal levels by endogenous PDGF-D, i.e., no further activation of the receptor is obtained with exogenously applied PDGF-D.

PDGF-ββ receptor engages two major signaling pathways; a pathway along a PI3 kinase/PDK1/Akt/Rac1 (Cdc42)/ROCK axis and another along a Ras/Raf/MEK/MEK/MAP kinase axis (Fig. 7). For all the cell types, spontaneous cell growth was prevented by the Akt inhibitor MK2206, to a smaller extent except for NCI-H2052 cells, or the ROCK inhibitor Y27632, to a greater extent. Then, one would think that ROCK is activated via the former pathway downstream PDGF-β receptor. An unexpected result, however, was that no inhibition of the cell growth for all the cell types was obtained with the PI3 kinase inhibitor wortmannin. This denies the participation of PI3 kinase at the initial entrance for a pathway along a PI3 kinase/PDK1/Akt/Rac1 (Cdc42)/ROCK axis. Furthermore, the PDK1 inhibitor BX912 suppressed the cell growth for NCI-H28 cells alone or the Rac1 inhibitor NSC23766 attenuated it for NCI-H2052 cells alone. Overall, these results indicate that ROCK as a downstream target of PDGF-β receptor plays a crucial role in spontaneous mesothelioma cell proliferation but that ROCK may be activated via a pathway independent of a PI3 kinase/PDK1/Akt/Rac1 (Cdc42) axis, i.e., via an as of yet unknown pathway.

Spontaneous mesothelioma cell growth, alternatively, was significantly prevented by the MEK inhibitor PD98059 or the ERK1/2 inhibitor FR180204 for all the cell types. This suggests that MEK/MAP kinase is another downstream target of PDGF-ββ receptor responsible for spontaneous mesothelioma cell proliferation, although the implication of Ras, Raf, and MEKK in the activation of MEK and MAP kinase has not been ascertained here. How ROCK is activated in mesothelioma cells also remains to be explored. A plausible explanation for this is that ROCK might be activated by a MAP kinase cascade, regardless of PI3 kinase, PDK1, Akt, or Rac1 (Cdc42). To address this question, we are currently attempting further experiments.

In the present study, malignant mesothelioma cells more highly expressed the uPA mRNA than non-malignant Met5A cells, while there was no difference in the expression of the PAI-1 mRNA between malignant and non-malignant mesothelioma cells. This, taken together with the fact that much higher expression of PDGF-ββ receptor is found in malignant mesothelioma cells as compared with in non-malignant mesothelioma cells, with
no difference in the expression of PDGF-D between them [15], raises the possibility that uPA-mediated production of the active form of PDGF-D from the inactive form of PDGF-D in malignant mesothelioma cells is greater than in non-malignant mesothelioma cells, to endogenously activate PDGF-β receptor. The expression levels of uPA, therefore, may be a critical index to determine malignancy for mesothelioma cells.

In conclusion, the results of the present study show that PDGF-D promotes mesothelioma cell proliferation by activating ROCK via a pathway independent of PI3 kinase/PDK1/Akt/Rac1 (Cdc42) axis or MAP kinase, possibly along a Ras/Raf/MEKK/MEK axis, through autocrine activation of PDGF-β receptor. The former may represent a novel pathway for mesothelioma cell proliferation.

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