Full Paper

Inhibitory Effects of Docetaxel on Platelet-Derived Growth Factor (PDGF)-BB–Induced Proliferation of Vascular Smooth Muscle Cells Through Blocking PDGF-Receptor β Phosphorylation

Eun Seok Park¹, Jae-Myung Yoo², Yong Lim³, Munkhtsetseg Tudev¹, Hwan-Soo Yoo¹, Jin Tae Hong^{1,4}, and Yeo-Pyo Yun^{1,*}

¹College of Pharmacy, CBITRC, ⁴College of Pharmacy and Medical Research Center, Chungbuk National University, Cheongju 361-763, Korea

²College of Pharmacy, Chungnam National University, Taejeon 305-764, Korea

³Department of Clinical Laboratory Science, Dong-eui University, Busan 305-333, Korea

Received October 26, 2010; Accepted April 10, 2011

Abstract. The abnormal proliferation of vascular smooth muscle cells (VSMCs) in arterial wall is an important pathogenic factor for vascular disorders such as atherosclerosis and restenosis after angioplasty. The present study was designed to investigate the inhibitory effects of docetaxel on VSMC proliferation, as well as the molecular mechanism of this inhibition. Docetaxel at 10, 20 and 40 μ M significantly inhibited both the proliferation and the DNA synthesis of fetal bovine serum (FBS)- and platelet-derived growth factor (PDGF)-BB–stimulated VSMCs in a concentration-dependent manner. In accordance with these findings, docetaxel blocked the FBS- and PDGF-BB–induced progression of synchronized cells through the G0/G1 phase of the cell cycle. Docetaxel also decreased the expressions of cell cycle-related proteins, including cyclin-dependent kinase (CDK) 2, cyclin E, CDK4, cyclin D1, retinoblastoma protein, and proliferative cell nuclear antigen in PDGF-BB–stimulated VSMCs. Docetaxel significantly inhibited the phosphorylation of extracellular signal-regulated kinase 1/2, Akt, and phospholipase C- γ 1, downstream molecule in the PDGF-BB signaling pathway. Docetaxel suppressed the phosphorylation of PDGF receptor (PDGF-R) β , the upstream molecule in PDGF-BB signaling cascade, suggesting that the inhibitory effect of docetaxel on the proliferation of VSMCs may occur by blocking PDGF-R β phosphorylation. Thus, docetaxel may be a potential antiproliferative agent for the treatment of atherosclerosis and angioplasty restenosis.

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.10276FP>]

Keywords: docetaxel, vascular smooth muscle cell, platelet-derived growth factor receptor β (PDGF-R β), proliferation

Introduction

Cardiovascular diseases are associated with a multitude of pathophysiological conditions such as inflammation, pulmonary hypertension, and coronary artery restenosis following balloon angioplasty (1). In a normal artery, vascular smooth muscle cells (VSMCs) are in a non-proliferative quiescent state and express a differenti-

ated contractile phenotype. After a vascular injury occurs, VSMCs dedifferentiate and re-enter the cell cycle. Injuries that induce this change include direct physical trauma, angioplasty, hypertension, and shear stress. This dedifferentiated phenotype plays a major pathophysiologic role in the development of atherosclerosis, restenosis after angioplasty or bypass, and hypertension (2). Under the influence of growth factors and cytokines, VSMCs adapt to a synthetic phenotype, commence proliferation, and then migrate across the intimal layer.

Although many growth factors and cytokine are involved in the proliferation of VSMCs, one of the principal

*Corresponding author. ypyun@chungbuk.ac.kr

Published online in J-STAGE

doi: 10.1254/jphs.10276FP

regulators of mitogenesis in VSMCs is platelet-derived growth factor (PDGF) (3). PDGF is produced by activated macrophages, VSMCs, and endothelial cells; and it consists of three isoforms (AA, AB, BB). PDGF-BB is a considerably more potent as a proliferative stimulus for VSMCs than PDGF-AA (4). The PDGF-BB-induced mitogenesis signaling pathways have been well characterized. The binding of PDGF-BB to the PDGF receptor (PDGF-R) leads to PDGF-R phosphorylation on multiple tyrosine residues. Then, it activates the extracellular signal regulated kinase (ERK) 1/2, phospholipase C (PLC)- γ 1, and phosphatidylinositol 3-kinase (PI3K) / Akt pathways, resulting in cell cycle progression (5). The upregulations of PDGF and its receptor are associated with the development and progression of cardiovascular diseases such as hypertension (6) and atherosclerosis (7). Therefore, the inhibition of VSMC proliferation by regulation of the PDGF signaling pathway could be a key pharmacological strategy for the prevention of atherosclerosis.

Docetaxel (Fig. 1), a microtubule-stabilizing agent, has been approved for the treatments of breast and prostate cancers and small cell carcinoma of the lung (8). Docetaxel promotes microtubulin assembly and stabilizes the polymers against depolymerization, thereby inhibiting microtubule dynamics (9). Docetaxel and paclitaxel bind to β tubulin, which leads to cell-cycle arrest (10). Docetaxel is approximately 2-fold more potent than paclitaxel as a tubulin assembly promoter and microtubule stabilizer (11). Paclitaxel has been shown in numerous studies to exhibit beneficial effects on the control of atherosclerosis and heart disease (12, 13). Thus, docetaxel may also affect vascular diseases through the regulation of smooth muscle cell proliferation.

In the present study, we sought to elucidate the anti-proliferative activity and the mechanistic target of docetaxel in the PDGF-BB-stimulated signaling pathway. Our findings provide evidence that docetaxel can inhibit both VSMC proliferation and cell cycle progression via

the cell cycle-related proteins by regulating PDGF-R β in VSMCs.

Materials and Methods

Cell culture

Rat aortic VSMCs were isolated by enzymatic dispersion using a slight modification of the method reported by Chamley et al. (14). Briefly, the cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplement with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 8 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)], and 2 mM L-glutamine (all obtained from Gibco-BRL, Gaithersburg, MD, USA) at 37°C in a humidified 5% CO₂ incubator. Passages 5 – 9 of the VSMCs were used in this study.

Cell proliferation and DNA synthesis

Cell proliferation and DNA synthesis were determined as previously described (15). In brief, VSMCs were seeded into 12-well culture plates at 4×10^4 cells/mL and then cultured in DMEM containing 10% FBS at 37°C for 24 h until 70% confluence. The medium was then replaced by serum-free media containing docetaxel (Sigma Chemical Co., St. Louis, MO, USA; dissolved in dimethyl sulfoxide (DMSO) and added to DMEM to a maximum final concentration of 0.05%) for 24 h. Cells were stimulated with 5% FBS or 25 ng/mL PDGF-BB (Upstate Biotechnology, Lake Placid, NY, USA) for 24 h, trypsinized with trypsin-EDTA, and counted using a hemocytometer. For [³H]thymidine incorporation experiments, VSMCs were seeded on 24-well culture plates at 5000 cells/well and then allowed to grow for 3 – 4 days in DMEM. The medium was then replaced with serum-free media, which consisted of DMEM containing various concentration of docetaxel (10 – 40 μ M). After 24 h, the cultures were exposed to 5% FBS or 25 ng/mL PDGF-BB for 20 h before adding 1 μ Ci/well [³H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) to the medium. Four hours later, the labeling reaction was terminated by aspirating the medium and subjecting cultures to sequential washes on ice with $1 \times$ phosphate-buffered saline (PBS) containing 10% trichloroacetic acid and ethanol/ether (1:1 v/v). Acid-insoluble [³H]thymidine was extracted into 250 μ L of 0.5 M NaOH per well, and 100 μ L of the extract was mixed with a liquid cocktail (UltimaGold; Packard Bioscience, Shelton, CT, USA) and counted in a scintillation counter (model LS3801; Beckman, Krefeld, Germany).

Cell cycle progression analysis

The cell cycle phase was determined as previously

Fig. 1. Chemical structure of docetaxel.

described (15). VSMCs were seeded into 6-well culture plates at 1×10^5 cells/mL and then cultured in DMEM containing 10% FBS at 37°C for 24 h until 70% confluence. The medium was then replaced with serum-free media containing docetaxel (10–40 μ M). Cells were stimulated with 5% FBS or 25 ng/mL PDGF-BB for 24 h, trypsinized, and then centrifuged at $1500 \times g$ for 7 min. The obtained pellets were suspended in 1 mL of $1 \times$ PBS, washed twice, and re-centrifuged. The pellets were suspended in 70% ethanol and fixed overnight at 4°C. The fixed VSMCs were briefly vortexed and centrifuged at $15,000 \times g$ for 5 min. The ethanol was discarded and the pellets were stained with 0.4 mL of propidium iodide (PI) solution (50 μ g/mL PI in buffer containing 100 μ g/mL of RNase A). Samples were incubated for 1 h at room temperature before analysis by flow cytometry. The PI-DNA complex in each cell nucleus was measured using a FACSCalibur (BD Biosciences, San Jose, CA, USA). The proportion of cell in G0/G1, S and G2/M phases were determined using the computer program ModFitLT (Verity Software House, Topsham, ME, USA).

Western blot analysis

The immunoblotting of VSMCs was performed as previously described (15). Briefly, VSMCs were seeded into 6-well culture plates at 1×10^5 cells/mL and then cultured in DMEM containing 10% FBS at 37°C for 24 h until 70% confluence. The medium was then replaced by serum-free medium containing docetaxel (10–40 μ M). Cells were stimulated with 25 ng/mL PDGF-BB for 24 h and then trypsinized and lysed. The harvested pellets were lysed, and the protein of interest was resuspended in a protein lysis buffer. The cell lysates were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10%–15% acrylamide gels) and then transferred to PVDF membranes (Amersham Pharmacia Biotech). The membranes were then incubated with a 1:1000 dilution of the following antibodies: phospho-PDGF-R β , phospho-ERK1/2, phospho-Akt, phospho-PLC- γ 1, PDGF-R β , ERK1/2, Akt, PLC- γ 1, phospho-Rb (all from Cell Signaling Technology Inc., Beverly, MA, USA) and proliferative cell nuclear antigen (PCNA), cyclin-dependent kinase (CDK) 2, CDK4, cyclin D₁, and cyclin E (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blots were washed with TBS/T and then incubated with a 1:5000 dilution of horseradish peroxidase–conjugated IgG secondary antibody (New England Biolabs, Ipswich, MA, USA). The proteins were detected using a chemiluminescent reaction (ECL plus kit; Amersham Pharmacia Biotech), followed by exposure of the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech). The band intensities

were quantified using Scion-Image Software for windows.

Statistical analysis

The experimental results are expressed as means $\pm$ S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparisons (Prism version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). If there was a significant variation between treated groups, the Dunnett test was applied. The data were considered significant when $P < 0.05$.

Results

Inhibitory effects of docetaxel on proliferation and DNA synthesis of VSMCs

VSMC proliferation plays an essential role in the development of cardiovascular diseases (16). To determine whether docetaxel inhibits FBS- and PDGF-BB-stimulated VSMC proliferation, we assessed the antiproliferative activities of docetaxel by direct cell counting and [³H]thymidine incorporation. The increase in the number of VSMCs induced by FBS and PDGF-BB was significantly inhibited by docetaxel in a concentration-dependent manner. The inhibition of FBS-stimulated cell proliferation by docetaxel at 10, 20, and 40 μ M was approximately 21.6% ($n = 4$), 60.8% ($P < 0.01$, $n = 4$), and 90.2% ($P < 0.01$, $n = 4$), respectively; and the inhibition of PDGF-BB-induced cell growth was 10.1% ($n = 4$), 62.9% ($P < 0.01$, $n = 4$), and 88.9% ($P < 0.01$, $n = 4$), respectively (Fig. 2: A and C). The level of [³H]thymidine incorporation into the DNA was also measured as an index of cell proliferation. Treatment of VSMCs with either 5% FBS or 25 ng/mL PDGF-BB caused a significant increase in the level of [³H]thymidine-labeled DNA synthesis. Docetaxel at 10, 20, and 40 μ M inhibited this DNA synthesis by 55.6% ($P < 0.01$, $n = 4$), 78.5% ($P < 0.01$, $n = 4$), and 94.5% ($P < 0.01$, $n = 4$), respectively, in FBS-stimulated cells and by 47.1% ($P < 0.01$, $n = 4$), 74.8% ($P < 0.01$, $n = 4$), and 99.5% ($P < 0.01$, $n = 4$) in PDGF-BB-induced cells (Fig. 2: B and D). Since antiproliferative is often accompanied by cytotoxicity, we also measured the cytotoxic effects of docetaxel on VSMCs and human umbilical vein endothelial cells and found no evidence of cytotoxicity at 40 μ M (Supplementary Fig. 1: available in the online version only). Therefore, our results indicate that docetaxel inhibits FBS- and PDGF-BB-induced VSMCs proliferation without cytotoxicity.

Docetaxel-induced G0/G1 phase arrest in cell cycle progression

We analyzed the effects of docetaxel on cell cycle

Fig. 2. Effects of docetaxel on proliferation and DNA synthesis induced by FBS or PDGF-BB in VSMCs. A and C: The VSMCs were precultured in the serum-free medium in the presence or absence of docetaxel (10 – 40 μM) for 24 h and then stimulated by 5% FBS or 25 ng/mL PDGF-BB for 24 h. The cells were trypsinized and counted using a hemocytometer. B and D: The VSMCs were cultured in serum-free medium in the presence or absence of docetaxel (10 – 40 μM) for 24 h and then exposed to 5% FBS or 25 ng/mL PDGF-BB for 20 h before 1 μCi/mL [³H]thymidine was added to the medium. Four hours later, the labeling reaction was quenched and quantified using a liquid scintillation counter. The cell morphology was observed under a light microscope with 200 × magnification. The scale bar indicates 50 μm. The data are reported as the mean ± S.E.M. from 4 different sets of experiments. **P* < 0.05 and ***P* < 0.01 vs. the PDGF-BB stimulation alone.

progression by using flow cytometry analysis. A concentration-dependent increase in the cell population in the G1 phase of the cell cycle and a concentration-dependent decrease in the cell population in the S phase of the cell cycle were observed after addition of docetaxel (10–40 μ M) to FBS- or PDGF-BB-stimulated VSMCs. The serum-deprivation of VSMCs for 24 h resulted in an approximately $2.8 \pm 1.4\%$ synchronization of the cell cycle in the S phase. FBS or PDGF-BB treatment of the VSMCs increased the S phase population to $27.3 \pm 2.2\%$ and $14.5 \pm 1.2\%$, respectively, at 24 h. However, docetaxel at 10, 20, and 40 μ M reduced the cell population in the S phase to about $19.6 \pm 3.5\%$ ($P < 0.01$, $n = 3$), $12.3 \pm 2.7\%$ ($P < 0.01$, $n = 3$), and $4.5 \pm 1.4\%$ ($P < 0.01$, $n = 3$) and to about 10.4% ($P < 0.05$, $n = 3$), 6.9% ($P < 0.01$, $n = 3$), and 3.4% ($P < 0.01$, $n = 3$), respectively, in FBS- or PDGF-BB-stimulated cells (Fig. 3).

Inhibitory effects of docetaxel on G0/G1 phase regulatory proteins

Docetaxel arrested PDGF-BB-stimulated VSMCs in

the G0/G1 phase of the cell cycle (Fig. 3). At 24 h after stimulation of the cells with PDGF-BB, there was an increase in the expression of CDK2, cyclin E, CDK4, and cyclin D1, whereas the pretreatment of VSMCs with docetaxel at 40 μ M significantly inhibited the increased expression by 72.0% ($P < 0.01$, $n = 3$), 59.0% ($P < 0.01$, $n = 3$), 57.7% ($P < 0.01$, $n = 3$), and 56.3% ($P < 0.01$, $n = 3$), respectively (Fig. 4). Docetaxel at 10, 20, and 40 μ M inhibited both the phosphorylation of Rb, a regulatory protein in the G1/S phase by 34.7% ($P < 0.01$, $n = 3$), 53.0% ($P < 0.01$, $n = 3$), and 74.7% ($P < 0.01$, $n = 3$) and inhibited the expression of PCNA, a phospho-Rb-mediated gene product, by 10.4% ($n = 3$), 14% ($n = 3$), and 52.6% ($P < 0.01$, $n = 3$), respectively. Therefore, docetaxel may regulate the activity of G0/G1-checkpoint proteins, leading to the cell cycle arrest in VSMCs.

Inhibitory effects of docetaxel on ERK1/2, Akt, and PLC- γ 1 phosphorylation

Phosphorylation of ERK1/2, Akt, and PLC- γ 1 is as-

Fig. 3. Effect of docetaxel on FBS- and PDGF-BB-stimulated cell cycle progression. The cells were pre-incubated in the presence or absence of docetaxel in serum-depleted medium for 24 h. The cells were stimulated with 5% FBS (A) or 25 ng/mL of PDGF-BB (B). After 24 h, individual nuclear DNA content was measured by fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment where data from at least 10,000 events were obtained. In the lower panels in A and B, the data are expressed as mean values from 3 independent experiments.

Fig. 4. Inhibitory effects of docetaxel on PDGF-BB-stimulated CDK2, CDK4, cyclin D1, cyclin E, PCNA expression, and Rb phosphorylation. Quiescent VSMCs were stimulated with 25 ng/mL PDGF-BB in the presence or absence of docetaxel at 10, 20, and 40 μM for 24 h. The cells were then lysed and proteins were analyzed using 12% SDS-PAGE. Western blot analysis was performed with antibodies specific for CDK2, CDK4, cyclin D1, cyclin E, PCNA, and phospho-Rb. α -Actin was used for normalization. A: Representative data from 3 different experiments are presented. B: The data were quantified with densitometry and are expressed as the mean $\pm$ S.E.M. ($n = 3$). ** $P < 0.01$ vs. the PDGF-BB stimulation alone.

sociated with PDGF-induced cell proliferation in VSMCs. Docetaxel inhibited PDGF-BB-induced ERK1/2, Akt, and PLC- γ 1 phosphorylation in a concentration-dependent manner. Docetaxel inhibited ERK1/2 phosphorylation induced by PDGF-BB in a concentration-dependent manner, with inhibition of 34.5% ($P < 0.05$,

$n = 3$), 47.5% ($P < 0.05$, $n = 3$), and 88.9% ($P < 0.01$, $n = 3$) at 10, 20, and 40 μM (Fig. 5A). Docetaxel also suppressed Akt phosphorylation induced by PDGF-BB by 27.8% ($P < 0.05$, $n = 3$), 38.2% ($P < 0.05$, $n = 3$), and 78.4% ($P < 0.01$, $n = 3$) at doses of 10, 20, and 40 μM , respectively (Fig. 5B). Similarly, PDGF-BB-induced

PLC- γ 1 phosphorylation was decreased by 15.8% ($n = 3$), 38.2% ($P < 0.05$, $n = 3$), and 87.6% ($P < 0.01$, $n = 3$) at doses of 10, 20, and 40 μ M, respectively (Fig. 5C). U0126 (ERK1/2 inhibitor, 10 μ M), LY294002 (Akt inhibitor, 50 μ M), and U73122 (PLC inhibitor, 10 μ M) were used as a positive control and inhibited PDGF-BB-

induced ERK1/2, Akt, and PLC- γ 1 phosphorylation. In addition, inhibition of ERK1/2, Akt, or PLC- γ 1 prevented PDGF-BB-induced DNA synthesis of VSMCs (Supplementary Fig. 2: available in the online version only). These results indicated that docetaxel inhibition of cell proliferation may occur through its effect on a PDGF-mediated signaling pathway.

Inhibitory effect of docetaxel on PDGF- $R\beta$ phosphorylation

Docetaxel inhibited the downstream components of the PDGF-BB signaling pathway, including ERK1/2, Akt, and PLC- γ 1 phosphorylation (Fig. 5). Thus, PDGF- $R\beta$ phosphorylation may be a target for docetaxel and lead to the inhibition of VSMC proliferation. Pretreatment with docetaxel significantly inhibited PDGF- $R\beta$ tyrosine phosphorylation induced by PDGF-BB by 44.5% ($P < 0.01$, $n = 3$), 79.9% ($P < 0.01$, $n = 3$), and 91.4% ($P < 0.01$, $n = 3$) at doses of 10, 20, and 40 μ M, respectively (Fig. 6). AG1295, a selective inhibitor of PDGF- $R\beta$ tyrosine phosphorylation, also prevented PDGF-BB-induced VSMCs proliferation as well as the phosphorylation of ERK1/2, Akt, and PLC- γ 1 (Supplementary Fig. 3: available in the online version only). These results indicate that docetaxel may inhibit VSMCs proliferation by blocking PDGF- $R\beta$ activation.

Discussion

VSMCs are major components of the arterial wall, and abnormal proliferation of VSMCs plays a pivotal role in the pathogenesis of atherosclerosis (16). In atherosclerotic lesions, VSMCs are exposed to mitogenic substances such as PDGF (17, 18). In studies of rabbits, birds, rats, and humans, increases in PDGF after arterial injury have been correlated with neointimal cellular proliferation (19–22), demonstrating an association between VSMC proliferation and PDGF. Therefore, an inhibition of VSMCs proliferation is a potentially impor-

Fig. 5. Effects of docetaxel on the phosphorylation of ERK1/2 (A), Akt (B), and PLC- γ 1 (C) in PDGF-BB-stimulated VSMCs. Confluent cells were pre-incubated for 24 h in the presence or absence of the indicated concentration of docetaxel and U0126 (ERK1/2 inhibitor, 10 μ M), LY294002 (Akt inhibitor, 50 μ M), or U73122 (PLC inhibitor, 10 μ M) in serum-free medium. The cells were stimulated with 25 ng/mL PDGF-BB for 5 min (for ERK1/2 and PLC- γ 1 phosphorylation) or 15 min (for Akt phosphorylation). The cells were lysed, and proteins were quantified using 7.5%–10% SDS-PAGE and immunoblotting. Quantification of band intensities from 3 independent experiments was performed with densitometry. Data are shown as the mean $\pm$ S.E.M. ($n = 3$) from 3 experiments performed for the expression of phospho-ERK1/2 / total-ERK1/2, phospho-Akt / total-Akt, and phospho-PLC- γ 1 / total-PLC- γ 1. * $P < 0.05$ and ** $P < 0.01$ vs. the PDGF-BB stimulation alone.

Fig. 6. Effect of docetaxel on PDGF-BB-induced PDGF-R β phosphorylation in VSMCs. Confluent cells were pre-cultured in the presence or absence of docetaxel (10–40 μ M) or AG1295 (PDGF-R β inhibitor, 20 μ M) in serum-free medium for 24 h and stimulated with 25 ng/mL PDGF-BB at 37°C for 1 min. The cells were lysed, and proteins were analyzed using 7.5% SDS-PAGE and immunoblotting. The total PDGF-R β was used for normalization. After densitometric quantification, the data were expressed as the mean $\pm$ S.E.M. (n = 3). ** P < 0.01 vs. the PDGF-BB stimulation alone.

tant therapeutic strategy for the treatment of cardiovascular diseases (23).

We investigated the antiproliferative effect of docetaxel on VSMCs and the mechanism of this effect. Docetaxel inhibited VSMC proliferation and DNA synthesis (Fig. 2), as paclitaxel does (24, 25). In addition, the antiproliferative activity of docetaxel was associated with cell cycle arrest in the G0/G1 phase (Fig. 3). Cell cycle progression is tightly controlled by a series of positive and negative regulators that act at sequential points throughout the cell cycle. CDKs play a central role in the cell cycle and promote the G-to-S phase transition by phosphorylation of the Rb protein (26). CDK2 and CDK4 mediate G0/G1-to-S phase progression of the cell cycle by forming complexes with cyclin A, E, and D1 (27–29). The CDK/cyclins complex phosphorylates a large number of proteins, resulting in hyperphosphorylation of Rb, that promote DNA synthesis (30). The inhibition of pRb phosphorylation by docetaxel correlated well with the inhibition of PDGF-BB-stimulated cell proliferation, DNA synthesis, and cell cycle progression (Fig. 4) (31). Expression of cell cycle regulatory proteins CDK2, cyclin E, CDK4, and cyclin D1 decreased after VSMCs were exposed to docetaxel (Fig. 4), indicating that cell

cycle arrest in the G0/G1 phase might be due to the down-regulation of CDKs/cyclins complex expression and pRb phosphorylation (30). The expression of PCNA, a gene product induced by phospho-Rb that is expressed in the early G0/G1 and S phase of the cell cycle, was also inhibited by docetaxel (Fig. 4).

ERK1/2, Akt, and PLC- γ 1 are downstream components of the PDGF signaling pathway. PDGF-BB potentially activates mitogen-activated protein kinase (MAPK) by triggering RAS-RAF activation, which activates MEK, which then phosphorylates ERK1/2 in VSMCs. MAPKs, such as ERK1/2 and JNK, are important players in the early intracellular mitogenic signal transduction for cell growth and have been implicated in the proliferation and migration of various cell types, such as L6 myoblasts and NIH3T3 fibroblasts induced by PDGF-BB (32, 33). PI3K/Akt is triggered by PDGF-BB, and this pathway is involved in the anti-apoptotic effect of PDGF-BB in VSMCs (34). PLC- γ 1 mediates a central downstream signal transduction route for various growth factors, including PDGF-BB (35). Docetaxel inhibited the PDGF-BB-induced ERK1/2 activation (Fig. 5A), indicating that ERK1/2 protein may be related to the antiproliferative activity of docetaxel. ERK1/2 was induced after arterial injury in rats (36), and either an ERK1/2 inhibitor (37) or the gene transfer of an ERK1/2 dominant-negative mutant (38) can suppress VSMC proliferation and block neointimal formation in balloon-injured arteries. Docetaxel produced a marked decrease in the PDGF-BB-stimulated phosphorylation of Akt and PLC- γ 1 (Fig. 5: B and C). PDGF-BB binding to PDGF-R β is associated with autophosphorylation, clustering, and activation of PDGF-R tyrosine kinase (39). Docetaxel inhibited PDGF-R β phosphorylation (Fig. 6), in agreement with other studies showing that docetaxel inhibits PDGF-R phosphorylation in peripheral blood leukocytes and prostate cancer (40, 41). Thus, PDGF-R β might be a potential target for docetaxel.

PDGF-R expression is upregulated during endothelial injury after angioplasty or in the early stage of atherosclerosis, whereas its expression is at low levels in arteries from healthy adults (42). Targeting PDGF with an anti-PDGF antibody or using inhibitors of PDGF signal transduction suppresses neointimal development (43–45). There are a couple of drugs that target PDGF-R β . Imatinib mesylate, an antitumor agent, was developed with the aim of preventing restenosis after coronary angioplasty through the inhibition of abnormal PDGF-R activation (46). In clinical trials, trapidil, a nonspecific PDGF-R inhibitor, can inhibit the stenosis caused by vascular injury (47). Docetaxel may also prove to be a potential therapeutic agent for the prevention, possibly, treatment of atherosclerosis.

Our results indicate that the inhibition of PDGF-R β expression stimulated by PDGF-BB may have contributed to the inhibition of VSMC proliferation by docetaxel, although the molecular mechanism through which docetaxel inhibits VSMC proliferation has yet to be fully clarified. There are mainly two possibilities in response to inhibition of PDGF-R β phosphorylation by docetaxel. One is that docetaxel may incorporate into the cell surface membranes, and then it reacts with PDGF-BB, causing it to react with non-receptor sites, resulting in a reduced PDGF binding to the respective receptors (48). Another possibility is that docetaxel may induce conformational changes at the ATP-binding sites involved in PDGF-R autophosphorylation, thereby inhibiting PDGF-mediated PDGF-R autophosphorylation (49). Inhibitors of PDGF signaling have successfully conferred resistance to the pathological processes associated with proliferative diseases such as atherosclerosis (50). Sirois et al. demonstrated that inhibition of PDGF-R β suppressed intimal thickening in the rat carotid artery following balloon injury (44). Administration of antibodies to PDGF-R β induced intimal atrophy in a baboon graft model (51). These observations support the pathogenic role of PDGF-R β in proliferative vascular diseases (52).

In summary, our study demonstrated that docetaxel inhibits PDGF-BB-induced VSMC growth and arrests cell cycle through inhibition of the PDGF-R β pathway. This beneficial property of docetaxel may make it a potential candidate for preventing or treating vascular diseases and restenosis after angioplasty.

Acknowledgment

This work was supported by the National Research Foundation of Korea [NRF] grant funded by the Korean government [MEST] (MRC, 2010-0029480).

References

- Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
- Rzucidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg*. 2007;45 Suppl A:A25–A32.
- Sachinidis A, Locher R, Vetter W, Tatje D, Hoppe J. Different effects of platelet-derived growth factor isoforms on rat vascular smooth muscle cells. *J Biol Chem*. 1990;265:10238–10243.
- Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, Ross R. Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. *Ann N Y Acad Sci*. 1995;766:416–430.
- Claesson-Welsh L. Platelet-derived growth factor receptor signals. *J Biol Chem*. 1994;269:32023–32026.
- Mulvany MJ. Structure and function of small arteries in hypertension. *J Hypertens Suppl*. 1990;8:S225–S232.
- Ross R. Cell biology of atherosclerosis. *Annu Rev Physiol*. 1995; 57:791–804.
- Crown J, O’Leary M. The taxanes: an update. *Lancet*. 2000;355: 1176–1178.
- Ringel I, Horwitz SB. Studies with RP 56976 (taxotere): a semi-synthetic analogue of taxol. *J Natl Cancer Inst*. 1991;83:288–291.
- Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. *Nature*. 1979;277:665–667.
- Gueritte-Voegelein F, Guenard D, Lavelle F, Le Goff MT, Mangatal L, Potier P. Relationships between the structure of taxol analogues and their antimitotic activity. *J Med Chem*. 1991;34: 992–998.
- Blagosklonny MV, Darzynkiewicz Z, Halicka HD, Pozarowski P, Demidenko ZN, Barry JJ, et al. Paclitaxel induces primary and postmitotic G1 arrest in human arterial smooth muscle cells. *Cell Cycle*. 2004;3:1050–1056.
- Kim SJ, Masaki T, Leypoldt JK, Kamerath CD, Mohammad SF, Cheung AK. Arterial and venous smooth-muscle cells differ in their responses to antiproliferative drugs. *J Lab Clin Med*. 2004; 144:156–162.
- Chamley JH, Campbell GR, McConnell JD, Groschel-Stewart U. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. *Cell Tissue Res*. 1977;177:503–522.
- Park ES, Lim Y, Hong JT, Yoo HS, Lee CK, Pyo MY, et al. Pterostilbene, a natural dimethylated analog of resveratrol, inhibits rat aortic vascular smooth muscle cell proliferation by blocking Akt-dependent pathway. *Vascul Pharmacol*. 2010;53:61–67.
- Gordon D, Reidy MA, Benditt EP, Schwartz SM. Cell proliferation in human coronary arteries. *Proc Natl Acad Sci U S A*. 1990; 87:4600–4604.
- Cui Y, Gutstein WH, Jabr S, Hsieh TC, Wu JM. Control of human vascular smooth muscle cell proliferation by sera derived from ‘experimentally stressed’ individuals. *Oncol Rep*. 1998;5: 1471–1474.
- Gutstein WH, Teresi JA, Wu JM, Ramirez M, Salimian F, Cui Y, et al. Increased serum mitogenic activity for arterial smooth muscle cells associated with relaxation and low educational level in human subjects with high but not low hostility traits: implications for atherogenesis. *J Psychosom Res*. 1999;46:51–61.
- Uchida K, Sasahara M, Morigami N, Hazama F, Kinoshita M. Expression of platelet-derived growth factor B-chain in neointimal smooth muscle cells of balloon injured rabbit femoral arteries. *Atherosclerosis*. 1996;124:9–23.
- Ding H, Wang R, Marcel R, Fisher DZ. Adenovirus-mediated expression of a truncated PDGF β receptor inhibits thrombosis and neointima formation in an avian arterial injury model. *Thromb Haemost*. 2001;86:914–922.
- Chang Y, Ceacareanu B, Zhuang D, Zhang C, Pu Q, Ceacareanu AC, et al. Counter-regulatory function of protein tyrosine phosphatase 1B in platelet-derived growth factor- or fibroblast growth factor-induced motility and proliferation of cultured smooth muscle cells and in neointima formation. *Arterioscler Thromb Vasc Biol*. 2006;26:501–507.
- Liu G, Changsirikulchai S, Hudkins KL, Banas MC, Kowalewska J, Yang X, et al. Identification of platelet-derived growth factor D in human chronic allograft nephropathy. *Hum Pathol*. 2008;39: 393–402.
- Jung F, Haendeler J, Goebel C, Zeiher AM, Dimmeler S. Growth factor-induced phosphoinositide 3-OH kinase/Akt phosphoryla-

- tion in smooth muscle cells: induction of cell proliferation and inhibition of cell death. *Cardiovasc Res.* 2000;48:148–157.
- 24 Axel DI, Kunert W, Goggelmann C, Oberhoff M, Herdeg C, Kuttner A, et al. Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery. *Circulation.* 1997;96:636–645.
 - 25 Blagosklonny MV, Demidenko ZN, Giovino M, Szydal C, Donskoy E, Herrmann RA, et al. Cytostatic activity of paclitaxel in coronary artery smooth muscle cells is mediated through transient mitotic arrest followed by permanent post-mitotic arrest: comparison with cancer cells. *Cell Cycle.* 2006;5:1574–1579.
 - 26 Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell.* 1995;81:32–330.
 - 27 Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999;13:1501–1512.
 - 28 Jirawatnotai S, Aziyu A, Osmundson EC, Moons DS, Zou X, Kineman RD, et al. Cdk4 is indispensable for postnatal proliferation of the anterior pituitary. *J Biol Chem.* 2004;279:51100–51106.
 - 29 Martin A, Odajima J, Hunt SL, Dubus P, Ortega S, Malumbres M, et al. Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1). *Cancer Cell.* 2005;7:591–598.
 - 30 Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med.* 2002;8:1249–1256.
 - 31 Seo JM, Jin YR, Ryu CK, Kim TJ, Han XH, Hong JT, et al. JM91, a newly synthesized indoleione derivative, inhibits rat aortic vascular smooth muscle cells proliferation and cell cycle progression through inhibition of ERK1/2 and Akt activations. *Biochem Pharmacol.* 2008;75:1331–1340.
 - 32 Graf K, Xi XP, Yang D, Fleck E, Hsueh WA, Law RE. Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. *Hypertension.* 1997;29:334–339.
 - 33 De Donatis A, Comito G, Buricchi F, Vinci MC, Parenti A, Caselli A, et al. Proliferation versus migration in platelet-derived growth factor signaling: the key role of endocytosis. *J Biol Chem.* 2008;283:19948–19956.
 - 34 Sandirasegarane L, Kester M. Enhanced stimulation of Akt-3/protein kinase B-gamma in human aortic smooth muscle cells. *Biochem Biophys Res Commun.* 2001;283:158–163.
 - 35 Heldin CH, Ostman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta.* 1998;1378:F79–F113.
 - 36 Lai K, Wang H, Lee WS, Jain MK, Lee ME, Haber E. Mitogen-activated protein kinase phosphatase-1 in rat arterial smooth muscle cell proliferation. *J Clin Invest.* 1996;98:1560–1567.
 - 37 Pyles JM, March KL, Franklin M, Mehdi K, Wilensky RL, Adam LP. Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. *Circ Res.* 1997;81:904–910.
 - 38 Izumi Y, Kim S, Namba M, Yasumoto H, Miyazaki H, Hoshiga M, et al. Gene transfer of dominant-negative mutants of extracellular signal-regulated kinase and c-Jun NH2-terminal kinase prevents neointimal formation in balloon-injured rat artery. *Circ Res.* 2001;88:1120–1126.
 - 39 Hughes AD, Clunn GF, Refson J, Demoliou-Mason C. Platelet-derived growth factor (PDGF): actions and mechanisms in vascular smooth muscle. *Gen Pharmacol.* 1996;27:1079–1089.
 - 40 Hofer MD, Rubin MA. Platelet-derived growth factor receptor inhibitor imatinib mesylate and docetaxel: a modular phase I trial in androgen-independent prostate cancer. *J Clin Oncol.* 2005;23:1332–1333; author reply 1333–1334.
 - 41 Mathew P, Fidler IJ, Logothetis CJ. Combination docetaxel and platelet-derived growth factor receptor inhibition with imatinib mesylate in prostate cancer. *Semin Oncol.* 2004;31:24–29.
 - 42 Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM. PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol.* 1990;111:2149–2158.
 - 43 Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science.* 1991;253:1129–1132.
 - 44 Sirois MG, Simons M, Edelman ER. Antisense oligonucleotide inhibition of PDGFR-beta receptor subunit expression directs suppression of intimal thickening. *Circulation.* 1997;95:669–676.
 - 45 Maresta A, Balducci M, Cantini L, Casari A, Chioin R, Fontanelli A, et al. The trapidil restenosis trial (STARC study): background, methods and clinical characteristics of the patient population. *Clin Trials Metaanal.* 1994;29:31–40.
 - 46 Buchdunger E, O'Reilly T, Wood J. Pharmacology of imatinib (STI571). *Eur J Cancer.* 2002;38 Suppl 5:S28–S36.
 - 47 Maresta A, Balducci M, Latini R, Bernardi G, Moccetti T, Sosa C, et al. Starc II, a multicenter randomized placebo-controlled double-blind clinical trial of trapidil for 1-year clinical events and angiographic restenosis reduction after coronary angioplasty and stenting. *Catheter Cardiovasc Interv.* 2005;64:375–382.
 - 48 Toki S, Agatsuma T, Ochiai K, Saitoh Y, Ando K, Nakanishi S, et al. RP-1776, a novel cyclic peptide produced by *Streptomyces* sp., inhibits the binding of PDGF to the extracellular domain of its receptor. *J Antibiot (Tokyo).* 2001;54:405–414.
 - 49 Banai S, Wolf Y, Golomb G, Pearle A, Waltenberger J, Fishbein I, et al. PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth in vitro and reduces neointimal formation after balloon angioplasty in swine. *Circulation.* 1998;97:1960–1969.
 - 50 Yang X, Thomas DP, Zhang X, Culver BW, Alexander BM, Murdoch WJ, et al. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neointima formation. *Arterioscler Thromb Vasc Biol.* 2006;26:85–90.
 - 51 Englesbe MJ, Hawkins SM, Hsieh PC, Daum G, Kenagy RD, Clowes AW. Concomitant blockade of platelet-derived growth factor receptors alpha and beta induces intimal atrophy in baboon PTFE grafts. *J Vasc Surg.* 2004;39:440–446.
 - 52 Li L, Blumenthal DK, Masaki T, Terry CM, Cheung AK. Differential effects of imatinib on PDGF-induced proliferation and PDGF receptor signaling in human arterial and venous smooth muscle cells. *J Cell Biochem.* 2006;99:1553–1563.