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Inhibitory Effects of YP 12, A Newly Synthesized Obovatol Derivative on Rat Aortic Vascular Smooth Muscle Cell Proliferation

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ABSTRACT - Platelet derived growth factor (PDGF)-BB is one of the most potent vascular smooth muscle cell (VSMC) proliferative factors, and abnormal VSMC proliferation by PDGF-BB plays an important role in the development and progression of atherosclerosis. The aim of this study was to assess the effect of YP 12, a newly synthesized obovatol derivative, on the proliferation of PDGF-BB-stimulated rat aortic VSMCs. The anti-proliferative effects of YP 12 on rat aortic VSMCs were examined by direct cell counting and by using [^{3}H] thymidine incorporation assays. It was found that YP 12 potently inhibited the growth of VSMCs. The pre-incubation of YP 12 (1-4 μM) significantly inhibited the proliferation and DNA synthesis of 25 ng/ml PDGF-BB-stimulated rat aortic VSMCs in a concentration-dependent manner. In accordance with these findings, YP 12 revealed blocking of the PDGF-BB-inducible progression through G0/G1 to S phase of the cell cycle in synchronized cells. Whereas, YP 12 did not show any cytotoxicity in rat aortic VSMCs in this experimental condition by WST-1 assay. These results also show that YP 12 may have potential as an anti-proliferative agent for the treatment of restenosis and atherosclerosis.

Key words : Atherosclerosis, proliferation, vascular smooth muscle cell, PDGF-BB

Introduction

Vascular smooth muscle cells (VSMCs) are the main component of arterial wall, and abnormal proliferation of VSMCs is implicated in the pathogenesis of atherosclerosis and restenosis after angioplasty, and possibly in the development of hypertension (Dzau, Braun-Dullaeus, & Sedding, 2002; Erlinge, 1998; Ross, 1990, 1999). Although various growth factors and cytokines are involved in the VSMCs proliferation, one of the principal regulators of mitogenesis in VSMCs is platelet derived growth factor (PDGF) (Sachinidis, Locher, Vetter, Tatje, & Hoppe, 1990). PDGF is produced by activated macrophages, VSMCs and endothelial cells, forming three isoforms (AA, AB, and BB). PDGF-BB is a much more potent proliferative stimuli to VSMCs than PDGF-AA (Bornfeldt et al., 1995). The PDGF-BB-stimulated mitogenesis signaling pathway has already been relatively well characterized. The binding of PDGF-BB to PDGF-receptor (PDGF-R) can activate three major signal transduction pathways; phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC)γ1 and extracellular regulated kinases 1/2 (ERK1/2) by triggering the activation of Raf-1 (Claesson-Welsh, 1994). It has been reported that ERK1/2 activation is required for its mitogenic signaling through a number of tyrosine kinase growth factor receptors, and is known to be associated with the development and progression of proliferative cardiovascular diseases, such as hypertension and atherosclerosis (Claesson-Welsh, 1994; Majesky et al., 1990; Mulvany, 1990; Ross, 1995).

Vascular proliferation contributes to the pathobiology of atherosclerosis and is linked to cellular processes such as inflammation, apoptosis and matrix alterations (Ross, 1995). Vascular proliferation represents an important contributor to the pathophysiology of instent restenosis, transplant vasculopathy and vein bypass graft failure (Braun-Dullaeus, Mann, & Dzau, 1998). Thus, a strategy for the treatment of those conditions is to inhibit cellular proliferation by targeting cell cycle regulation.

Obovatol was known to have anti-inflammation, anti-tumor and anti-platelet activities (S. K. Lee et al., 2008). It inhibited rat aortic vascular smooth muscle cells proliferation through the upregulation of $p21^{cip1}$ and downregulation of cell cycle-related proteins (Lim et al., 2010). We have screened better inhibitors than obovatol for the inhibition of VSMCs pro-

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liferation. In this study, we have investigated the anti-proliferative activity of YP 12 on PDGF-BB-stimulated VSMCs by measuring cell proliferation and [^{3}H]-thymidine incorporation into DNA. Furthermore, the cell cycle progression of YP 12 was also investigated.

Materials and Methods

Chemicals and reagents

YP 12, an obovatol derivative, was synthesized and kindly gifted by professor Jae-Kyung Jung in Chungbuk National University, Korea (M. S. Lee et al., 2007). The cell culture materials were obtained from Gibco-BRL (Rockville, MD, USA), and other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-thymidine was purchased Amersham Pharmacia Biotech (Buckingamshire, UK). Platelet-derived growth factor (PDGF)-BB was obtained from Upstate Biotechnolgy (Lake Placid, NY, USA). Other chemicals were of analytical grade.

Rat aortic VSMCs isolation and culture

Male Sprague-Dawley rats (Samtako Bio Korea Co., Ltd., Osan, Korea) weighing around 280 g were fed a normal chow diet and given water ad libitum. All protocols were approved by the Chungbuk National University Animal Care and Use Committee. Rat aortic vascular smooth muscle cells (VSMCs) were isolated by enzymatic dispersion as previously described (Lim et al., 2010) according to the modified method of Chamley et al. (Chamley, Campbell, McConnell, & Groschel-Stewart, 1977). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. The purity of rat aortic VSMCs culture was confirmed by immunocytochemical localization of α-smooth-muscle actin. The passage of rat aortic VSMCs used in this experiment was 5-9.

Measurement of cell proliferation and DNA synthesis

The cell proliferation and DNA synthesis of rat aortic VSMCs were measured as previously described (Lim et al., 2010). In brief, for cell counting, rat aortic VSMCs were seeded in 12-well culture plates at 4×10^4 cells/ml and cultured in DMEM with 10% FBS at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium consisting of YP 12. The cells were stimulated by 25 ng/ml PDGF-BB, and then trypsinized by trypsin-EDTA and counted using hemocytometer under microscopy. For [^{3}H]-thymidine incorporation experiments, rat aortic VSMCs were seeded in 24-well

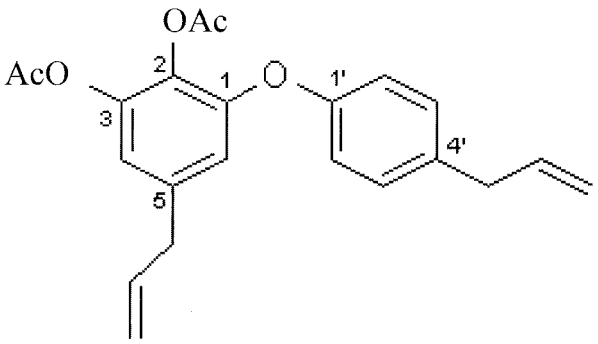

Fig. 1. Chemical structure of YP 12.

culture plates under the same conditions, and 1 μCi/ml of [^{3}H]-thymidine was added to the medium. The reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether [1:1, v/v] after 4 h. Acid-insoluble [^{3}H]-thymidine was extracted into 300 μl of 0.5 M NaOH/well, and this solution was mixed with 3 ml scintillation cocktail (Ultimagold, Packard Bioscience, CT, USA), and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

Cell cycle progression analysis

To estimate the proportion of cells in the various phases of the cell cycle, cellular DNA contents were measured by flow cytometry (FACS). Cells were harvested, fixed in 70% ethanol, and stored at −20°C. Cells were then washed twice with ice-cold PBS and incubated with RNase and propidium iodide (PI), a DNA-intercalating dye. Cell cycle phase analysis was performed using the FACSCalibur (Becton-Dickinson Co., San Jose, USA) and the proportion of cells within the G0/G1, S and G2/M phases was determined by analysis with ModFit LT V2.0 (Verity Software House, Topsham, USA).

Cell viability assay

For WST-1 assay (Premix WST-1, Takara, Japan), VSMCs were seeded in 96-well plates at 8×10^3 cells/well and pre-cultured in serum-free medium in the presence or absence of YP 12 for 24 h. WST-1 reagent was added at 22 h and further incubated for 2 h. Then the absorbance was determined in an ELISA at a wavelength of 450 nm.

Statistical analysis

The experimental results were expressed as mean ± S.E.M. A one-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett. Differences with $P < 0.05$ were considered statistically significant.

Results

Effect of YP 12 on the proliferation of VSMCs

The inhibitory effect of YP 12 on proliferation of PDGF-BB-induced VSMCs was measured by cell number. The number of cells was significantly increased by 25 ng/ml PDGF-BB for 24 h. The PDGF-BB-induced cell numbers were significantly decreased by pre-treatment of YP 12 for 24 h in a concentration-dependent manner. The percentage of inhibition exerted by 1, 2 and 4 μM YP 12 were 28.7, 40.6 and 78.8% respectively (Fig. 2).

Effect of YP 12 on the DNA synthesis of VSMCs

The effect of YP 12 on the DNA synthesis of VSMCs was examined. Stimulation of VSMCs by 25 ng/ml PDGF-BB potently increased [^{3}H]-thymidine incorporation from 2,481 to 52,140 cpm/well. YP 12 significantly inhibited the PDGF-BB-induced DNA synthesis in a concentration-dependent manner, and the inhibition percentages were 24.6, 48.4 and 73.2% at the concentrations of 1, 2 and 4 μM, respectively (Fig. 3). No significant difference in viability was detected between control cells and those treated with YP 12 (1-4 μM) when examined by WST-1 assay (Fig. 4).

Effect of YP 12 on cell cycle progression

Flow cytometric analysis (Table 1) demonstrated that YP 12 affects cell cycle progression induced by 25 ng/mL PDGF-BB. A primary culture of VSMCs was subjected to serum-deprivation for 24 h, which resulted in approximately 87.1 ± 0.5% of cells being synchronized in the G0/G1 phase. After incubation for 24 h in the presence of PDGF-BB, the percentage of cells in S phase increased from 0.9 ± 0.7 to 14.2 ± 1.0%. In contrast, YP 12 (1-4 μM) blocked PDGF-BB-induced cell cycle progressions in a concentration-dependent manner (Table 1). YP 12 (1, 2 and 4 μM) reduced the percentage of PDGF-BB-stimulated cells in S phase to 9.2 ± 1.2% ($P < 0.05$), 4.3 ± 0.4% ($P < 0.01$) and 2.1 ± 0.8% ($P < 0.01$), respectively (Table 1; n = 3, duplicate cultures). This finding indicates that YP 12 must act during early events in the cell cycle, since it is effective against DNA synthesis. YP12 arrested significant numbers of cells in the G1 phase of the cell cycle, suggesting

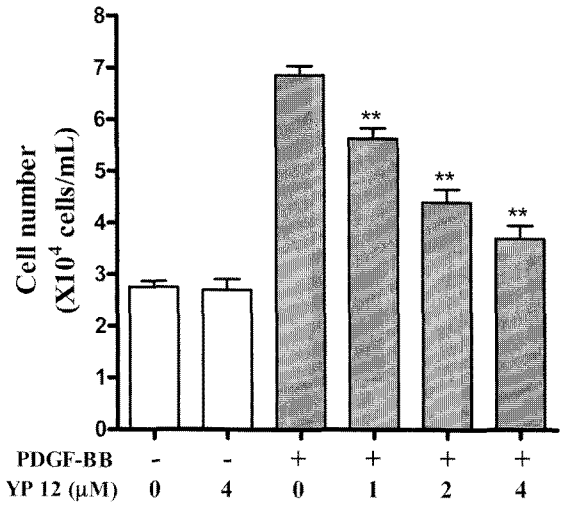

Fig. 2. Effect of YP 12 on the aortic vascular smooth muscle cell number induced by PDGF-BB. VSMCs were pre-cultured in serum-free medium at different concentrations (1, 2 and 4 μM) of YP 12 for 24 h, and then induced by 25 ng/mL PDGF-BB. After 24 h, cells were counted by hemocytometer. Data are expressed as mean ± S.E.M. from four different sets of experiments $^{**}P < 0.01$ *vs* PDGF-BB-induced VSMCs.

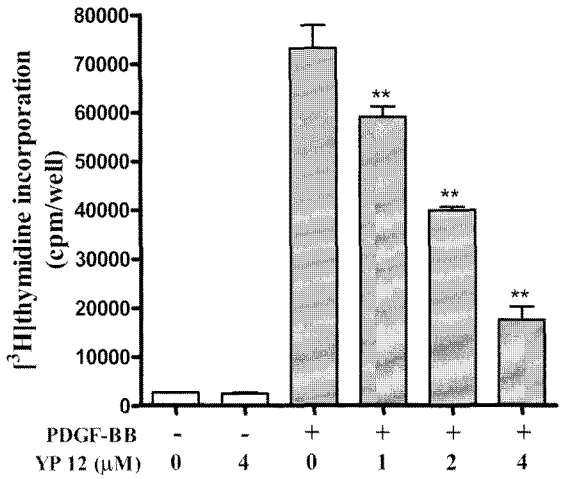

Fig. 3. Effect of YP 12 on DNA synthesis of vascular smooth muscle cells induced by PDGF-BB. VSMCs were precultured in serum-free medium at different concentrations (1, 2 and 4 μM) of YP 12 for 24 h, and then induced by 25 ng/mL PDGF-BB. [^{3}H]-thymidine incorporation assay was performed as described in materials and methods. Data are expressed as mean ± S.E.M. from four different sets of experiments $^{**}P < 0.01$ *vs* PDGF-BB-induced VSMCs.

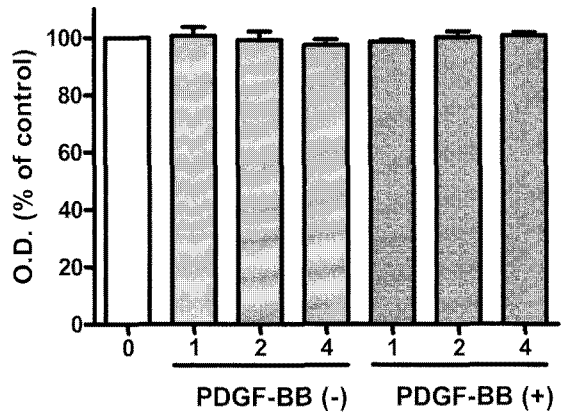

Fig. 4. Cytotoxic effect of YP 12. VSMCs were pre-cultured in serum-free medium at of different (1, 2 and 4 μM) concentrations of YP 12. After 24 h, cells were transferred to hemocytometer. WST-1 assay was performed as described in materials and methods.

Table 1. Effect of YP 12 on PDGF-BB-stimulated cell cycle progression

	G0/G1	S	G2/M
Control	87.1 ± 0.5	0.9 ± 0.7	12.0 ± 0.3
YP 12 (4 μM)	86.9 ± 0.2	1.0 ± 0.5	12.1 ± 0.5
PDGF-BB	73.0 ± 0.6$^{\#}$	14.2 ± 1.8$^{\#}$	12.8 ± 1.3
PDGF-BB + YP 12 (1 μM)	78.5 ± 1.4*	9.2 ± 1.7*	12.3 ± 0.2
PDGF-BB + YP 12 (2 μM)	83.8 ± 0.8**	4.3 ± 0.4**	11.9 ± 0.8
PDGF-BB + YP 12 (4 μM)	84.4 ± 0.5**	2.1 ± 0.8**	13.5 ± 0.9

The RASMCs were pre-cultured in the presence or absence of YP 12 (1-4 μM) in serum-depleted medium for 24 h, and then RASMCs were stimulated by 25 ng/ml PDGF-BB. After 24 h, individual nuclear DNA content was reflected 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. Results are means ± SD, n = 3. $^{\#}P < 0.01$ *vs.* PDGF-BB-unstimulated cells (Control). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* without YP 12 in PDGF-BB-stimulated cells.

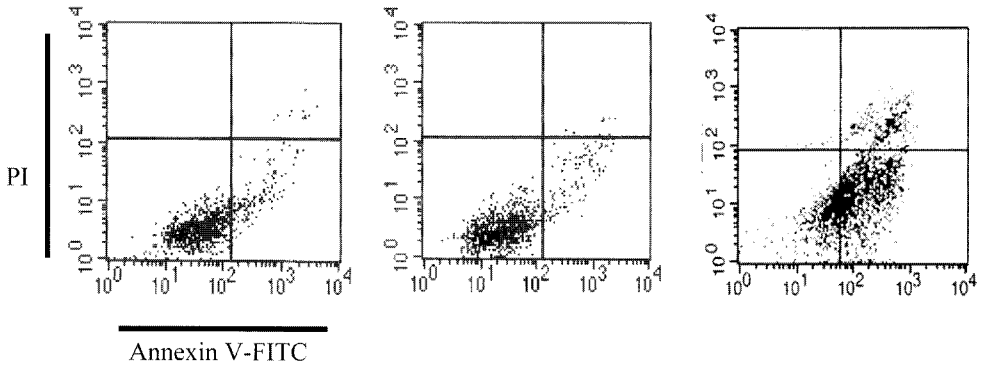

Fig. 5. Effect of YP 12 on apoptosis of PDGF-BB-stimulated VSMCs. Cells were pre-cultured in serum-free medium in the presence or absence of YP 12 (4 μM) or H_2O_2 (300 nM) for 24 h, and then stimulated with 25 ng/ml PDGF-BB for 24 h. Apoptotic (annexin V+/PI–) or necrotic cells (annexin V+/PI+) were identified by means of annexin V binding and PI dye detected by flow cytometry. Similar results of annexin V binding assay were found in three assays.

that its anti-proliferative effects in VSMCs are due to cell cycle arrest. Since anti-proliferative effects and cell cycle arrest are often accompanied by apoptosis, we measured VSMC apoptosis under the same conditions. Using Annexin-V staining, we observed no significant increase in apoptosis at the concentrations of YP 12 (1-4 μM) used in this study (Fig. 5). These results indicate the YP 12-mediated cell cycle arrest is not due to cellular toxicity or apoptotic cell death.

Discussion

The abnormal migration and proliferation of VSMCs in arterial walls are important pathogenetic factors of vascular disorders such as atherosclerosis and restenosis after angioplasty (Ross, 1993). Thus, inhibition of VSMCs proliferation represents a potentially important therapeutic strategy for the treatment of those diseases (Jung, Haendeler, Goebel, Zeiher, & Dimmeler, 2000). PDGF is one of the most potent mitogenic and chemotactic agents for VSMCs released by platelets, endothelial cells and VSMCs themselves, and is a major component of fetal bovine serum (Stroobant & Waterfield, 1984). The purposes of the present study were to find out whether YP 12, a newly synthesized obovatol derivate (Fig. 1), possesses an anti-proliferative effect on PDGF-BB-stimulated rat aortic VSMCs. In the present study, we demonstrated that YP 12 potently inhibited rat aortic VSMC proliferation (Fig. 2) and DNA synthesis (Fig. 3) induced by PDGF-BB. In addition, the anti-proliferative effect of YP 12 was associated with cell cycle arrest in the G0/G1 phase (Table 1) without any induction of apoptosis (Fig. 5). This indicates that YP 12 has a specific effect on cell cycle progression rather than decreasing the number of cells through apoptosis. It was recently reported that VSMCs are stimulated to divide in response to mitogens after a vascular injury, which results in their exit from the G1 phase and entry into the S phase (Ross, 1995). Some studies report that the G1 phase is a major point of control for cell proliferation in mammalian cells (Fang & Newport, 1991). Beyond this point, the cells are committed to DNA replication, and further cell cycle progression proceeds independently of growth factor stimulation.

Therefore, these results suggest that anti-proliferating agent can be a useful preventive or therapeutic agent for cardiovascular diseases including atherosclerosis, and YP 12 can be one of these candidate agents.

Acknowledgments

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