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INHIBITORY EFFECT OF COLCHICINE ON VASCULAR SMOOTH MUSCLE CELL PROLIFERATION INDUCED BY PLATELET-DERIVED GROWTH FACTOR-BB

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ABSTRACT

Abnormal proliferation of rat aortic vascular smooth muscle cells (VSMCs) is thought to play an important role in the pathogenesis of atherosclerosis and restenosis. The aim of this study was to elucidate the antiproliferative effects and molecular mechanism of colchicine on platelet-derived growth factor (PDGF)-BB-stimulated rat aortic vascular smooth muscle cells (VSMCs). Antiproliferative effects of colchicine on rat aortic VSMCs were examined by direct cell counting and by using [^{3}H] thymidine incorporation assays. It was found that colchicine potently the growth of VSMCs. Pre-incubation with colchicine (0.1∼10 μM) significantly inhibited proliferation. In accordance with these findings, colchicine blocked the PDGF-BB induced progression of synchronized cells through the G0/G1 phase of the cell cycle. Whereas, colchicine did not show any cytotoxicity in rat aortic VSMCs in this experimental condition by CCK-8 assay. Colchicine inhibited the PDGF-BB-stimulated phosphorylation of p38. Colchicine also inhibited the proliferating cell nuclear antigen (PCNA), a key regulator in the cell cycle. These findings suggest that the inhibition of colchicine to the cell proliferation. In conclusion, colchicine may be a potential anti-proliferative agent for the treatment of atherosclerosis and angioplasty restenosis.

KEYWORDS: Antiproliferative effects, Colchicine, Platelet-derived growth factor (PDGF)-BB, Vascular smooth muscle cells (VSMCs).

INTRODUCTION

Cardiovascular disease is increasing due to the recent rapid population aging and changes in dietary environment. According to the National Statistical Office (NSO) in Korea, deaths from cardiovascular diseases (heart disease, cerebrovascular disease, hypertension, diabetes, etc.) accounted for 25% of all deaths on 2015, followed by cancer. The number of medical personnel and medical expenses were 3.5 times and 1.4 times higher than cancer.[1] As the prevention and treatment of cardiovascular diseases become an important task in the age of aging, it is important to study the pathogenesis, mechanism, prevention and treatment of atherosclerosis, which is the most important risk factor of cardiovascular disease, in addition to hypertension, hyperlipidemia and diabetes.[2]

Medical angioplasty, such as balloon angioplasty or stent implantation, has been used to treat arteriosclerotic vascular disease, but approximately 10-15% of in-stent restenosis after stent implantation.[3] It still remains a problem. Proliferation of vascular smooth muscle cells (VSMCs) is an important step in neointimal hyperplasia in vascular disease, including arteriosclerosis, hypertension, and intimal stenosis following angioplasty.[4]

Platelet-derived growth factor-BB (PDGF-BB) is the most potent mitogen, a chemoattractant to vascular smooth muscle cells and plays a central role in the development of various vascular.[5] PDGF-BB activates various signal transduction pathways in vascular smooth muscle cells such as mitogen-activated protein kinase (MAPK), and plays an important role in cell proliferation, survival, apoptosis and the like by a cell response to stress.[6] Vascular smooth muscle cells remain stable in the G0 phase of the cell cycle under normal conditions, but when stimulated with growth factors such as cytokines or PDGF, they enter the cell cycle and cell.[7] In an effort to find a drug that inhibits the proliferation of vascular smooth muscle cells, it may be developed as a new drug using natural substances. However, this method has a disadvantage that the development period of the drug is considerably long. Therefore, drug repositioning techniques for generating another drug through the development of a new effect of the known drug are actively performed (24). Colchicine is an alkaloid-based ingredient that has been in use for a long time and is widely used worldwide in acute gout, psoriasis, scleroderma, and Behcet's disease.[8]

Colchicine has been studied for a long time, but its efficacy has been controversial. However, in 2009, the approval of the FDA has been actively studied for the safety and function of pharmacological agents for the past decade, and the interest in drug dosage and new indications has increased.[9] Colchicine has recently been studied for its potential as a therapeutic or prophylactic agent for cardiovascular disease and positive clinical studies have been reported.[10-11] These studies have only reported the preventive effects of cardiovascular disease according to dose, and no mechanism studies related to vascular smooth muscle cells have been reported.

In this study, we investigated the effect of colchicine on PDGF-BB induced vascular smooth muscle cell proliferation and investigated the possibility of using it for the treatment and prevention of vascular disease.

MATERIALS AND METHODS

Materials and reagents

Colchicine was purchased from Sigma-Aldrich (St. Louis, Mo., USA, 95% purity). Colchicine was prepared as a 200 mM stock solution with dimethyl sulfoxide (DMSO) and diluted with DMSO. The concentration of DMSO per medium did not exceed 0.1%. PDGF-BB was purchased from Koma Biotech. (Seoul, Korea, purity ≥ 98%) and dissolved in PBS (pH 7.4) to a final concentration of 50 ng/mL. The primary antibodies; anti-phospho-ERK1/2, anti-phospho-Akt, anti-phospho-p38 and anti-phospho-PDGFRβ was purchased from Cell Signaling Tech. (Danvers, MA, USA). Anti-PCNA and secondary antibody anti-mouse IgG horse radish peroxidase (HRP) were purchased from Santacruz Biotechnology (Santacruz, CA, USA). Inhibitors U0126, LY294002, SB203580, and AG1295 were purchased from Tocris Bioscience (Bristol, UK).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and trypsin. EDTA were purchased from Gibco-BRL (Grand Island, NY, USA). Unless otherwise noted, reagents and devices were purchased from Sigma-Aldrich and Nalge Nunc International (Naper Ville, IL, USA).

Cell culture

Aortic vascular smooth muscle cells of rats were purchased from Biobird (Seoul, Korea). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine, and cultured at 37°C and 5% CO_2. Cell culture was performed every 2-3 days and the cells were cultured in 6 ~ 10 passages under various conditions.

Measurement of cell proliferation

To measure vascular smooth muscle cell proliferation, 1 × 10^5 cell / mL was added to each well of a 12-well cell culture plate, and after 70% confluence, the medium was changed to a minimal medium and cultured for 24 hours. Thereafter, colchicine (0.1-10 μM) was added to the cell culture medium, and PDGF-BB 50 ng / mL was administered 24 hours later, and proliferation was induced for 24 hours. After inducing vascular smooth muscle cells, trypsin was added and the cells were transferred into a test tube and the number of cells was measured using a microscope and a hemocytometer.

Analysis of cell cycle progression

To analyze cell cycle progression, vascular smooth muscle cells were cultured in 6-well plates, washed with PBS, and cultured in minimal medium containing colchicine for 24 hours. PDGF-BB induced the proliferation of vascular smooth muscle cells for 24 hours. The cells were centrifuged at 1,200 rpm for 10 minutes, and the pellet was washed with 5 mL of PBS and centrifuged at 1,200 rpm for 10 minutes. The washed pellet was suspended in 70% ethanol and fixed at 4°C overnight. Fixed cells were suspended in the supernatant and centrifuged at 2,000 rpm for 5 minutes. The ethanol was discarded and stained with 0.5 mL of propidium iodide (PI) solution. After incubation for 1 hour at room temperature, the DNA-PI complexes of the nuclei of each sample prepared were measured with FACS Calibur (BD Biosciences Co., San Jose, CA, USA). G0/G1, S and G2/M ratio in the cell cycle were analyzed using ModFit LT V2.0 program (Verity Software House Inc., Topsham, ME, USA).

Cytotoxic effect analysis

To assess cytotoxic effects, vascular smooth muscle cells were inoculated in 96-well plates at a concentration of 5 × 10^3 cells / well in 200 μL of DMEM in 10% FBS. The cells were cultured for 48 hours at 37℃ in a 5% CO_2 atmosphere, and the medium was replaced with 100 μl of DMEM. After culturing for 24 h in minimal medium, colchicine at 10 μM was added and incubated for 24 h and treated CCK-8 (Dojindo Molecular Tech. Inc., Gaithersburg, MD, USA) reagent. The optical density (OD) at 450 nm wavelength was measured with an ELISA microplate reader and the degree of cytotoxicity was examined by comparing the OD value with the control group.

Statistical analysis

The results of all experiments were expressed as "mean ± standard error of the mean (SEM)" of representative values derived from independent experiments over three iterations. ANOVA was used for statistical comparison among the groups, statistical comparisons between the two groups were made by Student's t-test.

RESULTS

Effects of colchicine on PDGF-BB-induced vascular smooth muscle cell proliferation

In order to analyze the effect of colchicine on vascular smooth muscle cell proliferation, colchicine was administered to vascular smooth muscle cells at concentrations of 0.1, 1.0, and 10 μM, and 24 h later, 50 ng / mL PDGF-BB was induced to proliferate. Cell counts of vascular smooth muscle cells were directly counted using a hemocytometer. PDGF-BB-induced

vascular smooth muscle cell count was significantly lower than that of the control group and decreased significantly at concentrations of 1.0 and 10 μM in the pretreated group of colchicine ($^{*}p < 0.05$, $^{**}p < 0.01$) (Fig. 1).

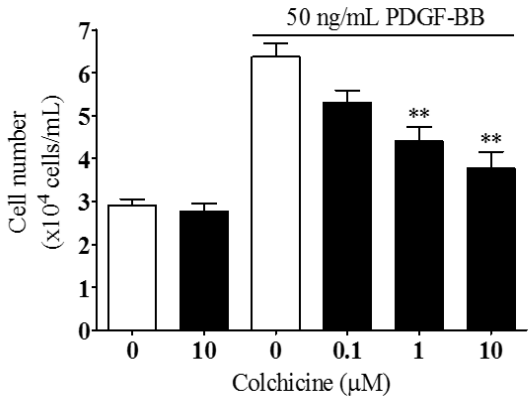

Fig. 1. Effect of colchicine on the PDGF-BB-induced vascular smooth muscle cell number. Rat aortic VSMCs were pre-cultured in serum-free medium in the presence or absence of colchicine (0.1~10 μM) for 24 hr, and then stimulated by 50 ng/mL PDGF-BB for a further 24 hr. Then the cells were trypsinized and were counted using a hemocytometer. The data were expressed as mean ± SEM (n = 4, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the PDGF-BB alone).

Effect of colchicine on cell cycle progression of vascular smooth muscle cells by PDGF-BB

Flow cytometric analysis was performed to evaluate the effect of colchicine on cell cycle progression of vascular smooth muscle cells. Cell cycle S levels were significantly increased by PDGF-BB compared to the control, but in the pretreatment group treated with colchicine, the S phase ratio was significantly decreased at 1.0 and 10 μM concentrations (Table 1).

This result confirms that G1/G1, which is the early stage of the cell cycle, does not progress and accumulates in the vascular smooth muscle cell proliferation. Thus, G1 checkpoint stage, which is an early stage of the cell cycle in vascular smooth muscle cell proliferation, The inhibition of proliferation of smooth muscle cells is shown in Table 1 and Fig. 3 ($^{*}P < 0.05$, $^{**}P < 0.01$).

Table 1: Effect of colchicine on PDGF-BB stimulated cell cycle progression.

PDGF-BB (50 ng/mL)	-	-	+	+	+	+
Colchicine (μM)	-	10	-	0.1	1	10
G0/G1	87.8±0.5	88.0±0.4	72.4±0.7	74.4±1.8*	77.7±0.8**	80.9±1.3**
S	1.3±0.4	1.4±0.5	14.4±1.6	12.6±2.1	9.5±0.9**	7.0±1.3**
G2/M	10.9±0.2	10.6±0.5	13.2±1.8	13.0±0.7	12.8±1.3	12.1±1.6

($^{*}p < 0.05$, $^{**}p < 0.01$)

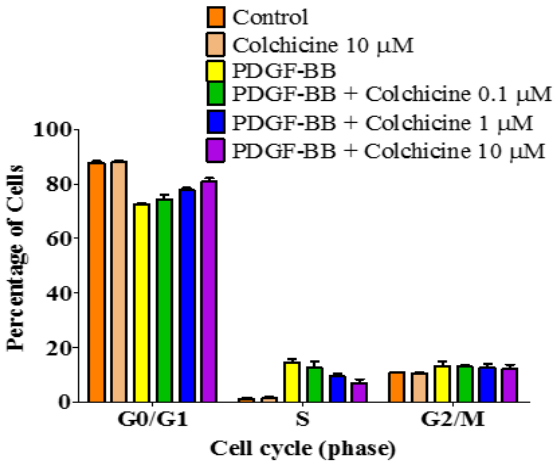

Fig. 2. Effect of colchicine on PDGF-BB-stimulated cell cycle progression. VSMCs were pre-cultured in the presence or absence of colchicine (0.1~10 μM) in serum-free medium for 24 hr, and then VSMCs were stimulated by 50 ng/mL PDGF-BB. After 24 hr, individual nuclear DNA content was reflected by fluorescence intensity of incorporated propidium iodide. Each item was derived from a representative experiment, where data from at least 10,000 events were obtained. The data were expressed as mean ± S.E.M from different set of experiments.

Effect of colchicine on vascular smooth muscle cell viability

The effect of colchicine on the viability and proliferation of vascular smooth muscle cells was investigated because the cell proliferation inhibitory effect can also be seen in cytotoxicity. Cell viability was measured for 24 h after treatment with the highest concentration of colchicine used for cell proliferation, 10 μM. As a result, no cytotoxicity was observed at a colchicine concentration of 10 μM (Fig. 3).

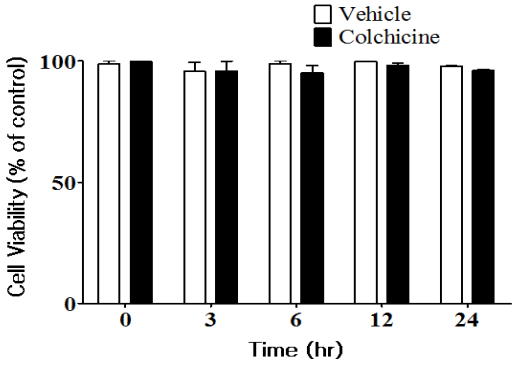

Fig. 3. Rat aortic vascular smooth muscle cell viability after treatment with colchicine. VSMCs viability of colchicine was determined by CCK-8 assay at 10 μM for 24 hr under serum starvation. The data were expressed as mean ± SEM (n = 3).

DISCUSSION

Colchicine inhibits the polymerization of microtubules by binding to tubulin, thus affecting all processes that require cytoskeletal changes, including cell mitosis and neutrophil. Recent studies have shown that colchicine can be useful in a wide range of cardiovascular diseases and is therefore increasingly interested in this drug.[12] The use of 0.5 mg colchicine twice daily for patients with chronic cardiovascular disease with high sensitivity C-reactive protein (hs-crp) despite the use of atorvastatin and aspirin resulted in an effective reduction of high sensitive C-reactive protein levels.[13] It has been reported that the use of colchicine once a day at 0.5 mg significantly reduces the incidence of cardiovascular disease and cardiac arrest or ischemic cardiovascular occlusion.[14] It has also been reported that intrathecal restenosis was significantly reduced by 0.5 mg of colchicine administered twice a day to patients with bare metal stents.[15] In the past few years, colchicine has been tested in a variety of clinical settings and has been actively studied for new indications including cardiovascular disease. However, the studies reported so far only report a correlation between the dose of colchicine and cardiovascular disease.

In this study, we investigated the effect of colchicine on vascular smooth muscle cell proliferation, a major cause of arteriosclerosis, by performing molecular biochemical experiments using colchicine in vascular smooth muscle cells induced by PDGF-BB.

PDFG is one of the important growth factors acting on vascular smooth muscle cell proliferation. PDGF-BB acts most strongly in vascular smooth muscle cells among many subtypes.[16] In this study, PDGF-BB was used as a growth factor of vascular smooth muscle cells and vascular smooth muscle cells were counted using a hemocytometer to investigate the effect of colchicine on colchicine proliferation in vascular smooth muscle cells. The number of vascular smooth muscle cells was significantly increased by PDGF-BB and decreased by colchicine in a concentration-dependent manner (Fig. 1). Vascular smooth muscle cell proliferation is manifested by several types of mitotic stimulating factors, which are important in cardiovascular disease. Since it is difficult to control the proliferation by many stimulating factors, studies are under way to regulate cell proliferation through cell cycle regulation, which is the final common pathway.[7] Recent studies have shown that arresting the cell cycle of vascular cells not only inhibits cell proliferation, but also alleviates changes in vascular cell phenotype and substantially reduces the susceptibility of certain blood vessels involved in atherosclerosis.[17] The effect of colchicine on the cell cycle of vascular smooth muscle cells was analyzed.

PDGF-BB decreased the percentage of cells in the G0/G1 phase and the ratio of G0/G1 phase significantly increased by colchicine (Fig. 2). Cell ratio of S phase was significantly increased by PDGF-BB and decreased by colchicine. In other words, it was confirmed that colchicine did not pass to S phase but accumulated in G1 period. These results suggest that colchicine is involved in the inhibition of vascular smooth muscle cell proliferation by stopping cell cycle G1.

The effect of colchicine on the survival of vascular smooth muscle cells was investigated because this cell proliferation inhibitory effect may also be caused by cytotoxicity by colchicine. Cell viability was measured for 24 hours after treatment with the highest concentration of colchicine used, 10 μM. Cytotoxicity did not appear as in Fig. 3. That is, colchicine is thought to inhibit vascular smooth muscle cell proliferation without cytotoxicity. MAPK is a protein kinase that is activated on the surface of cells and is activated by the stimuli outside the cell. Through the process of regulating transcription factors, MAPK is a representative example of regulating stress-induced cell responses such as cell differentiation, cell proliferation and cell apoptosis.[18] The MAPK pathway, including ERK1/2, JNK and p38, plays an important role in the initial intracellular cleavage for cell growth and is involved in the proliferation and migration of various cell types induced by PDGF-BB.[19]

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