EFFECT OF COLCHICINE FOR DNA SYNTHESIS AND EXPRESSION ON VASCULAR SMOOTH MUSCLE CELL BY PLATELET-DERIVED GROWTH FACTOR-BB

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ABSTRACT

Vascular smooth muscle cell (VSMC) migration is regulated by a variety of factors. In this study, we investigated the effect of colchicine on the effect of colchicine on vascular smooth muscle cell DNA synthesis and proliferating cell nuclear antigen (PCNA) Expression by PDGF-BB. In order to determine the degree of DNA synthesis, vascular smooth muscle cells were plated on a 24-well culture plate and 70% confluence was applied, and colchicine (0.001 to 10 μM) was administered after 24 hours. PDGF (Platelet-derived growth factor (PDGF)-BB increased significantly the amount of DNA synthesis in vascular smooth muscle cells than in the control group. As colchicine concentrations were increased to 0.1, 1.0, and 10 μM, DNA synthesis was inhibited to 31.8, 48.4, and 56.0%, respectively. Among ERK (Extracellularly Regulated Kinase)1/2, Akt, and p38 MAPK (Mitogen Activated Protein Kinase) in the case of colchicine treatment, p38 MAPK showed a significant decrease in phosphorylation as the concentration of colchicine increased. The colchicine proliferation inhibitory effect was due to blocking of the p38 MAPK pathway and did not affect ERK1/2 and Akt pathway.

Keywords: colchicine, DNA synthesis, Mitogen activated protein kinase (MARK), Platelet-derived growth factor (PDGF)-BB.

INTRODUCTION

Globally, cardiovascular diseases (CVDs), which include coronary heart disease (CHD), strokes, rheumatic heart disease (RHD), cardiomyopathy, and other heart diseases, represent the leading cause of death (World Health Statistics 2009, 2009). Cardiovascular diseases (CVD) are predicted to be the major causes of morbidity and mortality in most developing nations around the world (Celermajer et al., 2012). The status in prevention and control of cardiovascular disease in this large and aging country with about 1/5 of the world total population bears a huge potential impact on global health (Wu et al., 2016).

Platelet-derived growth factor (PDGF), an agent with important mitogenic effects for bone cells, exists in three isoforms, PDGF-AA, -BB, and -AB. PDGF-AB and -BB are the prevalent circulating isoforms, whereas normal unstimulated cells of the osteoblast lineage synthesize primarily PDGF-AA (Rydziel et al., 1994). PDGF-BB induces the proliferation and migration of PASMCs and has been proposed to be a key mediator in the progression of pulmonary artery smooth muscle cells (PASMCs). Vascular smooth muscle cell (VSMC) migration is regulated by a variety of factors, including platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-I (IGF-I) (Ross, 1993). PDGF-BB is the most potent known chemoattractant for VSMCs in culture (Ross, 1993) and PDGF-induced

migration of VSMCs is thought to play a major role in neo-intima formation in atherosclerosis and in restenosis (Cospedal, 1999).

The Extracellularly Regulated Kinase/Mitogen Activated Protein Kinase (ERK/MAPK) signaling pathway is a critical regulator of cellular processes in adult and developing tissues. The signal starts when a signaling molecule binds to the receptor on the cell surface and ends when the DNA in the nucleus expresses a protein and produces some change in the cell, such as cell division. Depending on the cellular context, MAPK cascade can act as a rheostat, a switch, or an oscillator. The ERK/MAPK pathway, which is downstream of receptor tyrosine kinases, leads to phosphorylation, and hence activation, of ERK and has been shown to be important in branching morphogenesis in several systems (Liu et al., 2007).

Colchicine is an alkaloid isolated from *Colchicum autumnale*. Colchicine is a medication most commonly used to treat gout and anti-inflammatory activities. Colchicine binds to tubulin, thereby interfering with the polymerization of tubulin, interrupting microtubule dynamics, and disrupting mitosis (Lu et al., 2012). While colchicine may confer lower risk for a second CV event in the general population, there are sparse data in gout where colchicine may also be indicated for disease control, treatment and/or prevention. One study showed a reduced prevalence of MI among colchicine users compared with non-users (Solomon et al., 2016).

In this study, we investigated the effect of colchicine on the effect of colchicine on vascular smooth muscle cell DNA synthesis and proliferating cell nuclear antigen (PCNA) Expression by PDGF-BB. This dataset provides drug prescribing, pharmacy filling, hospitalizations, and laboratory data on treatment and prevention of vascular disease.

METHODOLOGY

Materials

Colchicine was purchased from Sigma-Aldrich (St. Louis, Mo., USA). Colchicine was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. 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 phosphate-buffered saline (PBS) 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 Biotech. (Santacruz, CA, USA). Inhibitors U0126, LY294002, SB203580, 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).

Measurement of DNA Synthesis

In order to determine the degree of DNA synthesis, vascular smooth muscle cells were plated on a 24-well culture plate and 70% confluence was applied, and colchicine (0.001 to 10 μM) was administered after 24 hours. Twenty-four hours after colchicine administration, 50 ng / mL of PDGF-BB was added and the proliferation was induced for 20 hours. 1 μCi/mL [^{3}H] - thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for labelling was added to the medium for 4 hours and then the medium was removed. PBS containing 10% trichloroacetic acid (TCA) on ice and ethanol/ether (1:1, v/v) were used for washing. [3H] -

thymidine not dissolved in TCA was extracted by adding 250 μL of 0.5 M NaOH per well. 100 μL of this solution was mixed with 5 mL of scintillation cocktail (Ultimagold, Packard Bioscience Co., Meriden, CT) and were counted with a liquid scintillation counter (LS3801, Beckmam, Düsseldorf, Germany).

Western Blot Analysis

The cells were washed once with cold PBS. Lysis buffer (20 mM Tris, pH 7.0, 137 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 0.2 mM PMSF, 1 μg/mL aprotinin, 20 μM leupeptin, 1 mM Na3VO4, 1 mM EGTA, pH 8.0, 1 mM pyrophosphate, 1 mM β-glycerophosphate) was added and reacted on ice for 10 minutes. Cells were centrifuged at 12,000 rpm at 4°C for 15 minutes to isolate the total protein in the supernatant. Proteins of the supernatant were quantified using the Bradford method (Bio-Rad, Hercules, Calif., USA). The samples were mixed with sample buffer (12 mM Tris-HCl, pH 8.0, 0.5% glycerol, 0.4% SDS, 2.88 mM 2-mercaptoethanol, 0.02% bromophenol blue) and heated at 95°C for 5 minutes. Samples were electrophoresed SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose adsorbent (Bio-Rad). Absorbent paper was placed in 5% non fat dry milk blocking solution and reacted for 1 hour at room temperature to suppress nonspecific immune response. After washing twice with Tris buffer containing 0.1% Tween 20 every 10 minutes, primary antibody was reacted at room temperature for 2 hours and then washed four times for 15 minutes each. Proliferating-cell nuclear antigen (PCNA) antibody was calibrated with α-actin antibody. The secondary antibody was reacted at room temperature for 1 hour and then washed four times for 15 minutes each. Secondary antibodies were detected using Enhanced chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK) kit.

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM), $*p < 0.05$, $** p < 0.01$. Statistical comparisons between the two groups were made by Student's t-test. The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups.

RESULTS

Effect of Colchicine on the Synthesis of Vascular Smooth Muscle Cell DNA y PDGF-BB

DNA synthesis was essential for cell proliferation and the degree of synthesis of [3H] -thymidine was confirmed in order to confirm the effect of colchicine on vascular smooth muscle cell DNA synthesis. PDGF-BB increased significantly the amount of DNA synthesis in vascular smooth muscle cells than in the control group. However, DNA synthesis in the experimental group stimulated with PDGF-BB pretreated with colchicine (0.001-10 μM) 8, which inhibited DNA synthesis in a concentration-dependent manner. As colchicine concentrations were increased to 0.1, 1.0, and 10 μM, DNA synthesis was inhibited to 31.8, 48.4, and 56.0%, respectively ($^{*}p < 0.05$, $^{**}p < 0.01$) (Fig. 1).

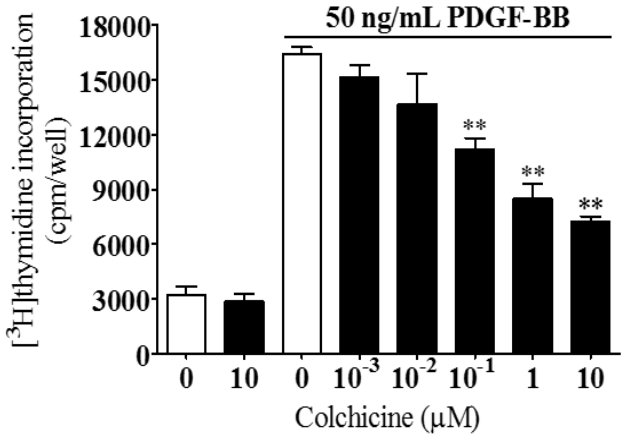

Figure 1. Effect of colchicine on DNA synthesis induced by PDGF-BB in rat aortic vascular smooth muscle cells. VSMCs were pre-cultured in serum-free medium in the presence or absence of colchicine (0.001~10 μM) for 24 hr, and then stimulated with 50 ng/mL PDGF-BB for 20 hr. [^{3}H]-thymidine (1 mCi/mL) was added to the medium, and cells were incubated for 4 hr. Radioactivities were determined using a liquid scintillation counter. The data were expressed as means ± SEM (n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the PDGF-BB alone).

Effect of Colchicine on the Protein Expression of Cell Proliferation Signal Transduction System by PDGF-BB

MAPK is an intracellular signaling system and Western blot analysis was performed to investigate the association of colchicine-induced MAPK pathway with PDGF-BB-induced vascular smooth muscle cells. Among ERK1/2, Akt, and p38 MAPK in the case of colchicine treatment, p38 MAPK showed a significant decrease in phosphorylation as the concentration of colchicine increased (Fig. 2). However, ERK1/2 and Akt showed activity even when colchicine concentration increased. The colchicine proliferation inhibitory effect was due to blocking of the p38 MAPK pathway and did not affect ERK1/2 and Akt pathway.

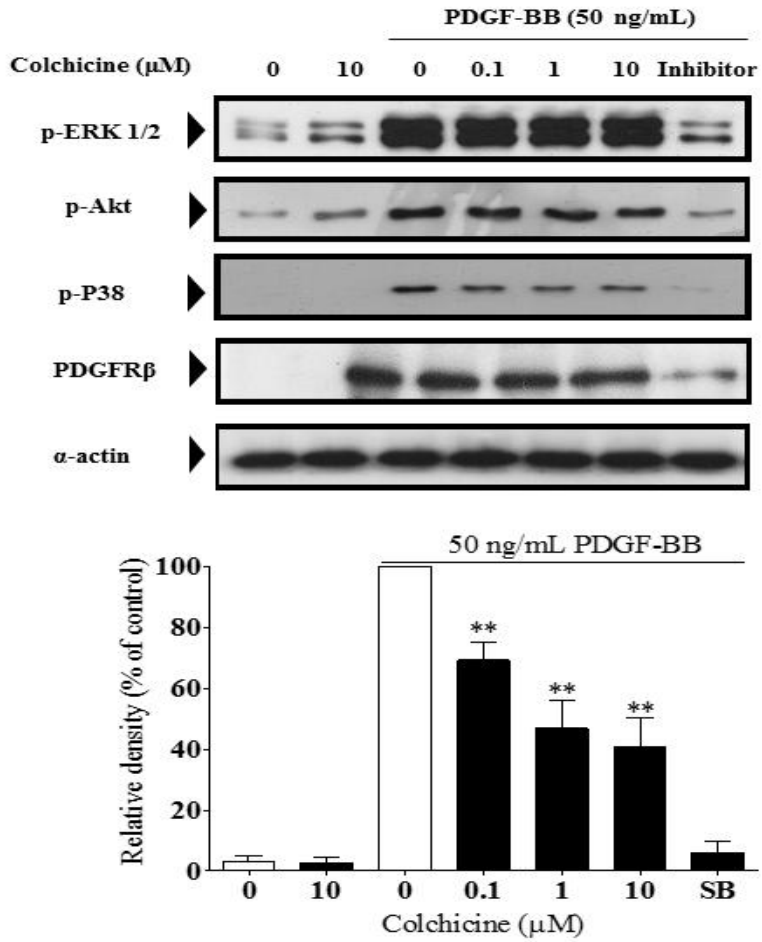

Figure 2. Effect of colchicine on ERK1/2, Akt, p38 and PDGF-Rβ phosphorylation in VSMCs. Confluent cells were pre-cultured in the presence or absence of colchicine (0.1-10 μM), U0126 (an ERK1/2 inhibitor, 10 μM), LY294002 (a PI3K/Akt inhibitor, 50 μM), SB203580 (a p38 inhibitor, 20 μM), or AG1295 (a PDGF-Rβ inhibitor, 20 μM) in serum-free medium for 24 hr. The cells were stimulated by 50 ng/ml PDGF-BB for 5 min for ERK1/2, 15 min for Akt and p38, and 1 min for PDGF-Rβ assay at 37℃, respectively. The cells were lysed, and proteins were analyzed using SDS–PAGE and immunoblotting (n = 3).

Effect of Colchicine on PCNA Expression by PDGF-BB

When an abnormality occurs in the accuracy and timing of the cell cycle, an abnormality occurs in the control function of the cell cycle, destroys the homeostasis of each tissue and organism, and eventually causes various diseases including cancer. Cells have checkpoints to maintain homeostasis that tightly controls the cell cycle, confirming that the cells have met the conditions for replication and cleavage. The G1 checkpoint, which is the process of determining the G1/S period, that is, the preparation process of DNA replication and the environment in which the cells can proliferate, is blocked. Thereby inhibiting cell proliferation by blocking the cell cycle (Fig. 3). These results indicate that PCNA expression is suppressed. PDGF-BB-induced PCNA expression was significantly increased compared to the control group, but significantly decreased at 1.0 and 10 μM concentrations in the pretreatment group treated with colchicine ($^{*}p < 0.05$, $^{**}p < 0.01$).

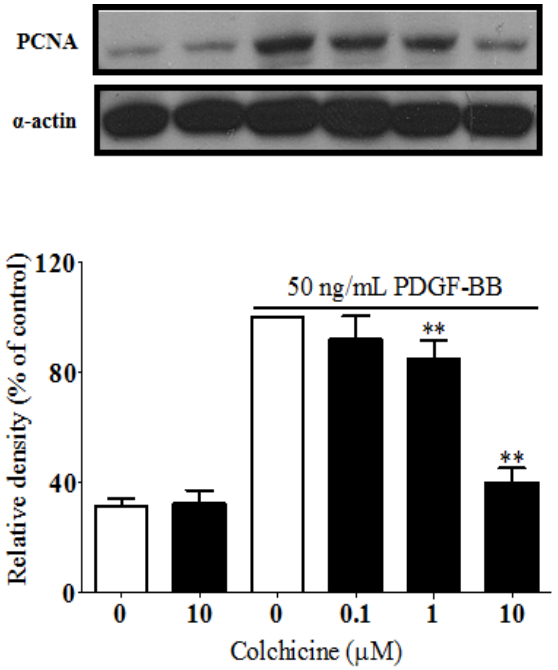

Figure 3. Effect of colchicine on expression of PCNA in VSMCs treated with PDGF-BB. Confluent cells were pre-cultured in the presence or absence of various concentrations of colchicine for 24 hr, and then stimulated by 50 ng/mL PDGF-BB at 37℃ for 24 hr. The cells were lysed, and proteins were analyzed using 12.5% SDS–PAGE and immunoblotting. After densitometric quantification, the data were expressed as mean ± SEM (n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the PDGF-BB alone).

DISCUSSION

DNA synthesis is essential for cell division. [^{3}H]-thymidine is used as an indicator of DNA synthesis because it is used in nuclear DNA synthesis in cell cycle S and displays all cells passing through S phase (Jiang et al., 2013). [^{3}H]-thymidine assay on the DNA synthesis of vascular smooth muscle cells of colchicine showed that the proliferation rate, which was increased by PDGF-BB, was also decreased in dependence on colchicine concentration (Fig. 1).

We investigated the effect of colchicines on protein expression in the cell proliferation signaling system. Western blot analysis was performed to observe changes in MAPK by colchicine in PDGF-BB-induced vascular smooth muscle cells. Activation of both ERK1/2, Akt, p38 MAPK and PGDFRβ by PDGF-BB stimulation was observed. However, phosphorylation was significantly decreased with increasing concentration of colchicine only in p38 MAPK. It was also found that ERK1/2, Akt and PGDFRβ did not change with increasing colchicine concentration (Fig. 2). These results suggest that colchicine suppresses vascular smooth muscle cell proliferation through inhibition of p38 MAPK signaling pathway in PDGF-BB stimulated vascular smooth muscle cell proliferation. Recent studies have shown that p38 MAPK pathway can control checkpoint control and cell cycle in G0, G1 / S and G2/M transitions depending on cell type (Speir & Epstein. 1992). The p38 MAPK signaling pathway is involved in the regulation of vascular smooth muscle cell proliferation by regulating the expression of cell cycle related proteins (Simons et al., 1994). Our results

are consistent with previous studies in which inhibition of the p38 MAPK signaling pathway inhibits cell proliferation by stopping the cell cycle.

In other words, p38 MAPK is an important protein that mediates the proliferation of vascular smooth muscle cells induced by PDGF-BB, and these proteins can be a target molecule for the development of a new therapeutic agent to prevent angioproliferative diseases.

PCNA is involved in DNA synthesis in the S phase of the cell cycle, and is known to bind DNA polymerase to regulate the synthesis of DNA, and has been reported to play a pivotal role in cell proliferation and regulation (Robbins et al.,1987). Expression of PCNA increases during cell cycle division and has been regarded as an important target to inhibit vascular smooth muscle cell proliferation both in vitro and in vivo. In addition, a marked increase in PCNA expression was demonstrated after balloon angioplasty in the carotid injury model (Speir & Epstein. 1992; Simons et a.,1994). Analysis of the effect of colchicine on PCNA expression revealed that PCNA expression, which was increased in PDGF-BB, was significantly increased compared to the control, but colchicine was decreased in a dose-dependent (Fig. 3). In the present study, colchicine inhibited the phosphorylation of p38 MAPK and terminated the cell cycle G0/G1 stage, resulting in decreased DNA synthesis and decreased expression of PCNA, leading to the proliferation of PDGF-BB-induced vascular smooth muscle cells. These results suggest that colchicine is a candidate for the management and prevention of arteriosclerosis and stent restenosis, and further studies on colchicine such as in vivo studies and in vivo experiments are needed.

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