ejpmr, 2018,5(9), 133-137

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EFFECT OF VASCULAR SMOOTH MUSCLE CELL PROLIFERATION OF URSOLIC ACID

Jun Hui Park[1], Man Kyu Huh[2] and Yong Lim[1*]

[1]Department of Clinical Laboratory Science, Dong-eui University, Busan 47340, Republic of Korea.
[2]Food Engineering and Technology Major, Dong-eui University, Busan 47340, Republic of Korea.

***Corresponding Author: Yong Lim**

Department of Clinical Laboratory Science, Dong-Eui University, Busan 47340, Republic of Korea.

Article Received on 10/07/2018 **Article Revised on 30/07/2018** **Article Accepted on 20/08/2018**

ABSTRACT

Ursolic acid is present in many plants, such as *Mirabilis jalapa*, as well as in many fruits and herbs. Ursolic acid has been reported to exhibit multiple biological effects including anticancer, antioxidant, anti-inflammatory, and antiviral efficacy. In this study, we investigated the effect of ursolic acid cell proliferation in rat vascular smooth muscle cells (VSMCs). Ursolic acid was found to inhibit cell proliferation and DNA synthesis in cultured VSMCs. Pretreatment with ursolic acid (10~50 μM) suppressed platelet-derived growth factor-BB (PDGF-BB) that stimulated cell number in a concentration-dependent manner. Moreover, ursolic acid inhibited [^{3}H]-thymidine incorporation into DNA in VSMCs in response to 50 ng/mL PDGF-BB. PDGF-BB stimulated DNA synthesis was significantly reduced by 61.6% and 87.2% at concentrations of 30 and 50 μM, respectively. Thus, ursolic acid blocked the PDGF-BB–inducible progression through G0/G1 to S phase of the cell cycle in synchronized cells. Ursolic acid appeared to activated platelets Induced growth factors such as PDGF, which may prevent the excessive proliferation of VSMCs.

KEYWORDS: Platelet-derived growth factor-BB, ursolic acid, vascular smooth muscle cell (VSMC).

INTRODUCTION

The blood vessels are composed of an intima layer (composed of endothelial cells), a medium (composed of smooth muscle cells and elastic tissues), and adventitia (composed of connective tissue of fibroblasts). VSMCs (VSMCs) are the main cells that make up the blood vessel walls together with endothelial cells. They regulate blood vessel tension by contracting, blood pressure, and blood flow.[1]

Atherosclerosis is a form of chronic inflammation associated with a number of cytokines secreted by T cells, macrophages, endothelial cells and VSMCs.[2] VSMCs stimulate leukocytes to activate, induce smooth muscle cell proliferation, inhibit endothelial cell function, and secrete a special cytokine that stimulates the production of extracellular components. The major cytokines are platelet-derived growth factor (PDGF), transforming growth factor-β, macrophage inhibitory factor (MIF), interferon gamma (IFNγ), and monocyte chemoattractant protein (MCP-1).[3] PDGF is the most important growth factor secreted in activated platelets. The association of PDGF with vascular smooth muscle cell proliferation has been confirmed in animal studies and increased levels of PDGF-B after arterial injury have been associated with neointimal hyperplasia.[4] PDGF has strong chemotaxis in VSMCs, leading to migration and proliferation of VSMCs.[5] In addition, when PDGF binds to the PDGF receptor, autophosphorylation of the receptor leads to tyrosine kinase activity and phosphorylation of exogenous proteins, resulting in the synthesis of new DNA.[6] MAPK (mitogen-activated protein kinase) pathway is also involved.[7] In other words, during the progression of atherosclerotic lesions, VSMCs migrate from the normal position to the middle membrane, proliferate, and when the lesion enlarges with extracellular lipid deposition, it causes vascular occlusion.[3]

Ursolic acid (sometimes referred to as urson, prunol, malol, or 3-beta-3-hydroxy-urs-12-ene-28-oic-acid), is a pentacyclic triterpenoid identified in the epicuticular waxes of apples as early as 1920 and widely found in the peels of fruits. Ursolic acid is present in many plants, such as *Mirabilis jalapa*, as well as in many fruits and herbs. A number of potential biochemical effects of ursolic acid have been investigated, but there has been no clinical study demonstrating benefits to human health. *In vitro*, ursolic acid inhibits the proliferation of various cancer cell types by inhibiting the STAT3 activation pathway,[8-9] and may also decrease proliferation of cancer cells and induce apoptosis.[10]

Ursolic acid (UA)-treated cells were more sensitive to transforming growth factor- (TGF-) β-mediated apoptosis than were the control cells. In vivo

experiments showed that hepatitis B viruses HBV (hepatitis B virus)-induced tumors were significantly lower in UA-treated animals when compared to controls.[11] These interesting studies showed that UA could block the pathological effects of HBV in cell lineages, suggesting that new classes of antiviral drugs could be developed using UA.[12]

The present study investigates the potential use of ursolic acid against the inhibitory effect of platelet-derived growth factor-BB (PDGF-BB)-mediated VSMCs, and the future perspectives about the use of compounds for human or even animal health are also discussed.

MATERIALS AND METHODS

Reagents and instruments

Ursolic acid (CAS No. 77-52-1; Sigma-Aldrich, St. Louis, Mo., USA) was prepared with 200 mM stock solution with dimethyl sulfoxide (DMSO). The concentration of DMSO per medium did not exceed 0.1%. PDGF-BB was purchased from Koma Biotech. (Seoul, Korea) and dissolved in PBS (pH 7.4) to a final concentration of 50 ng / mL. Dulbecco's Modified Eagle Medium, FBS, and trypsin-EDTA were purchased from Gibco-BRL (Grand Island, NY, USA), and reagents and devices were purchased from Sigma-Aldrich and Nalge Nunc International (Naper Ville, IL, USA).

Experimental animals and breeding conditions

New Zealand white male rabbits weighing 2 kg were purchased from Sam Taco Bio Korea (Osan, Gyeonggi-do) and weighed 2.3±0.3 kg after 10 days of circulation. Drinking water was infused with ultraviolet sterilized purified water. Animals were housed in a stainless steel cage (380 W × 490 L × 350 H mm) for the entire circulation periods and the experimental periods. The animals were maintained at a temperature of 23±2°C, a relative humidity of 50 ± 10%, a ventilation frequency of 10 to 15 times/hr, a lighting cycle of 12 hours (light) with lighting of 150 to 300 lux and 12 hours (dark). This study was conducted according to the guidelines of the Animal Experiment Ethics Committee of Dong-eui University.

Cell culture

Aortic VSMCs of rats used in the study were purchased from BioBird Co., Ltd. (Seoul, Korea). VSMCs were cultured in a DMEM medium containing 10% FBS and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere incubator at 37℃, and the medium was changed every 2-3 days.

Cell proliferation measurement

To measure vascular smooth muscle cell proliferation, 1×10^5 cell/mL was added to each well of a 12-well cell culture plate, followed by incubation for 24 hours with minimal medium replaced with 70% confluence. Ursolic acid (10, 30, and 50 μM) was added to the cell culture medium and 24 hours later and proliferation was induced with 50 ng/mL PDGF-BB. The VSMCs that were proliferated for 24 hours were treated with trypsin-EDTA and then counted using a hemocytometer.

Measurement of DNA synthesis

VSMCs were plated on 24 well culture plates and 70% confluence was applied. After replacing with minimal medium, uric acid was treated at each concentration (10, 30, and 50 μM). Twenty-four hours after the treatment with uric acid, 50 ng/mL PDGF-BB was added and [^{3}H] -thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to the medium. [^{3}H] -thymidine After 4 hours of reaction, the medium was removed and washed with PBS containing 10% trichloroacetic acid (TCA) and ethanol / ether (1 : 1, v/v) on ice. [^{3}H] -thymidine, which is not dissolved in TCA, is extracted by adding 250 μL of 0.5 M NaOH per well. To 100 μL of this solution is added 5 mL of scintillation cocktail (Ultima Gold, Packard Bioscience Co., Meriden, CT, USA) scintillation counter (LS3801, Beckman, Dusseldorf, Germany).

Cell cycle progression analysis

VSMCs were cultured in 6-well plates, washed with PBS, and cultured for 24 hours in a minimal medium supplemented with uric acid (10, 30, and 50 μM). PDGF-BB induced the proliferation of VSMCs for 24 hours. The cells were centrifuged at 1,200 rpm for 10 minutes, pelleted with 5 mL of PBS, centrifuged at 1,200 rpm for 10 minutes, and washed twice. The washed pellet was fixed with 70% ethanol and fixed overnight at 4°C. The fixed cells were centrifuged at 2,000 rpm for 5 minutes. The ethanol was discarded and stained with 0.5 mL of propidium iodide (PI) solution for 1 hour at room temperature. The ratio of cell cycle G0/G1, S and G2/M groups was determined by ModFit (BD Biosciences Co., San Jose, CA, USA) LT V2.0 program (Verity Software House Inc., Topsham, ME, USA).

Assessment of cytotoxic effect

VSMCs were inoculated into 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°C and 5% CO_2. After the culture medium was replaced with 100 μL of DMEM for 24 hours, the cells were treated with 50 μM of uric acid and cultured for another 24 hours. To evaluate the cytotoxic effect, OD value was measured at 450 nm wavelength using an ELISA microplate reader after Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA).

Statistical processing

The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The results were expressed as the mean ± SD. One-way analysis of variance was applied to determine differences in means. A P value <0.05 denoted statistical significance.

RESULTS

Inhibition of vascular smooth muscle cell DNA synthesis by ursolic acid

[^{3}H]-thymidine can be used to determine the proliferation of cells by inserting it into newly synthesized chromosomal DNA during cell mitosis. The degree of synthesis of [^{3}H]-thymidine was confirmed in order to examine the inhibitory effect of uronic acid on vascular smooth muscle cell proliferation. The amount of DNA synthesis of VSMCs induced by PDGF-BB for 20 hours was significantly increased compared to the control group. However, DNA synthesis induced by PDGF-BB pretreatment with uric acid was inhibited in a concentration-dependent manner compared to PDGF-BB alone without treatment with uric acid. At concentrations of uric acid 30 µM and 50 µM, DNA synthesis was strongly inhibited to 61.6% and 87.2%, respectively (Fig. 1)($^*P < 0.05$, $^{**}P < 0.01$).

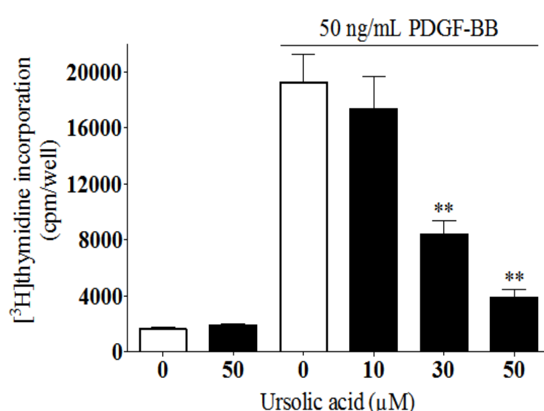

Fig. 1. Effect of ursolic acid DNA synthesis induced by PDGF-BB in rat aortic vascular smooth muscle cell.

VSMCs were pre-cultured in serum-free medium in the presence or absence of ursolic acid (10~50 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. Results were the means±SD of three independent experiments. $^{**}P < 0.01$ compared with PDGF-BB treatment alone.

Effects of ursolic acid on cell cycle progression of VSMCs

In order to investigate the effects of uric acid on the cell cycle progression of VSMCs, the proportion of G0/G1 cells was decreased by PDGF-BB and the ratio of S-phase cells was significantly increased compared to the control group. However, in the experimental group pretreated with uric acid, the cell ratio of G0/G1 was recovered and the cell ratio of S phase was decreased in a concentration dependence (Table 1).

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

PDGF-BB (50 ng/mL)	-	-	+	+	+	+
Ursolic acid (µM)	-	50	-	10	30	50
G0/G1	84.7±1.3	85.3±0.4	66.7±0.7	70.0±1.8**	71.9±0.9**	78.1±1.3**
S	2.1±1.0	1.9±1.1	19.3±0.8	16.4±1.3*	14.6±0.8**	9.3±1.7**
G2/M	13.2±0.8	12.8±1.6	14.0±2.2	13.6±0.9	13.5±1.3	12.6±1.7

Effect of ursolic acid on viability of VSMCs

Cell viability was measured because the effect of inhibiting cell proliferation by uric acid may also occur in cytotoxicity. Cell viability was measured for 24 hours after treatment with the highest concentration of uric acid used in cell proliferation (50 µM), and no cytotoxicity was observed at the highest concentrations used in the experiment (Fig. 2).

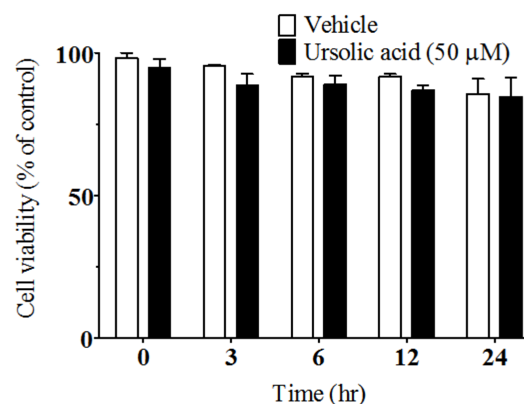

Fig. 2. Rat aortic vascular smooth muscle cell viability after treatment with ursolic acid.

VSMCs viability of ursolic acid was determined by CCK-8 assay at 50 µM for 24 hr under serum starvation. Data are expressed as mean±SD (n = 3).

DISCUSSION

Cell proliferation was induced by PDGF-BB in order to examine the proliferation inhibitory effect of VSMCs of uric acid. PDGF is the most abundant growth factor in α-granules of platelets, and PDGF-BB acts most strongly in vascular smooth muscle cell proliferation.[3] The number of VSMCs induced only by PDGF-BB was significantly increased compared with the control group. In the group induced by pretreatment with uric acid, the number of cells decreased in a concentration-dependent manner, indicating that uric acid inhibited vascular smooth muscle cell proliferation. [^{3}H] -thymidine can be used to determine the proliferation of cells by inserting it into newly synthesized chromosomal DNA during cell mitosis. In order to confirm the effect of inhibiting proliferation of VSMCs by uric acid, the degree of [^{3}H] -thymidine synthesis was confirmed.[13] PDGF-BB significantly increased the amount of DNA synthesis in VSMCs than in the control group. However, DNA synthesis in the experimental group induced by PDGF-BB pretreatment with uric acid was inhibited in a concentration-dependent manner. Vascular smooth muscle cell proliferation is manifested by several types of mitotic stimulating factors, which are important in cardiovascular disease. Because cell proliferation occurs when cell cycle progresses, cells have checkpoints to maintain homeostasis that strictly controls the cell cycle, confirming that the cells have met the conditions for replication and division. Since cell proliferation is regulated by a number of stimulating factors, studies are under way to regulate cell proliferation through regulation of the cell cycle, a common pathway.[14] The effects of uric acid on the cell cycle of VSMCs were significantly increased by PDGF-BB compared to the control group, but decreased in a concentration-dependent manner in the test group pretreated with uric acid. UA exerts anti-proliferation effects in rat primary VSMCs, which is associated with the inhibition of miRNA-21 expression and modulation of PTEN/PI3K signaling pathway.[15] These results suggest that smooth muscle cell proliferation is inhibited by regulating G1 checkpoint, which is an early stage of cell cycle in vascular smooth muscle cell proliferation. In order to determine whether the inhibitory effect of uric acid on cell proliferation is related to cytotoxicity, cell viability was measured and the cytotoxicity was not observed at the concentrations used in the experiment. These results suggest that uricosan can inhibit proliferation of VSMCs by regulating cell cycle through G1 check point without cytotoxicity. Vascular smooth muscle cell proliferation has been involved in the pathophysiology of a variety of maladies, including atherosclerosis and post-angioplasty restenosis.[16-17] Coronary stenting has drastically reduced the rates of restenosis and this effect was potentiated by the association of drug-carrying polymers to stents.[18] The so called VSMC antiproliferative drug-eluting stents have brought restenosis down to one-digit rates. However, in addition to significant costs involved in the procedures, particularly when more than one coronary artery lesion is treated, such therapeutic modality is not devoid of life-threatening side effects, such as coronary thrombosis.[19-21] Drug-releasing stent coated drugs used for atherosclerotic vascular lesions are the most developed to target cell cycle control and arrest. It is also a future task to find out the appropriate application concentration of uric acid, since the cell cycle control may vary depending on the dosage of the drug. In a study of the effect of uric acid on atherosclerosis, it was shown that voricular damage can be prevented by administration of uricol, and the effect of migration and proliferation on vascular injury model and VSMCs was evaluated. Chemotaxis was suppressed and PCNA (proliferating cell nuclear antigen) expression was decreased.

CONCLUSION

These results suggest that ursolic acid can inhibit proliferation of VSMCs by regulating cell cycle through G1 check point without cytotoxicity. However, different works have since demonstrated the wide array of pharmacological activities inherent in the natural compound.

ACKNOWLEDGEMENT

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A1A1008367).

REFERENCES

1. Timpl R. Macromolecular organization of basement membrane. Current Opinion in Cell Biology, 1996; 8: 618-24.
2. Falk E. Pathogenesis of atherosclerosis. Journal of the American College of Cardiology, 2006; 47: C7-C12.
3. Doran AC, Meller N, McNamara CA. The role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arteriosclerosis, Thrombsis, and Vascular Biology, 2008; 28: 812-9.
4. 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.
5. Grant MB, Wargovich TJ, Ellis EA, Caballero S, Mansour M, Pepine CJ. Localization of insulin-like growth factor I and inhibition of coronary smooth muscle cell growth by somatostatin analogues in human coronary smooth muscle cells. A potential treatment for restenosis? Circulation, 1994; 89: 1511-7.
6. Pazin MJ, Williams LT. Triggering signaling cascades by receptor tyrosine kinases. Trends in Biochemical Sciences, 1992; 17: 374-8.
7. Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, Ross R. Insulin-like growth factor-1 and

platelet derived growth factor-BB induce direct migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. Journal of Clinical Investigation, 1994; 93: 1266-74.

8. Shishodia S, Majumdar S, Banerjee S, Aggarwal BB. Ursolic acid inhibits nuclear factor-kappaB activation induced by carcinogenic agents through suppression of IkappaBalpha kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. Cancer Research, 2003; 63: 4375-83.
9. Pathak AK, Bhutani M, Nair AS, Ahn KS, Chakraborty A, Kadara H, Guha S, Sethi G, Aggarwal BB. Ursolic acid inhibits STAT3 activation pathway leading to suppression of proliferation and chemosensitization of human multiple myeloma cells. Molecular Cancer Research, 2007; 5: 943-55.
10. Wang X, Zhang F, Yang L, Mei Y, Long H, Zhang X, Zhang J, Qimuge-Suyila, Su X. Ursolic acid inhibits proliferation and induces apoptosis of cancer cells in vitro and in vivo. Journal of Biomedicine and Biotechnology, 2011; 2011: Article ID419343.
11. Shyu MH, Kao TC, Yen GC. Oleanolic acid and ursolic acid induce apoptosis in HuH7 human hepatocellular carcinoma cells through a mitochondrial-dependent pathway and downregulation of XIAP. Journal of Agricultural and Food Chemistry, 2010; 58: 6110-8.
12. Jesus JA, Lago JGL, Laurenti MD, Yamamoto ES, Passero LFD. Antimicrobial Activity of Oleanolic and Ursolic Acids: An Update. Evidence-Based Complementary and Alternative Medicine, 2015; 2015, Article ID620472.
13. Duque A, Rakic P. Different effects of BrdU and 3H-Thymidine incorporation into DNA on cell proliferation, position and fate. Journal of Neuroscience, 2011; 31: 15205-17.
14. Charron T, Nili N, Strauss BH. The cell cycle: a critical therapeutic target to prevent vascular proliferative disease. Canadian Journal of Cardiology, 2006; 22: 41B-55B.
15. Jiang Q, Han Y, Gao H, Tian R, Li P, Wang C. Ursolic acid induced anti-proliferation effects in rat primary VSMCs is associated with inhibition of microRNA-21 and subsequent PTEN/PI3K. European Journal of Pharmacology, 2016; 781: 69-75.
16. Ross R. The pathogenesis of atherosclerosis-an update. The New England Journal of Medicine, 1986; 314: 488-500.
17. Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. Circulation Research, 1986; 58: 427-44.
18. Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT, Turco M, Caputo R, Bergin P, Greenberg J, Popma JJ, Russell ME. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. The New England Journal of Medicine, 2004; 350: 221-31.
19. Finn AV, Nakazawa G, Joner M, Kolodgie FD, Mont EK, Gold HK, Virmani R. Vascular responses to drug eluting stents. Importance of delayed healing. Arterioscler, Thrombosis, and Vascular Biology, 2007; 27: 1500-10.
20. Win HK, Caldera AE, Maresh K, Lopez J, Rihal CS, Parikh MA, Granada JF, Marulkar S, Nassif D, Cohen DJ, Kleiman NS. Clinical outcomes and stent thrombosis following off-label use of drug-eluting stents. JAMA, 2007; 297: 2001-9.
21. Fogo AS, Antonioli E, Calixto JB, Campos AH. Tormentic acid reduces vascular smooth muscle cell proliferation and survival. European Journal of Pharmacology, 2009; 615: 50-4.