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ANTI-ATHEROSCLEROTIC EFFECT OF URSOLIC ACID

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ABSTRACT

Vascular smooth muscle cell (VSMC) proliferation is a key event in the development of hypertension, in stent restenosis and other cardiac disorders. Drug-eluting coronary stents may delay vascular healing and increase late stent thrombosis. Platelet aggregation and VSMC proliferation are essential events in the pathogenesis of atherothrombotic diseases. This study investigated the anti-atherosclerotic effect of ursolic acid. Ursolic acid significantly inhibited collagen induced washed rabbit platelet aggregation in a concentration-dependent manner. The inhibition rate of coagulation compared to the control group was found to be 36.0%, 56.1% and 77.2% at 10 μM, 30 μM and 50 μM concentrations of uricol, respectively ($^{*}p < 0.05$, $^{**}p < 0.01$). There was no difference in the activated partial thromboplastin time (APTT) and the prothrombin time (PT) between uricosylate and control, indicating that uric acid did not affect blood coagulation. The number of vascular smooth muscle cells induced by the platelet-derived growth factor-BB (PDGF-BB) was significantly increased compared with the control group, and the number of vascular smooth muscle cells treated with uric acid was decreased in a concentration-dependent manner at 30 μM and 50 μM. Taken together, these data provide new evidence that ursolic acid is able to inhibit VSMC proliferation and platelet aggregation, which may be a novel resource for the development of anti-atherosclerotic agents.

KEYWORDS: Blood coagulation, platelet-derived growth factor-BB (PDGF-BB), ursolic acid, vascular smooth muscle cell (VSMC).

INTRODUCTION

The development of medical technology has increased life expectancy, and the prevalence of chronic diseases is increasing due to rapid aging and western lifestyle habits.[1] WHO reports that chronic diseases such as heart disease, stroke, cancer, chronic respiratory disease and diabetes are the leading causes of death worldwide, and 68% of the world's deaths in 2012 were due to these diseases.[2] Atherosclerosis, which causes serious cardiovascular diseases such as myocardial infarction and angina pectoris, is a risk factor for hypertension, diabetes, hyperlipidemia, and smoking. Atherosclerosis is also a major cause of atherosclerosis-related atherosclerosis, stenosis and thrombus.[3] Although the percutaneous coronary intervention such as plain old balloon angioplasty or bare-metal stent is used one of the goals of treatment for arteriosclerotic vascular lesions, vascular restenosis related to rapid proliferation of vascular smooth muscle cells and the extracellular matrix secretion results in myocardial ischemia.[4] In order to prevent vascular restenosis, a drug-eluting stent is developed and used by applying a proliferation inhibitor or an immunosuppressant to a conventional stent.

Although drug-eluting stents have reduced vascular restenosis, it is a problem such as stent thrombosis which is a fatal complication that leads to death accompanied by acute myocardial infarction. Thus, new and safe drugs should be developed.[5] Among the areas of drug development research, natural materials are easy to obtain from nature and a large amount of natural materials can be obtained because of the development of extraction technology. Thus, natural materials are useful candidates for the development of new drugs.

When blood vessels are damaged by trauma or disease, they are recovered through complicated processes involving blood vessels, platelets, coagulation factors, coagulation inhibitors, and fibrinolysis in order to stop the bleeding. In this process, platelets participate in hemostasis through complex reactions based on adhesion, activation, and aggregation of platelets. When the blood vessels are damaged, subendothelial collagen is exposed to induce platelet activation and adhesion. At this time, von Willebrand Fator (vWF), which acts as an adhesive, binds to collagen and binds to the GPIb-V-IX complex present in the platelet membrane.[6] Adenosine diphosphate (ADP), serotonin, and Ca^{2+} secrete from the activated platelets, and arachidonic acid is liberated from the phospholipid of the membrane. TXA2 (thromboxane A2) is synthesized by the action of prostaglandin synthase such as cyclooxygenase. Secreted ADP

activates platelets again, and TXA2 contributes to hemostasis by platelet aggregation and strong vasoconstriction. When ADP is bound to platelet receptors, platelet activation activates platelet membrane GPIIb/IIIa complexes with fibrinogen and further enhances aggregation of platelets to form clots. The antagonism of TXA2 with platelet activation and PGI2 (prostacyclin), a platelet aggregation inhibitor secreted by vascular endothelium, maintains a balance of platelet-induced hemostasis.[7]

Ursolic acid is a pentacyclic triterpenoid that is isolated from the stem, leaf, and fruit shell of a plant and has a wide range of phytogenic physiological activity.[8] Numerous studies have revealed the anticancer,[9] antioxidant,[10] anti-inflammatory,[11] and antiviral[12] efficacy of uronic acid. In particular, NF-κB (nuclear factor kappaB), Bcl-2 (B-cell lymphoma 2), Caspase, COX-2 (cyclooxygenase-2), cyclin, matrix metalloproteinases (MMPs), intercellular adhesion molecules vascular endothelial growth factor (VEGFR), and epidermal growth factor receptor (VEGFR), thereby inhibiting the growth, proliferation and metastasis of cancer cells.[13]

This study investigated the inhibitory effect of uronic acid on platelet aggregation as an agonist for collagen, an activator of platelet aggregation. In order to investigate the anti-atherosclerotic effect of uronic acid, the inhibitory effect of platelet-derived growth factor-BB (PDGF-BB) -mediated vascular smooth muscle cells on vascular smooth muscle cell proliferation was investigated.

MATERIALS AND METHODS

Reagents and instruments

Ursol 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℃, a relative humidity of 50±10%, a ventilation frequency of 10 to 15 times/hr, a lighting cycle of 12 hours (dark)/12 hours (light) and lighting of 150 to 300 Lux. This study was conducted according to the guidelines of the Animal Experiment Ethics Committee of Dong-eui University.

Cell culture

Aortic vascular smooth muscle cells of rats used in the study were purchased from BioBird Co., Ltd. (Seoul, Korea). Vascular smooth muscle cells were cultured in a DMEM medium containing 10% FBS and 2 mM L-glutamine in a 5% CO_2 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 vascular smooth muscle cells that were proliferated for 24 hours were treated with trypsin-EDTA and then counted using a hemocytometer.

Measurement of platelet aggregation ability

The platelet aggregation ability was measured using Aggregometer (Chrono-Log Co., Havertown, PA, USA) for the difference in permeability according to platelet aggregation. The principle of measurement is to adjust the platelet count of platelets rich plasma (PRP) to about 3 × 10^8/mL and add collagen (Chrono-Log Co.). Blood samples were collected from rabbits with normal platelet function at a ratio of 1: 9 in an anticoagulant (3.8% sodium citrate) tube. To obtain PRP, centrifugation was carried out at 1,000 rpm for 10 minutes, and the platelet count was adjusted to 3 × 10^8/mL using HEPES buffer. The temperature was fixed at 37℃, the number of revolutions was set at 1,000 rpm, and the blank was corrected with platelet poor plasma (PPP). Platelet-rich plasma and ursolic acid (10, 30, and 50 μM) were transferred to a measuring instrument and allowed to react for 5 minutes before collagen was added.

Blood coagulation measurement

The activated partial thromboplastin time (APTT) and the prothrombin time (PT) tests were performed to confirm the effect of uric acid on the delayed and shortened blood clotting time. PT due to APTT and the extrinsic pathway due to the intrinsic pathway during the measurement of blood coagulation activity was measured using an automatic blood coagulation analyzer ACL-7000 (Instrumentation Laboratory, Bedford, Mass., USA). The control group was 10% DMSO.

Statistical processing

The results of all experiments were expressed as "mean ± standard error of mean (SEM)" of representative values derived from independent experiments over three replicates. Statistical comparisons among the groups were made by ANOVA Statistical comparisons between the groups were made by Student's *t*-test to be significant when the *p* value was less than 0.05.

RESULTS

Inhibitory effect of ursolic acid on platelet aggregation

In order to investigate the inhibitory effect of uronic acid on platelet aggregation, platelet aggregation ability induced by collagen was measured. The maximum aggregation rates of the test group with uric acid were 71±5%, 52±6% and 28±3% at 10 μM, 30 μM and 50 μM concentrations, respectively. Concentration-dependent aggregation inhibition was observed in which the coagulation rate decreased as the concentration increased (Fig. 1). The inhibition rate of coagulation compared to the control group was found to be 36.0%, 56.1% and 77.2% at 10 μM, 30 μM and 50 μM concentrations of ursol, respectively ($^{*}p < 0.05$, $^{**}p < 0.01$).

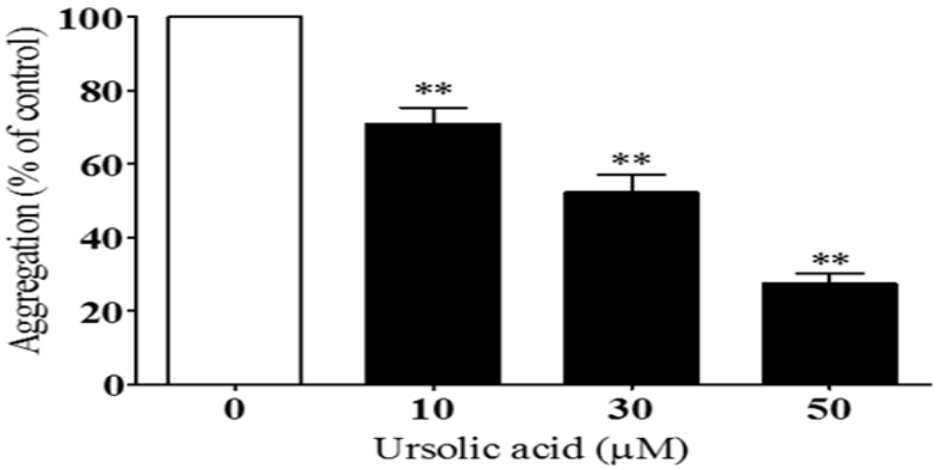

Fig. 1: Effects of ursolic acid on rabbit platelet aggregation. Washed rabbit platelets were incubated at 37℃ in an aggregometer with stirring at 1,000 rpm and then ursolic acid (10~50 μM) was added. After 5 min preincubation, platelet aggregation was induced by addition of collagen (10 μg/mL). The aggregation percent was presented as % of maximal aggregation induced. The data were expressed as the mean±SEM (n = 4, $^{**}p < 0.01$ *vs*. control).

Effects of ursolic acid on blood coagulation

PT and APTT were evaluated to investigate whether the inhibition of platelet aggregation by uric acid treatment affects the exogenous or endogenous pathway (Fig. 2). In the control group, the APTT and PT results were 33.9±1.3 and 10.2±0.8 sec, respectively. At 50 μM, the APTT and PT were 35.0±1.7, 9.6±0.6 sec, respectively. At 100 μM, the APTT and PT were 34.2±0.4, 10.2±0.8 sec, respectively. There was no difference in APTT and PT between uricosylate and control, indicating that uric acid did not affect blood coagulation.

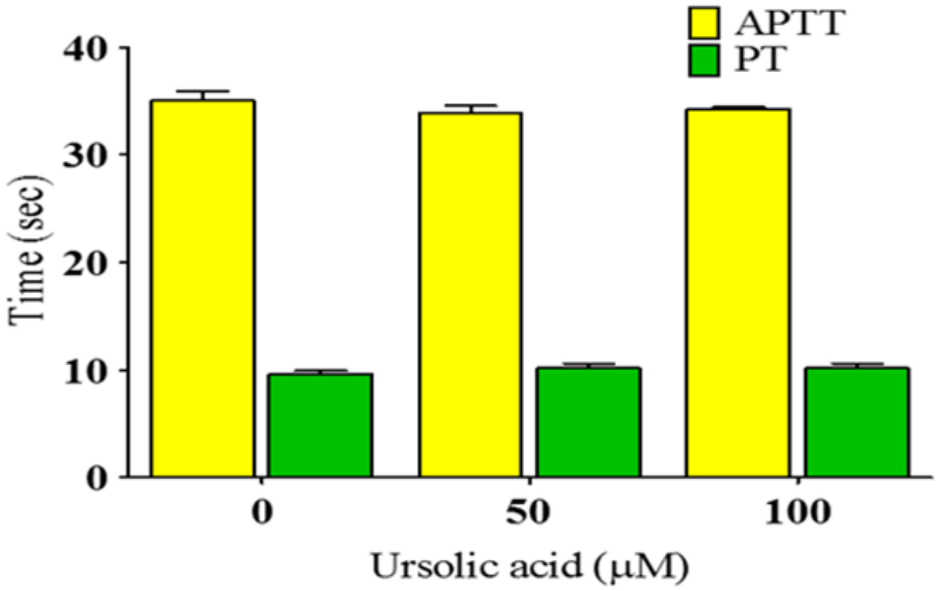

Fig. 2: Effects of ursolic acid on coagulation time. Each bar represents the means ± SEM.

Effect of ursolic acid on platelet viability

The platelet survival rate was measured because the effect of inhibiting platelet aggregation by uric acid may also occur in cytotoxicity. Cell viability was measured after 2 hours of treatment with the highest concentration of uric acid (50 μM) used for cell proliferation, and no cytotoxicity was observed at the highest concentrations used in the experiment (Fig. 3).

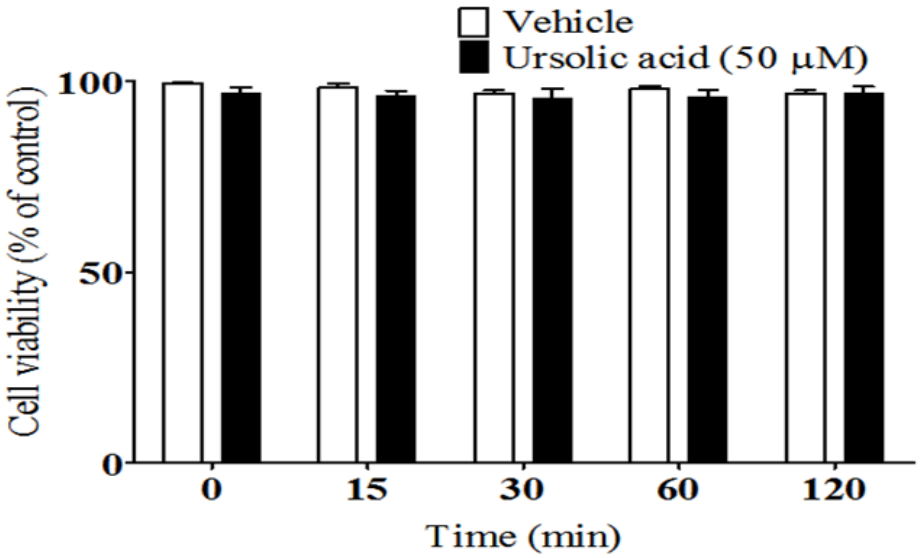

Fig. 3: Cytotoxicity of ursolic acid on the rabbit washed platelets. The cytotoxicity was assessed by CCK-8 assay at 50 μM. Data are expressed as mean ± SEM (n = 3).

Inhibitory effect of ursolic acid on vascular smooth muscle cell proliferation

The effect of uronic acid were investigated on vascular smooth muscle cell proliferation. Vascular smooth muscle cells cultured in minimal medium were pretreated with 10 μM, 30 μM, and 50 μM ursol acid and the number of vascular smooth muscle cells induced with PDGF-BB for 24 hours were counted. The number of vascular smooth muscle cells induced by PDGF-BB was significantly increased compared with the control group, and the number of vascular smooth muscle cells treated with uric acid was decreased in a concentration-dependent manner at 30 μM and 50 μM (Fig. 4) ($^{*}P < 0.05$, $^{**}P < 0.01$).

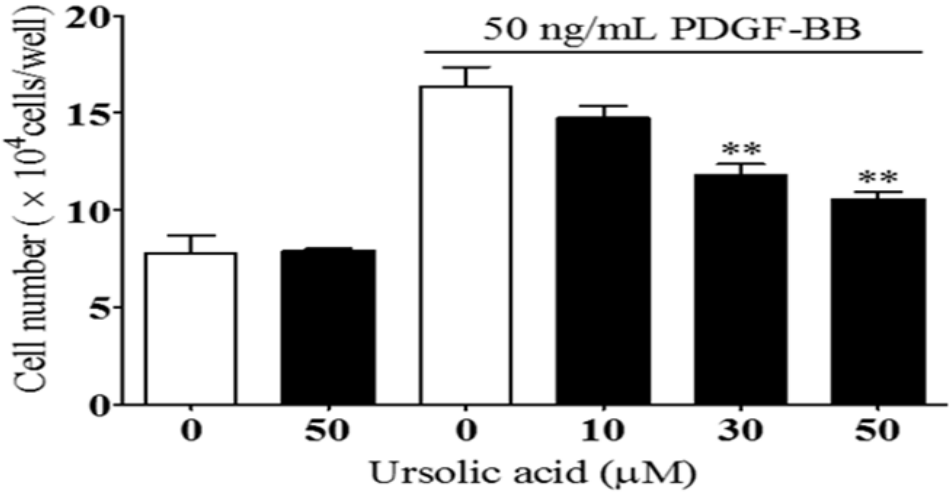

Fig. 4: Effect of ursolic acid on the PDGF-BB-induced vascular smooth muscle cell number. Rat aortic VSMCs were pre-cultured in serum-free medium in the presence or absence of ursolic acid (10~50 μM) for 24 hr, and then stimulated by 50 ng/mL PDGF-BB for a further 24 hr. Then the cells were trypsinized,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

DISCUSSION

Dissatisfaction with currently used antihyperlipidemic and vascular smooth muscle cell proliferation inhibitors and drug side effects triggered a search for new generation effective and safe drugs from natural sources. Natural materials, which are widely distributed in nature,

are an important material in the development of new drugs.[14] Recently, a large number of triterpenes and derivatives such as uric acid derived from natural products have been reported to significantly inhibit platelet aggregation.[15,16] On the other hand, collagen had no effect on platelet aggregation, contrary to the action of ADP and thrombin that uricosanic acid induces platelet aggregation.[17] Therefore, we investigated whether urosolic acid inhibited aggregation of platelets causing atherosclerotic thrombosis by using collagen to induce the early action of platelet aggregation. Platelet aggregation occurs when GPIIa / IIIa, which is a glycoprotein complex in the cell membrane, is activated after binding of agonists such as ADP, collagen, and thrombin to platelet receptors, and GPIIa / IIIa is bound to fibrinogen.[18] Platelet-rich plasma was isolated and induced to collagen, which promotes platelet aggregation. As a result of measuring the inhibitory effect of platelet aggregation of uric acid, it was confirmed that the coagulation inhibition rate was increased in a concentration-dependent manner by the maximum aggregation ratio by concentration. In addition, platelet aggregation after treatment with uric acid did not affect the exogenous pathway or endogenous pathway. As a result, there was no difference in PT and APPT between uronic acid treatment group and control group, and uric acid did not affect platelet aggregation. As the platelet aggregation inhibition effect may also be caused by platelet cytotoxicity, the platelet survival rate was measured, and it was not cytotoxic at the highest concentration used in the experiment. Other studies have also shown that the inhibition of uric acid against thrombin and ADP-induced platelet aggregation in a dose-dependent platelet aggregation inhibition assay, in which four types of triterpenes, including uronic acid, were induced to platelet agonists (thrombin, ADP, epinephrine).[19] These results are consistent with those reported in other previous studies.[16] As a result of this study and other previous studies, it has been shown that uronic acid inhibits platelet aggregation, which results in vascular damage due to arteriosclerosis and subsequent platelet deposition, thrombosis resulting from the rupture of fragile plaques, and thrombosis of the coronary stent. This may be a natural candidate for the development of antiplatelet agents necessary for the prevention of arterial thrombosis. Excessive platelet aggregation and vascular stenosis can have serious consequences for the progression of atherosclerosis and the adverse effects of coronary intervention. The inhibition of vascular smooth muscle cell proliferation is essential for the prevention and treatment of vascular diseases. Therefore, the effect of uronic acid on vascular smooth muscle cell proliferation was investigated. Atherosclerosis is an abnormal hyperactivity of blood cells (monocytes, macrophages, T-lymphocytes) and endothelial cells (smooth muscle cells) due to damage of blood vessel walls. Growth factors affecting their interaction and growth are important.[20] The proliferation of vascular smooth muscle cells occurs not only in atherosclerosis but also in restenosis after coronary intervention and intimal hyperplasia after vascular grafting in response to vascular wall injury. In vivo experiments, neointimal hyperplasia was significantly suppressed in the carotid balloon catheter injury model of rats treated with uric acid. The administration of ursol at a dose of 6 mg/kg body weight per day for 10 days demonstrated an 80% reduction in the ratio of intima-to-media contrast ratio and stenosis and suppression of PCNA expression in the neointimal and mesentery.[21] Another study reported that diabetic mice administered with uric acid inhibit mononuclear dysfunction and delay the progression of atherosclerosis.[22] These results suggest that uronic acid has potential therapeutic value in vascular injury and may prevent restenosis after coronary angioplasty and progression of atherosclerosis. Although uronic acid has been studied as a safe and useful phytochemical in studies of the effects of ursolic acid on cardiovascular.[23,24] There have been reports of potential adverse effects in arteriosclerosis.[25] However, the results of this study suggest that uronic acid inhibits aggregation of collagen-activated platelets exposed from damaged arteries that are damaged by arteriosclerosis, and that activated platelets are secreted by activated platelets Induced growth factors such as PDGF could inhibit excessive proliferation of vascular smooth muscle cells. Therefore, we confirmed the possibility that ursol can be used for the prevention and treatment of thrombosis and vascular restenosis in arteriosclerotic lesions and stent side effects. Therefore, future studies on specific mechanism of action of uric acid and clinical efficacy for safe use of natural products such as uric acid should be essential.

CONCLUSION

These results suggest that uronic acid has potential therapeutic value in vascular injury and may prevent restenosis after coronary angioplasty and progression of atherosclerosis. Although uronic acid has been studied as a safe and useful phytochemical in studies of the effects of ursolic acid on cardiovascular, there have been reports of potential adverse effects in arteriosclerosis.

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