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Inhibitory Effects of Ginseng Saponins on c-fos mRNA Expression and the Proliferation of Rat Aortic Vascular Smooth Muscle Cells Stimulated by Angiotensin II

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To evaluate the possibility that the ginseng saponins could be developed as an anti-arteriosclerotic agent, we examined the inhibitory effects of ginseng saponins (total saponin[TS], panaxatriol[PT], panaxadiol[PD]) on the expression of c-fos mRNA and the proliferation of cultured rat aortic vascular smooth muscle cells (VSMCs) stimulated by angiotensin II (Ang II). TS and PT (1.0 mg/ml) suppressed c-fos mRNA induction in VSMCs stimulated by 10^{-5} M Ang II. The order of inhibitory potency was PT>TS. Ginseng saponins (0.01~1.0 mg/ml) inhibited the proliferation of VSMCs stimulated by Ang II in a concentration dependent manner, the inhibitory potency was TS>PT>PD at 0.1~1.0 mg/ml. These results suggest that ginseng saponins may suppress Ang II-stimulated proliferation of aortic VSMCs which can be seen in atherosclerosis, hypertension and restenosis.

Key Words: Ginseng saponins, c-fos, Proliferation, Angiotensin II, Vascular smooth muscle cells

INTRODUCTION

Vascular smooth muscle cell (VSMC) proliferation is thought to play a key role in the pathogenesis of atherosclerosis and restenosis after baloon coronary angioplasty (Ross, 1993). VSMCs have a kind of receptors for growth factors and vasoactive hormones, such as platelet-derived growth factor (PDGF), endothelin-1, and angiotensin II (Ang II), which can mediate cell proliferation. VSMC proliferation, especially associated with vascular injury and hypertension, is thought to be induced by Ang II (Taubman et al, 1989).

The induction of c-fos mRNA is suggested to be a primary event and the earliest known effect on gene expression by many growth stimulators such as serum, growth factors, phorbol esters, and the reagents which can increase the intracelluar calcium level (Muller et al, 1984). The level of c-fos mRNA and protein is very low in quiescent or unstimulated cells. Stimulation of VSMCs with Ang II leads to a dramatic induction of expression of both c-fos mRNA and protein within a few minutes (Taubman et al, 1989).

If the c-fos expression is suppressed, the cell proliferation can be inhibited even after the stimulation with growth factors. Antisense c-fos oligonucleotide treatment can suppress proliferation of exponentially growing cells after serum stimulation (Simonson, 1994). Estrogen and progesterone can inhibit both the induction of c-fos and the proliferation of human umbilical VSMCs stimulated by endothelin-1 (Morey et al, 1997). These reports suggest that an intracellular signal transduction pathway which control c-fos expression might play an important role in regulating the proliferation of VSMCs.

Ginseng has been traditionally used for a kind of cardiovascular disorders, such as hypertension, ischemic heart disease, thrombotic disease, and athero-

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sclerosis. However, the mechamism of action is not well defined. The ginseng saponins are mainly triterpenoid dammarane glycosides and are a major constituent of ginseng. The ginseng saponins are more classified according to their non-sugar (aglycone) structure, as panaxatriol, panaxadiol and etc. The ginseng saponins are specific for ginseng species and are thought to be a major compound which have cardiovascular protective actions such as a decrease in heart rate, a biphasic effect on blood pressure, endothelium dependent vascular relaxation, and an inhibitory effect on atheroma formation (Kaku et al, 1975).

If ginseng saponins could inhibit the proliferation of VSMCs, they could be developed as anti-arteriosclerotic agent. In this study, we intended to examine whether ginseng saponins (total saponin[TS], panaxatriol[PT], panaxadiol[PD]) show anti-proliferative effect on Ang II-stimulated VSMCs, and whether this effect could be correlated with the suppression of c-fos mRNA increase.

METHODS

Cell cultures

Vascular smooth muscle cells (VSMCs) were obtained by collagense digestion of aortae from 7-week-old Sprgue-Dawley rats (Pang & Venance, 1992). The resulting cells were routinely grown in Dulbecco's modified Eagle media (DMEM) containing 8 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS). VSMCs were subcultured weekly and were characterized with hill-and-valley shape and by immunofluorescence with smooth muscle-specific anti-α-actin antibody (data not shown). The average passage of VSMCs used in these experiments was six to ten.

Northern analysis

VSMCs were made quiescent by incubating the cells in starving medium (DMEM with 0.4% FBS) for 24 hours. Quiescent cells were treated with ginseng saponins for 1 hour and stimulated with 10^{-5} M angiotensin II (Ang II) for another 30 minutes. Total cellular RNA was isolated by acid-phenol-guanidinium isothiocyanate method from these VSMCs. Equal amounts (20 μg) of RNA was seperated in 1% agarose-formaldehyde gel. The gel was stained with 0.1 mg/ml ethidium bromide to examine the integrity of RNA. Equal amount RNA loading was confirmed by comparing the intensity of 28S and 18S ribosomal RNA band in each lane (Fig. 2A). The RNA was capillary transferred to a nylon membrane and the blotted membrane was probed with [α-^{32}P]dCTP labelled 1.1 kb Pst I fragment of v-fos DNA to detect c-fos mRNA. Autoradiogram was obtained by exposing the hybridized membrane to Fuji XR film and was analyzed with a video densitometer (Pharmacia, USA)

[^{3}H]thymidine uptake

The antiproliferative activity of each ginseng saponins was determined by measuring [^{3}H]thymidine incorporation into the DNA of VSMCs cultured in 24 well plates. VSMCs were seeded at a density of 2 X 10^3 cells/well and at 70~80% confluency, quiescent cells were obatined by incubating cell in a starving medium for 24 hours. After 1 hour incubation with ginseng saponins, VSMCs were stimulated with 10^{-5} M Ang II for another 20 hours. 1 μCi of [^{3}H]thymidine was added into each well and VSMCs were labelled for 4 hours in the presence of ginseng saponins and Ang II. VSMCs were rinced with ice-cold PBS twice and treated with 10% trichloroacetic acid (TCA). TCA-insoluble pellet was rinced twice with ethanol-ether mixture (1 : 1 by volume) and was solublized with 0.25 M sodium hydroxide and its radioactivity was measured by β-counter (Beckman, USA)

Reagents

Ginseng saponins were donated from the Korea Ginseng and Tobacco Reaserch Institute (Taejon, Korea). [^{3}H]thymidine (925 GBq/mmol) and [α-^{32}P] dCTP (100 TBq/mmol) were purchased from Amersham, and all other chemicals from Sigma, otherwise mentioned.

Statistics

All data are represented as means ± standard errors of means. The mean values of each group were compared to that of unstimulated non-treated control or Ang II only stimulated group by Student's *t*-test. Differences were considered to be statistically signi-

ficant when p values were less than 0.05.

RESULTS

Morphological observation in VSMCs

Single VSMC can be characterized by a prominent nucleus and large cytoplasmic area (Pang & Venance, 1992). VSMCs usually grew in culture as packed colonies, and it was difficult to distinguish cell-cell boundaries (Fig. 1A). It was, however, easy to identify the prominent nuclei of each cell (arrow, Fig. 1H). The VSMCs were pretreated with either vehicle alone or with ginseng saponins (1.0 mg/ml each) 1 hour before Ang II stimulation. There was no significant changes in the morphology of VSMCs at 30 minutes after Ang II stimulation. At 24 hours, PT-treated VSMCs showed the shrinkage of cell volume, which resulted in the decrease of cytoplasmic area (Fig. 1G). Other treatment did not seem to exert any change to VSMCs 24 hours after Ang II stimulation.

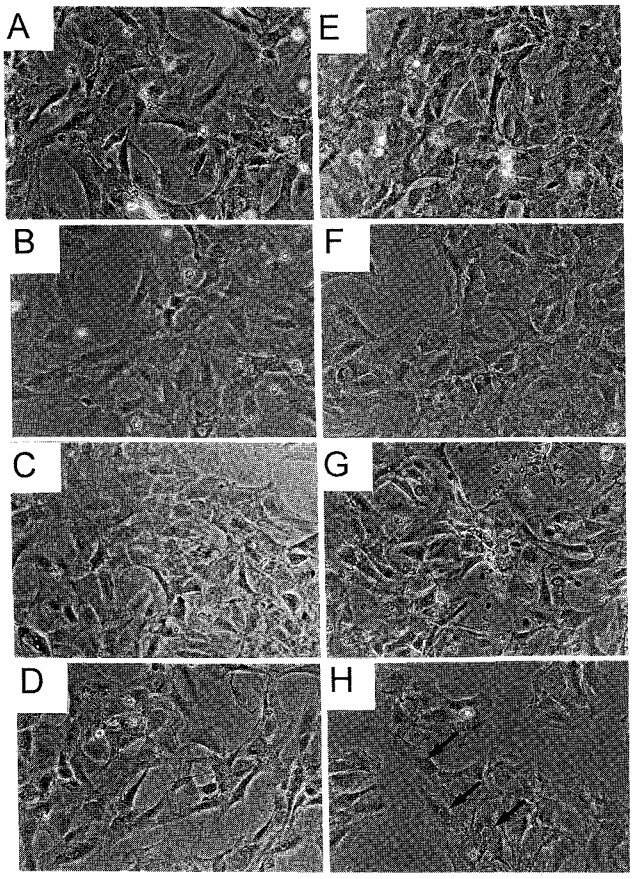

Fig. 1. Phase-constrast photomicrographs of VSMCs. The VSMCs were treated with either vehicle alone (A, E) or with ginseng saponins (B, F: TS, C, G: PT and D, H: PD) before Ang II stimulation. The photographs were taken under 100X field, at 30 minutes (A, B, C, D) or at 24 hours (E, F, G, H) after Ang II stimulation. (arrow in H: the nucleus of each VSMC)

Ginseng saponins suppressed the increase of c-fos mRNA expression stimulated by angiotensin II (Ang II)

The changes of c-fos mRNA expression was examined 30 miniutes after stimulation by Ang II. This is because the expression was maximal at 30 mintutes

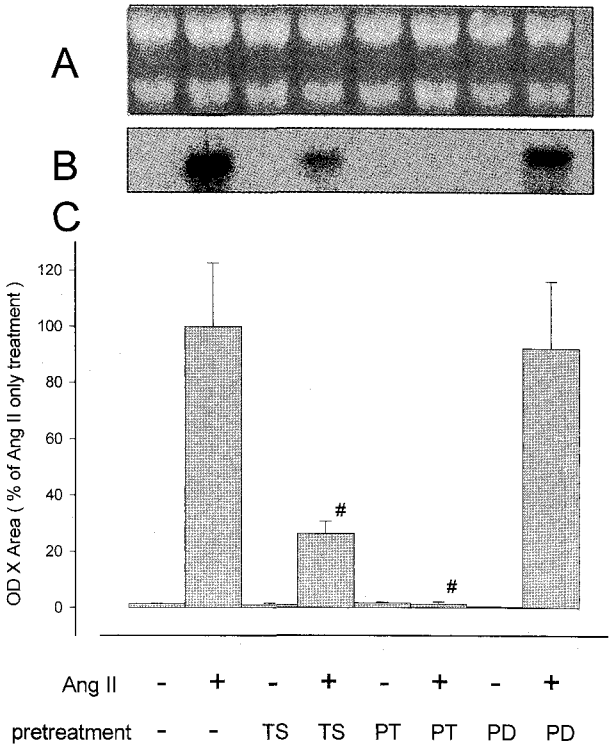

Fig. 2. Pretreatment with ginseng saponins suppressed c-fos mRNA increase stimulated by Ang II in VSMCs. One hour before Ang II stimulation, VSMCs were treated with ginseng saponins (1.0 mg/ml). Total cellular RNA seperated on agarose gel (A) was transferred onto a nylon membrane; the membranes were hybridized with v-fos DNA to get autoradiograms (B), which were densitometrically analyzed (C). Area-integrated optical density (OD X area) was expressed as the percentile to the mean OD X area of Ang II only stimulated group. (+) and (−) in the row of Ang II indicate the presence and absence of Ang II stimulation. Each bar represents the mean and standard errors of mean (N=6). # Significant different from the Ang II only stimulated cells ($p<0.05$).

after Ang II treatment at $10^{-7} \sim 10^{-5}$ M concentration; there is no more increase in c-fos mRNA expression when the concentration of Ang II is over 10^{-5} M (data not shown).

TS, PT, and PD was added to vascular smooth muscle cells (VSMCs) at a final concentration of 1.0 mg/ml, 1 hour prior to 30 minute stimulation by Ang II; TS and PT showed significant suppression of c-fos mRNA expression stimulated by Ang II (78% and 98% inhibition respectively). PD also appeared to exert an inhibitory effect (10% inhibition by mean), but this was not statistically significant. TS, PT, and PD themselves did not show any changes to the basal expression of c-fos mRNA (Fig. 2).

Ginseng saponins inhibited the proliferation of VSMCs stimulated by Ang II

[^{3}H]thymidine incorporation into the DNA can be used as an index for the cell proliferation, since it represents the new DNA synthesis during the cell proliferation. Each ginseng sponins (0.01 ~ 1.0 mg/ml) inhibited [^{3}H]thymidine incorporation stimulated by 10^{-5} M Ang II in a dose-dependent manner (Fig. 3). At a concentration of 1.0 mg/ml, all ginseng saponins (TS, PT, and PD) suppressed the [^{3}H]thymidine incorporation to a level below that of unstimulated control VSMCs. The inhibitiory potency was TS>PT >PD (Fig. 3). At a concentration of 0.1 mg/ml, TS treatment lowered the level of [^{3}H]thymidine incorporation below that of unstimulated control VSMCs. In PT treated cells, the [^{3}H]thymidine incorporation level was similar to that of unstimulated control VSMCs and PD treatment slightly inhibited the [^{3}H]thymidine incorporation below that of Ang II only stimulated cells. So the relative inhibitory potency was TS>PT>PD. At a concentration of 0.01 mg/ml, PT and PD showed similar extent of inhibition; [^{3}H]thymidine incorporation of PT and PD treated VSMCs were less than that of Ang II only-stimulated cells. TS appears to show an inhibitory effect; but, this was not statistically significant.

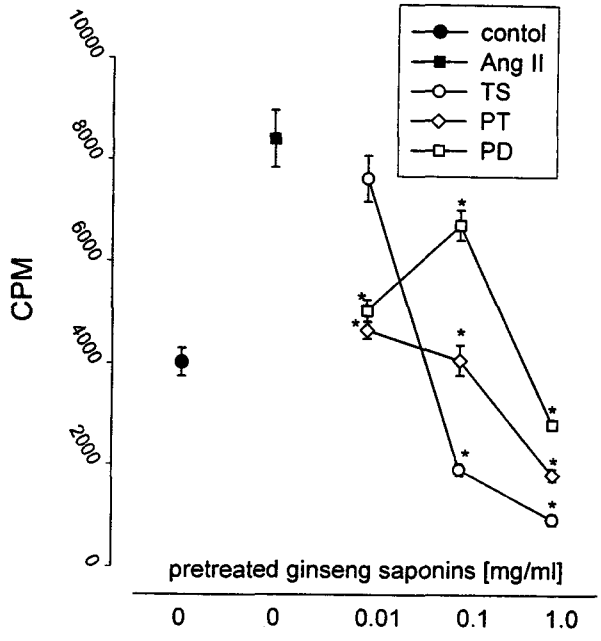

Fig. 3. [^{3}H]thymidine incorporation after stimulation by Ang II in the presence of ginseng saponins. VSMCs were labelled with [^{3}H]thymidine in the presence of ginseng saponins (0.01 ~ 1.0 mg/ml) and Ang II. Count per minute (CPM) represents the radioactivity of incorporated [^{3}H]thymidine. Each point represents the mean and standard errors of mean (N=6). *Significant different from the Ang II only stimulated cells ($p<0.05$)

DISCUSSION

Ang II is produced during blood pressure regulating process controlled by renin-angiotensin-aldosterone system. Ang II may be responsible for the abnormal VSMC growth observed in hypertension either indirectly as a consequence of elevating blood pressure or directly as a receptor-mediated effects on VSMC growth. And, Ang II is thought to play an important role in stimulating migration and proliferation of VSMCs after vascular injury (Taubman et al, 1989).

Ang II is reported to rapidly induce the accumulation of c-fos mRNA as shown in Fig. 2. This is known to be dependent on mobilization of intracellular calcium and activation of several kinds of protein kinases such as protein kinase C (PKC), mitogen-activated protein kinase(MAPK), MAPK kinase(MAPKK) which are involved in intracellular signal transduction pathways (Taubman et al, 1989; Takahashi et al, 1996).

The degree of c-fos mRNA expression was thought to be the best index for expecting the Ang II-stimulated proliferation of VSMCs after ginseng saponin treatment. The inhibitory effects of ginseng saponins on c-fos mRNA expression, however, did not show a good correlation with their anti-proliferative effects represented as the inhibition of [^{3}H]thymidine

incorporation (Fig. 3).

At a concentration of 1.0 mg/ml of ginseng saponins, all the level of [^{3}H]thymidine incorporation was below that of unstimulated control. The c-fos mRNA expression, however, showed wide range (10 ~98%) of inhibition. There is a possibility that PD might exert its antiproliferative effect at the downstream of Ang II signal transduction pathway for c-fos mRNA expression, since PD did not show statistically significant inhibition on c-fos mRNA increase stimulated by Ang II.

Since TS showed more inhibitory effect on [^{3}H]thymidine incorporation than PT and PD, which are major constituents of ginseng total saponins, it can not be completely excluded that a minor component of TS, other than PT and PD might have a more potent antiproliferative effect. Although TS did not show a significant antiproliferative effect at 0.01 mg/ml of concentration, PT and PD suppressed Ang II stimulated VSMC proliferation to the level of unstimulated control. This suggests that the antiproliferative effect of TS at 1.0 mg/ml is unlikely due to the synergistic antiproliferative effects of PT and PD.

VSMCs treated with 0.1 mg/ml PD showed more [^{3}H]thymidine incorporation than VSMCs treated either with 0.01 mg/ml or with 1.0 mg/ml PD. Although it is hard to explain why PD showed such a bell-shaped inhibitory effect, it is unlikely that PD might interact with only one pathway which regulates the cell proliferation.

Ginseng has been known to have many clinical beneficial effects on the cardiovascular system. Recently, ginseng saponins, which are a major constituent of ginseng, have been reported to show several kinds of activies that could be attributed to their cardioprotective effects.

Ginseng saponins have estrogen-like activity. Saponins from american ginseng can induce estrogen-regluated protein, pS2 in MCF-7 breast cancer cell line (Grills, 1997) and there is a report that the use of ginseng facial cream leads to postmenopausal vaginal bleeding (Hopkins et al, 1988). It is reported that mortality from atherosclerotic cardiovascular disease is lower in premenopausal women than in age-matched men and Morey et al (1997) claimed that this is due to estrogen that can inhibit MAPK and MAPKK, of which the downstream target is c-fos expression and cell proliferation. There is a report that 17-β -estradiol reduced the maximal contractile response induced by Ang II (Mugge et al, 1997; Ravi et al, 1994). Above reports suggest that estrogen-like activity of ginseng saponins can inhibit the VSMC proliferation stimulated by Ang II.

The induction by Ang II of both the AP-1 (of which one component is c-fos) binidng activity and the proliferation of quiescent C2C12 myoblast is abolished by an antioxidant glutathione precursor, N-acetyl-L-cysteine(NAC). This observation suggests that a role for reactive oxygen intermediates(ROIs) in the intracelluar Ang II signal tranduction for c-fos mRNA induction and cell proliferation (Puri et al, 1995a; Puri et al, 1995b). Panaxadiol ginseng saponins can induce the transcription of Cu, Zn-superoxide dismuatase (SOD), which is one of the major antioxidant enzyme (Kim et al, 1996). Ginseng extract can directly inhibit decomposition of unsaturated fatty acid caused by iron and hydrogen peroxide-induced lipid peroxidation, involving a hydroxyl-radical scavenging mechanism (Zhang et al, 1996). Ginsenoside Rb1 (panaxatriol saponin) and Rg1 (panaxadiol saponin) could inhibit lipid peroxidation of microsomes and ginsenoside Rb1 could increase the activities of catalase and GSH peroxidase (Deng et al, 1991). These antioxidant activities of ginseng saponins imply that ginseng saponins might suppress the Ang II-stimulated intracelluar signal transduction which is partly mediated by ROIs.

Soe et al (1980) reported that ginseng saponins could activate adenylate cyclase in the cytosolic preparation. cAMP and cGMP could inhibit FBS-stimulated or vascular injury mediated proliferation of VSMCs (Takahashi et al, 1996; Pai & Bird, 1994). Treatment with cAMP-elevating agents leads to inhibition of Ang II-induced protein synthesis in rat aortic VSMCs (Indolfi et al, 1997; Assender et al, 1992), probably by inhibiting the stimulatory effect of Ang II on protein tyrosine phosphorylation (Giasson et al, 1997). Many of organ-protective actions of ginseng is known to be associated with its ability to enhance NO synthesis in endothelium (Chen, 1996). Dubey et al (1995) reported that nitric oxide(NO) could inhibit Ang-II induced proliferation and migration of VSMCs and that this effect seemed to be dependent upon cGMP.

Total ginseng saponins from *Panax notoginseng* is suggested to be a selective calcium channel blocker which interacts with the putative receptor-operated calcium channel (Kwan, 1995). Calcium channel blockers such as amlodipine, verapamil and diltiazem have an inhibitory effect on the cultured human

VSMC derived from saphenous vein and graft restenosis (Munro et al, 1994). Calcium channel blockers also have been shown to retard the progression of atherosclerosis (Duda et al, 1996).

All the above reports suggest that ginseng saponins might inhibit Ang II stimulated VSMC proliferation and c-fos mRNA expression at the multiple steps of intracelluar signal transduction cascades.

In conclusion, ginseng saponins could inhibit Ang II-stimulated proliferation of rat arotic VSMCs and this is partly dependent upon their inhibitory effect on c-fos mRNA expression. As PT showed the most potent antiproliferative activity at low concentration and as it was a good inhibitor of c-fos mRNA expression, PT may be used for the development of a preventive and therapeutic drug to inhibit the proliferation of VSMCs, which could be seen in many cardiovascular disorders.

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