Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 525 (2005) 41 – 47
www.elsevier.com/locate/ejphar

Astrapterocarpan isolated from *Astragalus membranaceus* inhibits proliferation of vascular smooth muscle cells

Susumu Ohkawara [a,b], Yasunobu Okuma [a,c], Takashi Uehara [a], Takashi Yamagishi [d], Yasuyuki Nomura [a,e,*]

[a] *Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*
[b] *Northern Advancement Center for Science and Technology, Sapporo 060-0807, Japan*
[c] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Choshi, Chiba 288-00285, Japan*
[d] *Kitami Institute of Technology, Koen-cho, Kitami, Hokkaido 090-0857, Japan*
[e] *Daiichi University, College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan*

Received 8 August 2005; accepted 8 August 2005

Abstract

The inhibitory effects of astrapterocarpan, formononetin, and calycosin isolated from *Astragalus membraneceus* on platelet-derived growth factor (PDGF)-BB-induced proliferative response in rat vascular smooth muscle cells (A10 cells) were investigated. Astrapterocarpan significantly inhibited PDGF-BB-induced cell proliferation and DNA synthesis in a concentration-dependent manner. This inhibition was not attributed to toxicity. In contrast, formononetin and calycosin had no effect. We next examined the effect of astrapterocarpan on PDGF-BB signal transduction. Astrapterocarpan inhibited PDGF-BB-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERIC1/2) mitogen-activated protein (MAP) kinase. However, this compound had no effect on phosphorylation of PDGF-β-receptor, Akt kinase and p38 MAP kinase. These results indicated that astrapterocarpan inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and that this effect may be mediated, at least in part, by inhibition of the ERK1/2 MAP kinase cascade.

Keywords: Astrapterocarpan; *Astragalus membraneceus*; Platelet-derived growth factor; Vascular smooth muscle cell; Cardiovascular disease

1. Introduction

Medicinal plants have been used as traditional remedies for hundreds of years. *Astragalus membranaceus* is a medicinal herb that has been widely used for the treatment of inflammatory disease (Yoshida et al., 1997), nephritis (Bensky and Gamble, 1993), tumors (Lau et al., 1994), and various cardiovascular diseases (Sinclair, 1998; Miller, 1998) in East Asian. Recent pharmacological studies have demonstrated that the crude extract of this plant has anti-arterogenic effects, such as inhibition of platelet aggregation (Huang et al., 1995), induction of prostacyclin (Deng et al., 1995), and radical scavenger activity (Hong et al., 1994).

Flavonoids are found in many foods and botanical medicines, appearing fruits, flowers, leaves and roots. Epidemiology studies have shown an association between dietary flavonoid intake and reduced risk of cardiovascular disease (Hertog et al., 1993). *Astragahs membranaceus* has been reported to contain several kinds of flavonoids, including 7-hydroxy-4'-methoxy isoflavane (formononetin), 3',7-dihydroxy-4'-methoxy isoflavone (Calycosin) and (6aR, 11 aR)-3-hydroxy-9,l *O*-dimethoxy pterocarpan (Astrapterocarpan) (Lin et al., 2000). Recent experimental evidence suggests that these flavonoids are responsible for the beneficial effects of the plant in the prevention of cardiovascular disease (Yim et al., 2000). However, the underlying mechanisms responsible for these effects are not yet completely understood.

Abnormal growth of vascular smooth muscle cell is a prominent feature of cardiovascular diseases such as atherosclerosis, myocardial infarction and restenosis after angioplasty (Ross, 1993). Furthermore, this process contributes to certain forms of

* Corresponding author. Daiichi University, College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan. Tel.: +81 92 541 0161; fax: +81 92 553 5698.
E-mail address: yn029@yahoo.co.jp (Y. Nomura).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.08.063

vascular remodeling in hypertension (Pauletto et al., 1996). Platelet-derived growth factor (PDGF)-BB released by the damaged intimal surface and platelet is implicated as mediators of these processes (Ferns et al., 1991). The effect of PDGF-BB is exerted by binding to a protein–tyrosine kinase receptor called β-receptor. Ligand binding can activate three major signal transduction pathways: mitogen-activated protein (MAP) kinase, phosphatidilinositol3(PI3) kinase, and phospholipase C γ 1 (PLC γ 1). It has been reported that extracellular signal-regulated kinasel/2 (EFK1/2), one of the MAP kinase pathways, plays a critical role in the regulation of vascular smooth muscle cell proliferation by PDGF-BB (Claesson-Welsh, 1994). Besides treatment of malignant tumor (Strawn et al., 1994; Buchdunger et al., 1995), which involve PDGF β-receptor activation, PDGF β-receptor kinase blockers could have therapeutic potential for the treatment of atherosclerosis and restenosis processes (Hart and Clowes, 1997; Golomb et al., 1997). Therefore, elucidation of the regulatory mechanism of PDGF-BB-induced vascular smooth muscle cell proliferation is important to understand the pathogenesis of cardiovascular disease.

In the present study, to elucidate the mechanism of cardiovascular protection by *Astragalus membranaceus*, we investigated the effects of astrapterocarpan, formononetin, and calycosin on PDGF-BB-induced vascular smooth muscle cell proliferation.

2. Materials and methods

2.1. Materials

Pterocarpan [(6aR, 11 aR)-3-hydroxy-9,l0-dimethoxyl-pterocarpan].

Pterocarpan was prepared from its glucoside by treatment with β-glucosidase (from Almond, 36.8 U/mg). The glucoside was isolated from methanol extract of Astraguk menbranaceus (obtained from Hokkaido Experimental Station for Medicinal Plants, National Institute of Health Sciences, Nayoro, Hokkaido, Japan in August 1998) by silica gel column chromatography using *n*-hexane and ethyl acetate as eluent. Pterocapan was isolated from ethyl acetate soluble portion of the reaction mixture by preparative HPLC and was recrystallized from methanol. Purity of pterocarpan was confirmed by comparison its physical data and spectral data with those of previous report (Anetai et al., 1994; Katsura and Yamagishi, 1987). The high performance liquid chromatography (HPLC) chromatogram of the isolated sample showed one peak at 280 nm. Pterocarpan was demonstated to be pure (>99% purity) as evidenced by HPLC and Nuclear Magnetic Resonance (NMR) spectrum.

A colorless needle from MeOH, mpf 89–190.5 °C, $[\alpha]_D$ −220° (*c*=O.26, MeOH), UV: λ max (MeOH, nm, log ε) : 285.5 (3.78), 281 (3.80),), Electron Impact Ionization Mass Spectrometer (EI-MS) *m/z* : 300 (M^+),285,267,253,225, 197,147. Hight Resolution Fast Atom Bomberdment Mass Spectrometry (HR-FAB-MS) *m/z* : 300.1001 (calcd. for $C_{17}H_{16}O_5$: 300.0998), ^{13}C-NMR(δ,$CDC1_3$): 40.8, 56.7, 60.9, 67.0, 80.4, 103.9, 105.8, 110.5, 112.2 119.5 123.0 133.0 134.7 152.1 153.9 157.6 156.7. ^{1}H-NMR(δ, $CDC1_3$): 4.23 (lH, dd, *J*=1.0.1, 4.3), 3.54 (ddd, *J*=6.4, 10.4, 4.2), 3.61 (lH, dd, *J*=10.4, lO.l), 3.82 (3H, s), 3.86 (3H, s), 6.31 (lH, d, *J*=2.5 Hz), 6.50(1H, dd, *J*=2.5,7.9), 6.53 (lH, d, *J*=8.2), 6.95 (lH, d, *J*=7.9), 7.33 (lH, d, *J*=8.2).

Formononetion (7-hydroxy-4-methoxy isoflavone) and Calycosin (7,3′-dihydroxy-4′-methoxyisoflavone) were purchased from Calbiochem-Novabiochem Corporation.

2.2. Cell culture

Al0 cells derived from the rat thoracic aorta were obtained from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin: GIBCO BRL). Cells were grown to 70% confluence at 37 °C in 5% CO_2 on culture dishes and then cells were deprived of serum by replacing with the DMEM with 0.2% serum for 48 h before the addition of test agents for all experiments.

2.3. DNA synthesis assay and cell counting

Quiescent Al 0 cells were stimulated with PDGF-BB (50 ng/ml) combined with various concentrations of pterocarpan. Then the cells were treated with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) for 2 h. BrdU incorporation into DNA was measured by utilizing clorimetric reaction with peroxidase-linked anti-BrdU antibody using a cell proliferation enzyme linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals). For cell counting, A10 cells were seeded in 60-mm culture dishes ($5\times1O^4$ cells/plate) and cultured at 37 °C for 2 days. The medium was then replaced with serum-free medium consisting of PDGF-BB (50 ng/ml). Cells were directly counted using a hemocytometer every 2 days.

2.4. Western blotting and immunoprecipitation

Quiescent Al0 cells in 10 cm dishes were incubated in serum-free DMEM in the presence and absence of pterocarpan for 24 h. Al0 cells were then stimulated for 30 min with PDGF-BB. After removal of the medium, cells were lysed with a cell lysis buffer consisting of 10 Tris–HCl, pH 7.5, I50 mM NaCI, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonylfluoride. Lysed cells were sonicated on ice for 15 s and were transferred to microcentrifuge tubes and centrifuged at 15,000 g for 20 min at 4 °C. The protein concentrations of the supernatants were measured using the Bio-Rad protein assay according to the method of Bradford. For irnmunoprecipitation, cell lysates were incubated with goat anti-PDGF receptor-fl antibody for 1 h at 4 °C and then incubated with 60 μ1 of proteinG-Sepharose for 2 h and washed 5 times with washing buffer (10 mM Tris–HCl, pH 7.8, 1% NP40, 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin). For

Western blot analysis, cell lysates and immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in a Tris buffered saline containing 0.l% Tween20 and 5% skim milk for 1 h at room temperature. The blots were then incubated for 24 h at 4 °C with specified antibodies for 1 h with a secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system. Primary antibodies used for Western blotting were as follows: anti-phospho-PDGF-receptor banti-phospho-PDGF-receptor b. (Santa Cruz Biotechnology), anti-Akt, anti-phospho-Akt, anti-p3 8 MAPK, anti-phospho-p38 MAPK, anti-ENS and anti-phospho-ERK (Cell Signaling Technology).

2.5. Cytotoxic assay

A10 cells grown in 96-well plates (5×10^3 cells/well) were incubated for 48 h in serum-free DMEM in the presence of astrapterocarpan (l–50 μM) or PDGF-BB (25 ng/ml). The medium was then collected and the cells were lysed with 1% Triton X-100. Lactate dehydrogenase (LDH) activity was measured using an LDH assay kit (Wako Chemical, Japan). The percentage of LDH release was calculated from the ratio of LDH activity in the medium to the sum of the LDH activity in the medium and in the cell lysate.

2.6. Statistics

Results were expressed as mean ± S.E.M. Statistical analysis was performed by using Dunnett's test.

3. Results

3.1. Effects of astrapterocarpan, formononetin and calycosin on PDGF-BB-induced cell proliferation

We assessed the effects of astrapterocarpan, formononetin and calycosin on cell proliferation in serum-free medium

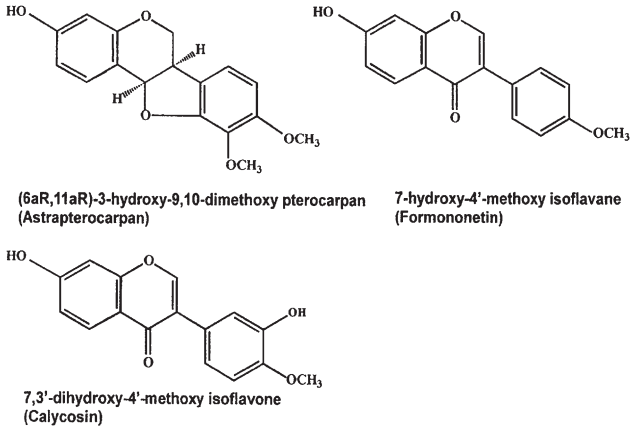

Fig. 1. Chemical structure of flavonoids isolated from *Astragalus membranaceus*.

Table 1
LDH release in the presence of PDGF-BB or Astrapterocarpan

	LDH-release (%)
Control	12.1 ± 0.7
PDGF-BB (25 ng/ml)	13.1 ± 1.0
PDGF-BB (25 ng/ml) + Astrapterocarpan (1 μM)	12.9 ± 0.9
PDGF-BB (25 ng/ml) + Astrapterocarpan (5 μM)	13.2 ± 1.1
PDGF-BB (25 ng/ml) + Astrapterocarpan (10 μM)	12.3 ± 0.6
PDGFnBB (25 ng/ml) + Astrapterocarpan (25 μM)	12.5 ± 0.7
PDGF-BB (25 ng/ml) + Astrapterocarpan (50 μM)	12.1 ± 0.8
Astrapterocarpan (50 μM)	12.3 ± 0.9

A10 cells grown were incubated for 48 h in serum-free DMEM in the presence of astrapterocarpan (l–50 μM) or PDGF-BB (25 ng/ml). The medium was then collected and the cells were lysed with 1% Triton X-100. The percentage of LDH release was calculated from the ratio of LDH activity in the medium to the sum of the LDH activity in the medium and in the cell lysate. Values are mean ± S.E.M. of four samples.

stimulated by PDGF-BB by a direct cell count of trypsinized cells. As shown in Fig. 1, incubation with PDGF-BB (25 ng/ml) resulted in a 5-fold increase in cell number compared with the basal value. Astrapterocarpan (l–50 μM) inhibited PDGF-BB-stimulated cell proliferation in a concentration-dependent manner with significant inhibition observed at 1 μM. The IC_{50} value for the inhibitory effect of astrapterocarpan was 10 μM. In contrast, formononetin and calycosin had no effect.

The viability of A10 cells stimulated with PDGF-BB combined with astrapterocarpan was confirmed by the quantity of LDH released from A10 cells in serum-free medium. Table 1 shows the viabilities of A10 cells under the conditions used in the present study. The viabilities of A10 cells in the control and astrapterocarpan-treated groups were not different, suggesting that the anti-proliferative effect of astrapterocarpan on A10 cells was not due to its cytotoxicity.

3.2. Effects of astrapterocarpan, formononetin and calycosin on PDGF-BB-induced DNA synthesis

As shown in Fig. 2, exposure of quiescent cells in serum-free medium to PDGF-BB (25 ng/ml) caused 8-fold increase in BrdU incorporation compared with the basal value. Astrapterocarpan (l–50 μM) inhibited PDGF-BB-stimulated BrdU incorporation in a concentration-dependent manner with significant inhibition observed at 1 μM. The IC_{50} value for the inhibitory effect of astrapterocarpan was 10 μM. In contrast, formononetin and calycosin had no effect.

3.3. Effect of astrapterocarpan on PDGF-BB-induced tyrosine phosphorylation of PDGF fl-receptor

The most proximal biological effect of PDGF-BB is activation of its receptor tyrosine kinase, which is essential for all of its biological functions. Thus, the inhibitory effect of astrapterocarpan on PDGF-BB-induced cell proliferation may have resulted from inhibition of tyrosine kinase activity of PDGF β-receptor. Therefore, we examined the effect of astrapterocarpan on PDGF-BB-induced tyrosine phosphorylation of the

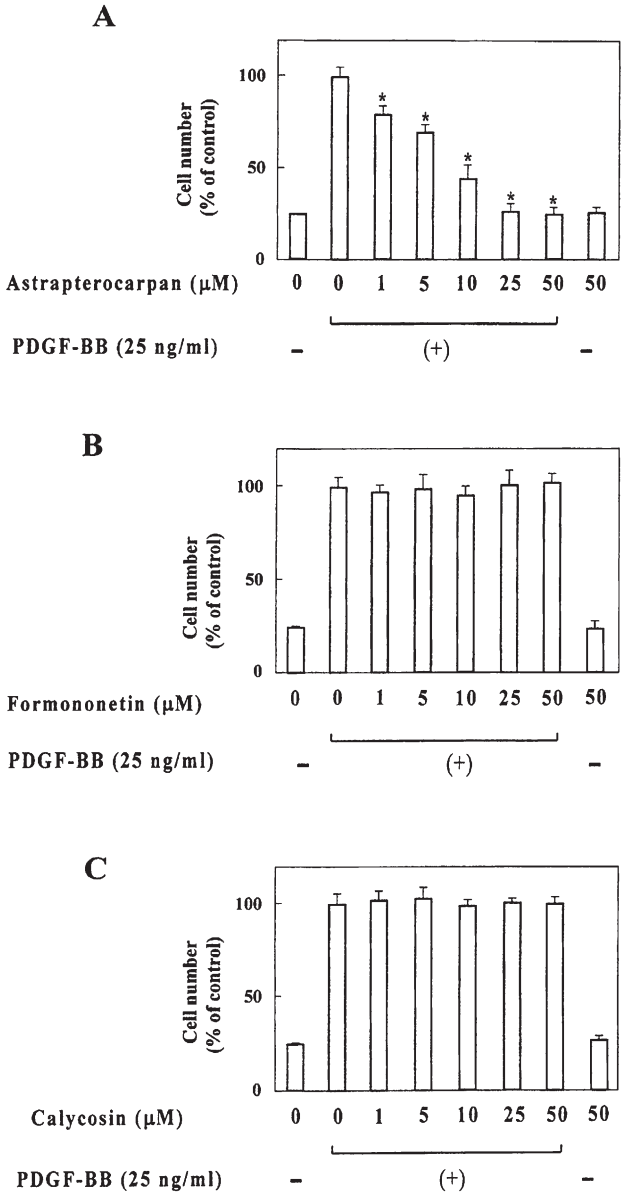

Fig. 2. Effects of astrapterocarpan, formononetin, and calycosin on cell prolifelation stimulated by PDGF-BB. Subconfluent cells were continuously cultured with PDGF-BB (25 25 ng/ml) in the absence or presence of the indicated concentrations of astrapterocarpan (A), formononetin (B), and calycosin (C). After 48 h in culture, the cells in triplicate dishes were trypsinized and counted as described in Methods. Values are mean ±S.E.M. of four samples. *Significantly different from the corresponding control ($P<05$).

PDGF P-receptor (Fig. 3). A10 cells in serum-free medium were treated with astrapterocarpan and then incubated with PDGF-BB (25 ng/ml). The cell lysates were immunoprecipitated with a PDGF β-receptor antibody, and phosphotyrosine-specific antibodies were used for immunoblotting to detect phosphorylated tyrosine on PDGF β-receptor. Treatment with PDGF-BB induced an increase in tyrosine–phosphorylated PDGF β-receptor, which was not affected by preincubation with astarapterocarpan. The inhibitory effect of astrapterocarpan on PDGF-BB-induced proliferation of A10 cells is not due to inhibition of the tyrosine phosphorylation of the PDGF β-receptor. Rather, the inhibition of cell proliferation by astrapterocarpan is likely to be due to post-PDGF β-receptor events (Fig. 4).

3.4. Effect of astrapterocarpan on PDGF-BB-induced ERK1/2 activation

We next examined whether astrapterocarpan inhibits cell proliferation by targeting the PI3K/Akt, ERKl/2 MAPK or p38 MAPK pathway. Al0 cells in serum-free medium were stimulated with PDGF-BB (25 ng/ml) for 20 min to examine the action of astrapterocarpan on PDGF-BB-induced activation and phosphorylation of Akt, ERK1/2 and p38 MAPK.

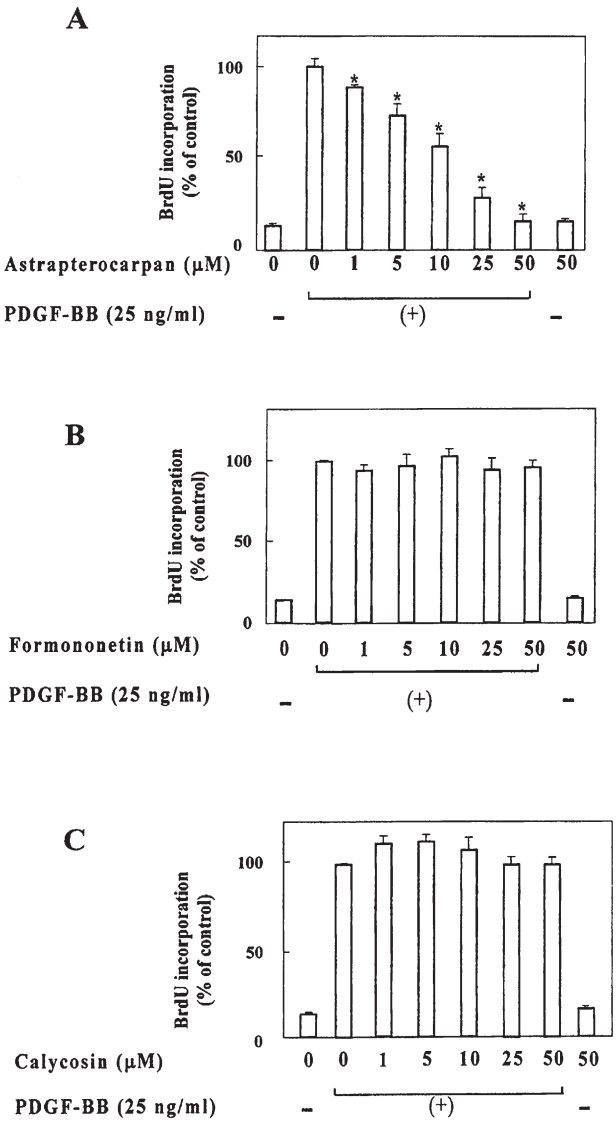

Fig. 3. Effects of astrapterocarpan, formononetin, and calycosin on DNA synthesis stimulated by PDGF-BB. Quiescent cells were continuously cultured with PDGF-BB (25 ng/ml) for 24 h in the absence or presence of the indicated concentrations of astrapterocarpan (A), formononetin (B), and calycosin (C). Then the cells were treated with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) for 2 h. BrdU incorporation into DNA was measured by utilizing clorimetric reaction with oxidase-linked anti-BrdU antibody using a cell proliferation ELISA kit. Values are mean ± S.E.M. of four samples. *Significantly different from the corresponding control ($P<0.05$).

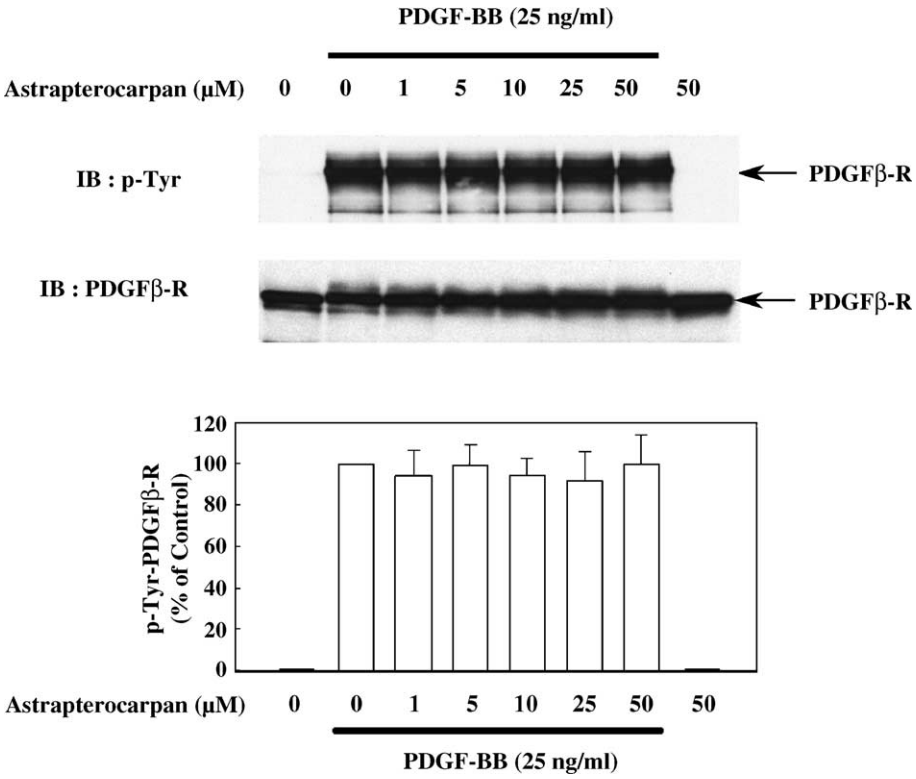

Fig. 4. Effect of astrapterocarpan on tyrosine phosphorylation of PDGF β-receptor. Quiescent Al0 cells were incubated with astrapterocarpan at the indicated concentration for 24 h. The cells were then stimulated briefly with 25 ng/ml PDGF-BB for 20 min. Equal amounts of lysates were immunoprecipitated with a PDGF β-receptor-specific antibody, and immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with an anti-phosphotyrosine antibody. The amounts of phosphorylated PDGF β-receptor (−R) are expressed as ratios of the densitometric measurements of the samples. Values are mean±S.E.M. of three samples.

Astrapterocarpan pretreatment inhibited ERK1/2 MAP kinase activation in a concentration-dependent manner with significant inhibition at 1 μM. The IC_{50} value for the inhibitory effect of astrapterocarpan was 10 μM. Surprisingly, the phosphorylation of other pathway, p38 MAP kinase and Akt, was not inhibited by astrapterocarpan except at a high concentration (50 μM). It is noteworthy that the concentration of astrapterocarpan necessary for inhibition of the ERK1/2 pathway was almost the same as that of astrapterocarpan for inhibition of A10 cell proliferation.

4. Disscussion

Pterocarpan derivatives, the main constituents of the legume have been reported to possess several pharmacological activities, such as antimicroviral activity (Bojase et al., 2002), anti-inflammatory activity (Miller et al., 1989) and antiplatelet aggregation activity (Goda et al., 1992). In the present study, astrapterocarpan, a pterocarpan derivative isolated from *Astragahs membranaceus*, was shown to inhibit rat vascular smooth muscle cell proliferation and DNA synthesis in response to PDGF-BB. The inhibitory actions of astrapterocarpan do not appear to result from nonspecific cellular toxicity, because the quantity of LDH released in the medium was not significantly affected by astrapterocarpan (Table 1) and the inhibitory effects of astrapterocarpan on growth were rapidly reversed when the compound was removed from the culture medium (data not shown). Therefore, in addition to the above-stated biological effects of pterocarpan derivatives, the present study provides new evidence that astrapterocarpan has antiprolifelative activity in vascular smooth muscle cells.

Prominent flavonoids, quercetin, genistein and gallates, have been reported to be tyrosine kinase inhibitors that block signal transduction pathways mediated by MAPK and P13K in various cells (Nicosia et al., 2003). Therefore, we speculate that astrapterocarpan inhibits vascular smooth muscle cell proliferation to modify the protein kinase-mediated intracellular signal pathway(s). Tyrosine phosphorylation of PDGF β-receptor creates a binding site for SH-2 domain-containing signaling proteins such as phospho1ipase Cγ, PI3-kinase p85 subunit, SOS and the adaptor molecule She or Grb2 (Heldin et al., 1998). Among these signaling proteins, binding of Grb2/sos or She in turn activates the small GTP-binding protein RAS that couples to the Raf/MEKl/z/EM or Rac/MEK3kYp38 cascade, which is considered to be a critical step for proliferation of vascular smooth muscle cells (Marshall, 1995). In our study, astrapterocarpan had no effect on tyrosine phosphorylation of PDGF-receptor, suggesting that the site of action for astrapterocarpan is downstream from these signaling proteins.

Matsumoto et al. (1999) reported that expression of dominant-negative Ras inhibited PDGF-BB-induced migration in

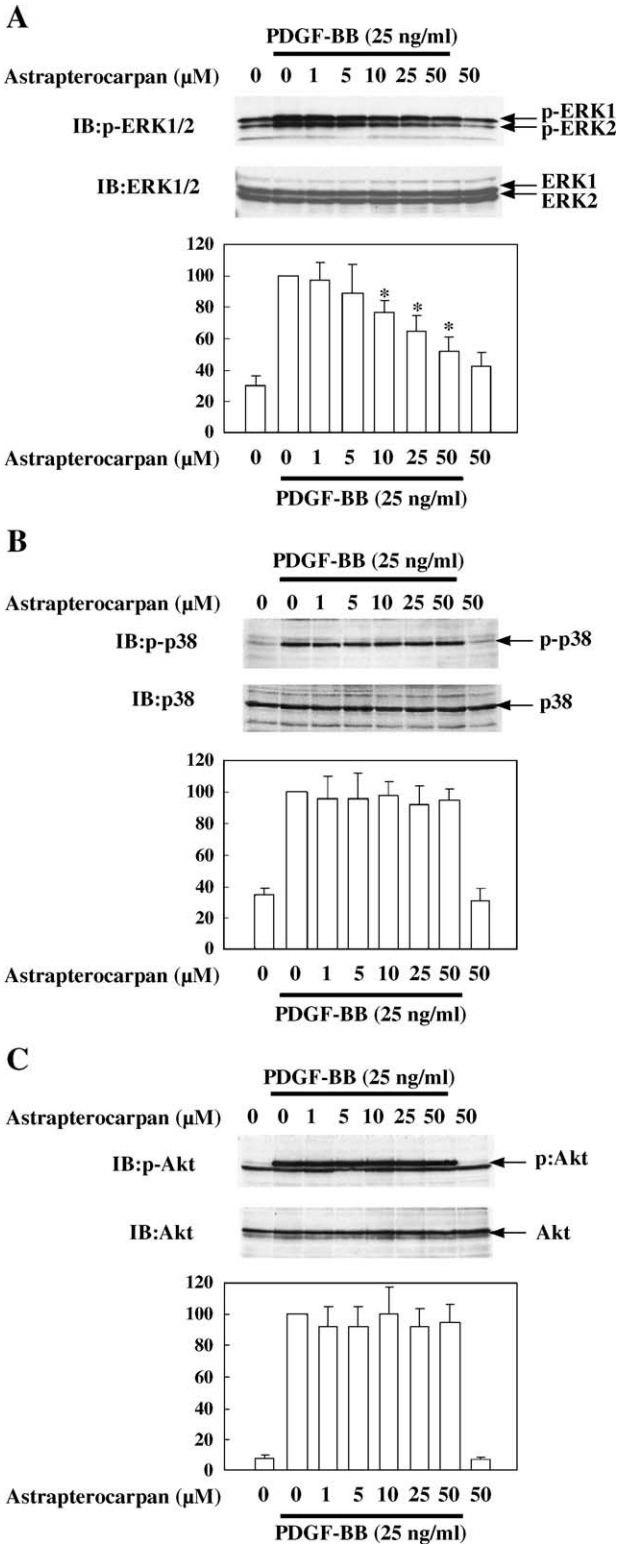

Fig. 5. Effects of astrapterocarpan on phosphorylation of ERK1/2, p38 MAP kinase and Akt. Quiescent A10 cells were incubated with astrapterocarpan at the indicated concentration for 24 h. The cells were then stimulated briefly with 25 ng/ml PDGF-BB for 20 min. The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with an anti-phospho-ERK1/2 antibody (A), anti-phospho-p38 MAP kinase antibody (B), and anti-phospho-Akt antibody (C). The amounts of phosphorylated kinases are expressed as ratios of the densitometric measurements of the samples. Values are mean ± S.E.M. of four to five samples. *Significantly different from the corresponding control ($P < 0.05$).

endothelial cells expressing wild-type PDGF β-receptor. They demonstrated that dominant-negative Ras inhibited PDGF-BB-induced activation of p38 MAP kinase as well as ERKl/2 map kinase. In the present study, since astrapterocarpan did not inhibit PDGF-BB-induced p38 phosphorylation, it is likely that astrapterocarpan did not interfere with thepathway from the PDGF P-receptor, via Ras, to p38 map kinase. These findings suggest that the site of astrapterocarpan action may be between Ras and ERK1/2. Further studies, including an investigation of the effects of astrapterocarpan on Raf and MEIW2 of vascular smooth muscle cells are needed to determine the exact target site of astrapterocarpan.

It has been reported that gallates block the signal transduction pathways by MAPK and PI3K in human smooth muscle cells (Ahn et al., 1999). In addition, it has been shown that quercetin selectively inhibits PI3K in rat aortic smooth muscle cells (Yoshizumi et al., 2001). Kinetic analysis has indicated that these flavonoids do not compete with ATP binding to signaling protein and most likely elicit their inhibitory effects through an allosteric mechanism (Dudley et al., 1995). Studies to elucidate the structure-activity relationship of the inhibitory effect of astrapterocarpan on ERIC activity are in progress in our laboratory.

Moreover, this compound significantly inhibited the tyrosine phosphorylation of ERKl/2 MAPK without having any effect on PDGF β-receptor tyrosine kinase activity, p38 MAP kinase or Akt kinase activity. The concentration of astrapterocarpan necessary for the inhibition of this ERK1/2 MAP kinase activity was similar to that for the inhibition of vascular smooth muscle cell proliferation (Fig. 5). Pyles et al. (1997) reported that MAPK was activated in response to balloon overstretch injury in porcine carotid arteries. Moreover, in a recent study using carotid artery ballooning injury, it has been demonstrated that MAPK signaling, particularly ERK ctivity, was increased and that medial cell replication following injury was reduced by D098059, an ERK/MAPK pathway inhibitor (Koyama et al., 1998). The above results imply that the ERIC/MAP pathway may be crucial in neosintimal thickening progression. Our results suggest that astrapterocarpan has preventative effects on vessel restenosis in addition to cardiovascular diseases such as atherosclerosis and myocardial infarction.

In summary, we have demonstrated for the first time that astraprotecarpan has an inhibitory effect on vascular smooth muscle cell proliferation and that its mechanism of action appears to be ediated, at least in part, by inhibition of ERKl/2 activation. The results suggest that astrapterocarpan is an attractive candidate for the protection of cardiovascular disease.

Acknowledgements

This study was supported by the Hokkaido collaboration of regional entities for the advancement of technological excellence, Japan Science and Technology Corporation. This research was also supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports Science, and Technology, Japan (Y.O., Y.N.).

References

Ahn, H.Y., Hadizadeh, K.R., Seul, C., Yun, Y.P., Vetter, H., Sachinidis, A., 1999. Epigallocathechin-3 gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma cells (A172). Mol. Biol. Cell 10, 1093–1104.

Anetai, M., Katsura, E., Katoh, Y., Yamagishi, T., 1994. Chemical evaluation of *Astragali radix*. Nat. Med. 48, 244–252.

Bensky, D., Gamble, A., 1993. Chinese Herbal Medicine: Materia Medica, Revised Edition. Eastland Press, Seattle, WA.

Bojase, G., Majinda, R.R., Gashe, B.A., Wanjala, C.C., 2002. Antimicrobial flavonoids from Bolusanthus speciosus. Planta Med. 68, 615–620.

Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Regenass, U., Lydon, N.B., 1995. Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein–tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. Proc. Natl. Acad. Sci. U. S. A. 92, 2558–2562.

Claesson-Welsh, L., 1994. Platelet-derived growth factor receptor signals. J. Biol. Chem. 269, 32023–32026.

Deng, C.Q., Ge, J.W., Wang, Q., 1995. Comparison of effect of Astragalus membranaceus and huoxuefang on thromboxane, prostacyclin and adenosine cyclic monophosphate in cerebral reperfusion injury in rabbits. Zhongguo zhong Xi Yi Jie He Za Zhi 15, 165–167.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., Saltiel, A.R., 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. U. S. A. 2, 7686–7689.

Ferns, G.A., Raines, E.W., Sprugel, K.H., Motani, A.S., Reidy, M.A., Ross, R., 1991. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 253, 1129–1132.

Goda, Y., Kiuchi, F., Shibuya, M., Sankawa, U., 1992. Inhibitors of prostaglandin biosynthesis from *Dalbergia odorifera*. Chem. Pharm. Bull. 40, 2452–2457.

Golomb, G., Fishbein, I., Banai, S., Mishaly, D., Moscovitz, D., Gertz, S.D., Gazit, A., Poradosu, E., Hart, C.E., Clowes, A.W., 1997. Platelet-derived growth factor and arterial response to injury. Circulation 95, 555–556.

Hart, C.E., Clowes, A.W., 1997. Platelet-derived growth factor and arterial response to injury. Circulation 95, 555–556.

Heldin, C.H., Ostman, A., Ronnstrand, L., 1998. Signal transduction via platelet-derived growth factor receptors. Biochim. Biophys. Acta 1378, F79–F113.

Hertog, M.G., Feskens, E.J., Hollman, PC., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342, 1007–1011.

Hong, C.Y., Lo, Y.C., Tan, F.C., Wei, Y.H., Chen, C.F., 1994. *Astragalus membranaceus* and *Polygonum multiflorum* protect rat heart mitochondria against lipid peroxidation. Am. J. Chin. Med. 22, 63–70.

Huang, W.M., Yan, J., Xu, J., 1995. Clinical and experimental study on inhibitory effect of sanhuang mixture on platelet aggregation. Zhongguo zhong Xi Yi Jie He Za Zhi 15, 465–467.

Katsura, E., Yamagishi, T., 1987. Quantitative determination of isoflavonoids in *Astragali radix* by high performance liquid chromatography. Rep. Hokkaido Inst. Public Health 37, 48–52.

Koyama, H., Olson, N.E., Dastvan, F.F., Reidy, M.A., 1998. Cell replication in the arterial wall: activation of signaling pathway following in vivo injury. Circ. Res. 82, 713–721.

Lau, B.H., Ruckle, H.C., Botolazzo, T., Lui, P.D., 1994. Chinese medicinal herbs inhibit growth of murine renal cell carcinoma. Cancer Biother. 9, 153–161.

Lin, L.Z., He, X.G., Lindenmaier, M., Nolan, G., Yang, J., Cleary, M., Qiu, S.X., Cordell, G.A., 2000. Liquid chromatography-electrospray ionization mass spectrometry study of the flavonoids of the roots of *Astragalus mongholicus* and *A. membranaceus*. J. Chromatogr. 876, 87–95.

Marshall, C.J., 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

Matsumoto, T., Yokote, K., Tamura, K., Takemoto, M., Ueno, H., Saito, Y., Mori, S., 1999. Platelet-derived growth factor activates p38 mitogen-activated protein kinase through a Ras-dependent pathway that is important for actin reorganization and cell migration. J. Biol. Chem. 274, 13954–13960.

Miller, A.L., 1998. Botanical influences on cardiovascular disease. Altern. Med. Rev. 3, 422–431.

Miller, D.K., Sadowski, S., Han, G.Q., Joshua, H., 1989. Identification and isolation of medicarpin and a substituted benzofuran as potent leukotriene inhibitors in an anti-inflammatory Chinese herb. Prostaglandins Leukot. Essent. Fat. Acids 38, 137–143.

Nicosia, S.V., Bai, W., Cheng, J.Q., Coppola, D., Kruk, PA., 2003. Oncogenic pathways implicated in ovarian epithelial cancer. Hematol./Oncol. Clin. North. Am. 17, 927–943.

Pauletto, P., Da Ros, S., Tonello, M., Capriani, A., Chiavegato, A., Sartore, S., Pessina, A.C., 1996. Anipamil prevents intimal thickening in the aorta of hypertensive rabbits through changes in smooth muscle cell phenotype. Am. J. Hypertens. 9, 687–694.

Pyles, J.M., March, K.L., Franklin, M., Mehdi, K., Wilensky, R.L., Adam, L.P., 1997. Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. Circ. Res. 8 (1), 904–910.

Ross, R., 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362, 801–809.

Sinclair, S., 1998. Chinese herbs: a clinical review of Astragalus, Ligusticum, and Schizandrae. Altern. Med. Rev. 3, 338–344.

Strawn, L.M., Mann, E., Elliger, S.S., Chu, L.M., Germain, L.L., Niederfellner, G., Ullrich, A., Shawver, L.K., 1994. Inhibition of glioma cell growth by a truncated platelet-derived growth factor-beta receptor. J. Biol. Chem. 269, 21215–21222.

Yim, T.K., Wu, W.K., Pak, W.F., Mak, D.H., Liang, S.M., Ko, K.M., 2000. Myocardial protection against ischaemia-repe~usion injury by a Polygonurn multiflorum extract supplemented 'Dang-Gui decoction for enriching blood', a compound formulation, ex vivo. Phytother. Res. 14, 195–499.

Yoshida, Y., Wang, M.Q., Shan, B.E., Yamashita, U., 1997. Immunomodulating activity of Chinese medicinal herbs and *Oldenlandia diffusa* in particular. Int. J. Immunopharmacol. 19, 359–370.

Yoshizumi, M., Tsuchiya, K., Kirima, K., Kyaw, M., Suzaki, Y., Tamaki, T., 2001. Quercetin inhibits Shesand phosphatidylinositol 3-kinase-mediated c-Jun N-terminal kinase activation by angiotensin II in cultured rat aortic smooth muscle cells. Mol. Pharmacol. 60, 656–665.