Seed of *Trichosanthes kirilowii* MAXIM Inhibits TNF-α-induced Migration in Human Aortic Smooth Muscle Cells Via MMP-9 Inhibition

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Atherosclerosis, slow progressing inflammatory lesion in arteries, is one of the major causes of cardiovascular diseases. As mortality due to cardiovascular disease keeps increasing in Korea, researches on pathological mechanism of atherosclerosis may be beneficial in fighting against cardiovascular diseases. It is known that migration and MMP-9 secretion of Vascular Smooth Muscle Cell(VSMC) play a significant part in pathogenesis of atherosclerosis, although detailed mechanism of entire process is not clarified. We investigated whether the seeds of *Trichosanthes kirilowii* maxim (TS). inhibit migration and MMP-9 production of HASMC(human aortic SMC), which were induced by TNF-α treatment. Migration assay showed that TS inhibited the migration of HASMC induced by TNF-α, in dose dependent manner. Also by Zymography MMP-9 production of HASMC was found to be reduced by TS, both in time and in dose dependent manner. Western blotting results suggest TS suppress activity of MAPkinases.

Key words : *Trichosanthes seed*(TS), anti-atherosclerotic, MMP-9, migration, HASMC, MAPKinase

Introduction

Synopsis of Golden Chamber (金匱要略, also translated as Medical Treasures of the Golden Chamber) is one of the most precious texts in traditional korean medicine. In its chapter 9 (Diagnosis and Treatment of Chest discomfort and pain), the descriptions of the symptoms reminds us of cardiovascular diseases such as angina pectoris. Among the herbal formulations prescribed in the chapter, gwaruhaebecbecju-tang, gwaruhaebecbanha-tang, and gwaruhaebecgyeji-tang stand out. These sereis of formulations commonly have the seed of Trichosanthes kirilowii MAXIM. (TS) in its components, which implies the possible application of TS in cardiovascular diseases.

Atherosclerosis is a disease affecting arterial blood vessels. It is a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophages and promoted by low density lipoproteins without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins (HDL)[1]. It has been understood that, in development of atherosclerosis, proliferation and migration of vascular smooth muscle cells (VSMC) are closely related[2]. In the early stages of atherosclerosis, aortic smooth muscle cells (SMC) increase in numbers, causing intimal thickening of the arterial walls[3]. Besides the increase in numbers, degradation or remodeling of extracellular matrix surrounding the cells should be preceded before migration of VSMC[4]. VSMC produces several extracellular matrix, including collagens. An imbalance between the accumulation and degradation of extracellular matrix may result in intimal thickening[5,6].

It is quite meaningful to understand mechanisms of SMC migration in developing therapeutic methods to prevent atherosclerosis caused by SMC migration and intimal thickening. Proteinase, especially matrix metalloproteinases(MMPs), needs to act in the course of matrix remodeling[7]. Among three groups of MMPs, type IV collagenases(or gelatinases), such as MMP-2 and -9, degrade denatured collages in the cells. The expression of MMP-9 seems related with the development of atherosclerotic lesions. It is generally assumed, based on the recent in vivo studies, that functions of MMP-9 that regulates VSMC migration and proliferation play core part in development of arterial lesions[8]. From a number of reports, it has been concluded that the level of MMP-9 in VSMC is normally low, and that its expression is induced by treating tumor necrosis factor-α (TNF-α)[9,10].

A number of natural products are known effective for

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vascular diseases, and certain traditional herbal prescriptions have been used in treating atherosclerosis[11-13].

Trichosanthes seed (TS) is a dried seed of *Trichosanthes kirilowii* MAXIM (*T. kirilowii*). Some of its pharmacological actions known so far include therapeutic effects of cardiovascular diseases such as vasodilatation of coronary artery, protective function against acute myocardial ischemia, apophlegmatic, and anti-bacterial function[14]. Major components of *T. kirilowii* studied are trichosanthin, trichomislin, karasurin, and trichokirin, most of which were found to have certain pharmacological effects above mentioned[15]. There are several studies on *T. kirilowii*, most of which are focused on its anti-cancer activities against hepatoma[16], retinoblastoma, choriocarcinoma[17], both in vitro and in vivo. Nonetheless, anti-migration mechanism of TS remains unclear in cardisovascular diseases field.

Therefore, this study was performed to elucidate the antiangiogenic mechanism of TS in HASMC through MMP-9 and MAPK pathways to evaluate the potent effeicacy of TS in cardiovascular field.

Materials and Methods

1. Preparation of the plant material extract

The authentic plant material, TS was our deposit, which was purchased from local market and identified by the botanical expert at the College of Oriental Medicine, Dongguk University (Gyeongju, Korea).

The dried TS was extracted with boiling water (Fig. 1). Briefly, a 100 g of TS was cut into small sections, boiled in 500 ㎖ of distilled water for 24 hrs. The residues were removed by filtration, and then the filtrate was evaporated to obtain the desired concentration, and then was lypophilized by freeze-drier to produce powder (yield, 2.35%). The dried extract was solved in distilled water to appropriate concentrations and the resolved extract was used for analysis.

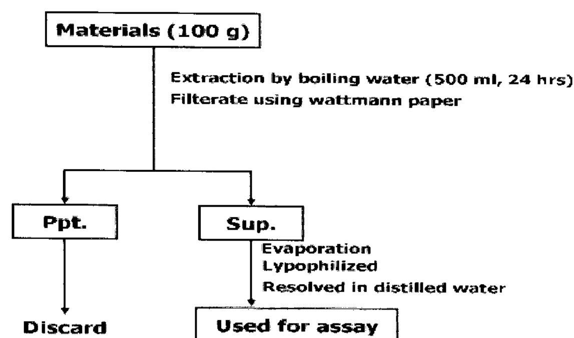

Fig. 1. Preparation of the water-extracts of the seeds of *Trichosanthes kirilowii* MAXIM.

2. Cell culture

Human aortic smooth muscle cells (HASMC) were our deposit, which were purchased from Sciencell (CA, USA). HASMC were cultured in Smooth Muscle Cell Medium(SMCM, ScienCell, CA, USA) containing essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals and 2% of fetal bovine serum. For all experiments, early passage of HASMC were grown to 80-90% confluence and made quiescent by serum starvation for at least 24 hrs. The serum-free medium, which contains secreted proteins such as MMP-9, was used for gelatin zymography.

3. Geltain zymography assay

Culture supernatants of HASMC treated with or without TNF-α(100 ng/㎖) were resuspended in a sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue and loaded without boiling in 7.5% acrylamide / bisacrylamide (29.2:0.8) separating gel containing 0.2% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 100 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (twice for 30 min) at room temperature and rinsed in distilled pure water. For inhibitory effect of TS on gelatinolytic activity of MMP-9, the samples were freshly solubilized in the Tris-HCl buffer used for incubation of gel to appropriate concentration. The gel slab was cut into slices corresponding to the lanes and was incubated at 37℃ for 20 hrs in the incubation buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-58 with or without the stated concentrations of TS in different tanks. The gel was then stained for 15-30 min in 0.1% (w/v) Coomassie blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie blue dye. Proteolysis was detected as a white zone in a dark field and the intensity of the bands obtained from zymogram studies was estimated with Scion Image (Scion Corp., MA, USA). The values are calculated by percent of control and expressed as means ± SE.

4. Cell viability assay

The cytotoxic effect of TS on HASMC was investigated using a commercially available proliferation kit (XTT II, Roche Diagnostics GmbH, Mannheim, Germany). Briefly, the cells were plated in 96-well culture plates at a density of $1x10^4$cells per well in SMCM culture medium and allowed to attach for 2 hrs. a: The TS was added to various final concentrations (and control: 0 ㎍/㎖) in triplicates. After 24 hrs of culture 50 ㎕ of XTT reaction solution (sodium 3'- [1-(phenyl-aminocarbonyl)-

3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the wells. b: Also, to investigate the time factor in the TS effect, XTT reaction solution was administered after 0h, 3h, 6h, 12h, and 24h respectively. After the plates were incubated for 4 hrs at the condition of 37℃ and 5% CO_2, the optical density at 490 nm was measured by an ELISA plate reader (Molecular device co., CA, USA). All determinations were confirmed using replication in at least three identical experiments. The data were shown mean SE as percent of control.

5. RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted by the TRIZOL Reagent (Invitrogen life technologies, USA) according to the manufacturer's instructions. For RT-PCR, cDNA was synthesized from 1 ㎍ of total RNA using AccuPower RT Premix Kit (Bioneer, Korea) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: MMP-9 (537 bp), 50-CGGAGCACGGAGACGGGTAT-30 (sense) and 50-TGAAGGGGAAGACGCACAGC-30 (antisense); β-actin (247 bp), 50-CAAGAGATGGCCACGGCTGCT-30 (sense) and 50-TCCTTCTGCATCCTGTCGGCA-30 (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

6. Migration assay

Matrigel-coated filter inserts (8 ㎛ pore size) that fit into 24-well invasion chambers were obtained from Corning (NY, USA). HASMC cells to be tested for invasion were detached from the tissue culture plates, washed, resuspended in conditioned medium (5×10^4 cells/200 ㎕), and then added to the upper compartment of the invasion chamber in the presence or absence of TNF-α (100 ng/㎖) and various concentration of TS (0, 50, 200 and 500 ㎍/㎖). The serum-free medium (500 ㎕), which incubated HASMC for 24 hrs, was added to the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37℃ for 24 hrs in 5% CO_2. After incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions (Corning, NY, USA). The cells that migrated through the Matrigel and were located on the underside of the filter were counted. Three to five invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

7. Western blotting assay

HASMC were treated with various concentrations of TS in the presence of 100 ng/㎖ TNF-α. Cellular lysates were prepared in a lysis buffer containing 20 mM Tris - HCl (pH 7.5), 1 mM Na_2EDTA, 150 mM NaCl, 1 mM EGTA, 1 % deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 5 mM NaF, 1 mM Na_3VO_4, 1 ㎍/㎖ leupeptin, 1 ㎍/㎖ aprotinin, and 1% NP-40. The cells were disrupted and extracted at 4 ℃ for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant was obtained as the cell lysate. Protein concentrations were measured using the Bio-Rad protein assay. Aliquots of cellular proteins (30 ㎍ /lane) were electrophoresed on 10% SDS - polyacrylamide gel electrophoresis (PAGE) and transferred to an Hybond-ECL Nitrocellulose membrane, (Amersham Biosciences, Buckshire, UK). The membrane was allowed to react with a specific antibody and detection of specific proteins was carried out by enhanced chemiluminescence following the manufacturer's instructions. Loading differences were normalized using polyclonal beta-actin antibodies.

Results

1. Cytotoxicity of TS on HASMC cells

The cytotoxicity of the TS on the HASMC cells were evaluated using XTT cell proliferation assay kit. The 5×10^4 cells were incubated for 24 hrs in cultures in 96-well microplates (volume 100 ㎕/well) with various final concentration of TS (0, 50, 100, 200, 500, 1000, 2000, 4000 and 8000 ㎍/㎖). Time-dependent cytotoxic effect of TS was shown in Fig. 2. TS showed little cytotoxic effect on HASMC viability even after 24 hours. Dose-dependent cytotoxic effect of TS against HASMC was shown in Fig. 3. TS has a weak cytotoxic effect on HASMC cells in a typical concentration.

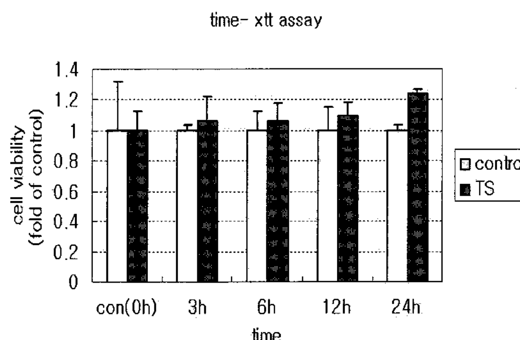

Fig. 2. Cytotoxicity of TS, by XTT assay(in time-dependent manner).
TS concentration: 500 ㎍/㎖

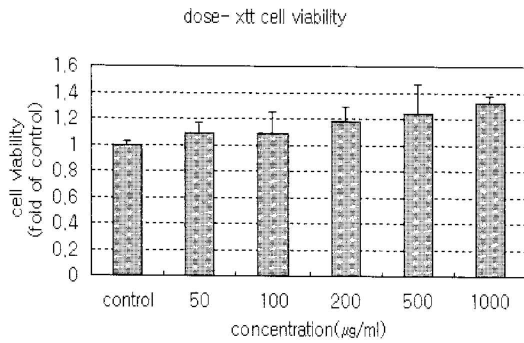

Fig. 3. Cytotoxicity of TS, by XTT assay (in dose-dependent manner).

2. Inhibitory effect of TS on HASMC cells migration

HASMC was suspended in conditioned medium (5×10^4 cells/200 μℓ), added to the upper components of Matrigel invasion chamber supplemented with various final concentrations of TS (0, 50, 200 and 500 μg/mℓ) in presence of TNF-α (100 ng/mℓ). The cells were incubated for 24 hrs at 37℃ and 5% CO_2. As shown in Fig. 4 the total number of cells that invaded to the underside of the filters was significantly decreased by TS treatment, in a dose dependent manner.

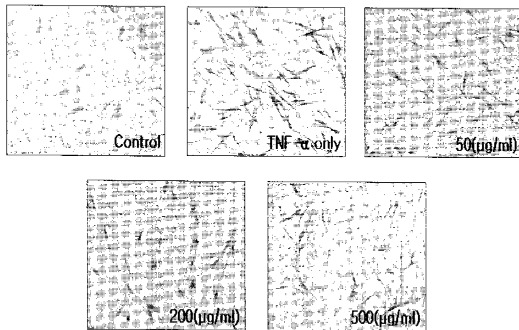

Fig. 4. Inhibitory effect of TS on HASMC cell migration induced by TNF-α.

3. Gelanitolytic activity of MMP-9 induced by TS

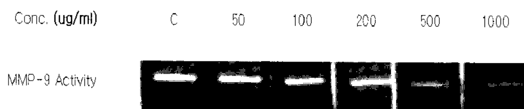

Fig. 5. MMP-9 activity by TS, with Hep3B cells.

4. Inhibitory effect of TS on the MMP-9 activity - zymography

To examine the inhibitory activity of TS against MMP-9 activity, the cultured conditioned media obtained from TNF-α (100 ng/mℓ)-treated HASMC cells have been subjected to the gelatin zymography in the presence of various concentrations of TS (0, 50, 100, 200 and 500 μg/mℓ) and in various treatment times. As shown in Fig. 6 and Fig. 7 TS inhibited the MMP-9 activity, which were induced by TNF- α treatment, both in a dose-dependent manner and in a time-dependent manner. And then, the effects of specific kinase inhibitors on the expression of MMP-9 in TNF-α-induced HASMC were analyzed by zymography. TNF-α-induced MMP-9 secretion was inhibited by selective inhibitors of the ERK1/2 (PD98059), p38 MAPK (SB203580), or SAPK/JNK (SP600125) pathways, although the degrees of inhibition were various(Fig. 8).

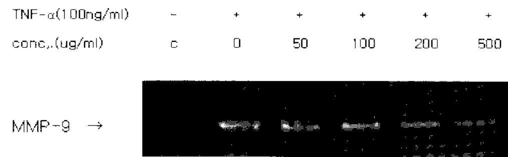

Fig. 6. Inhibitory effect of TS on MMP-9 activity induced by TNF-α (in concentration-dependent manner).

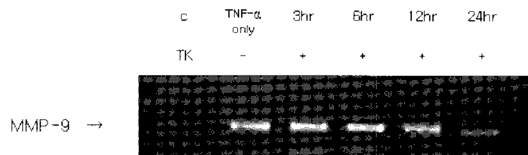

Fig. 7. Inhibitory effect of TS on MMP-9 activity induced by TNF-α (in time-dependent manner).

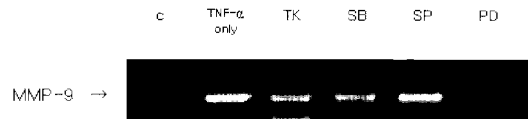

Fig. 8. Comparison of inhibitory effect of TS on MMP-9 activity with various MAPK inhibitors.

5. Inhibitory effect of TS on the MMP-9 activity - RT-PCR results

TS suppresses TNF-α-induced MMP-9 secretion through inhibition of its transcriptional activity in HASMC. To determine whether the inhibition of MMP-9 secretion by TS was due to a decreased level of transcription, we performed RT-PCR. Treatment of cells with TS decreased the levels of TNF-α-stimulated MMP-9 mRNA expression(Fig. 9). These results show that TS suppresses TNF-α-induced MMP-9 secretion through inhibition of its transcriptional activity in HASMC. Fig. 10 is a RT-PCR result of the experiment to find out and compare the effects of specific kinase inhibitors(see also Fig. 8).

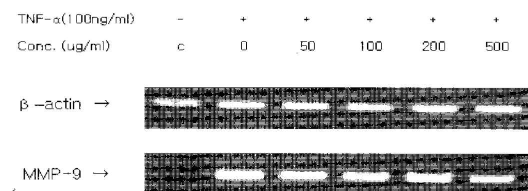

Fig. 9. Inhibitory effect of TS on MMP-9 activity(RT-PCR results, in concentration-dependent manner).

6. Inhibitory effect of TS on the MAPK activation

The subsequent experiments were designed to elucidate which of these signal transduction pathways is involved in TNF-α-stimulated MMP-9 expression and TS inhibition of the MMP-9 expression in HASMC. MAPK signaling pathways are involved in the inhibition of TNF-α-induced MMP-9 expression by TS in HASMC. MMP-9 gene expression can be activated via a number of signal transduction pathways including those involving ERK1/2, p38 MAPK, and SAPK/JNK. Then we investigated whether TS inhibited MMP-9 secretion by blocking activation of the ERK1/2, p38 MAPK, or SAPK/JNK pathways. TNF-α induced phosphorylation of all of three members of the MAPKs. TS suppressed the TNF-α-induced phosphorylation of ERK1/2, p38 MAPK, and JNK pathways in a dose-dependent manner(Fig. 11). These results suggest that the specific inhibitions of MAPK signaling pathways are directly involved in the regulation of TNF-α-induced MMP-9 expression by TS in HASMC.

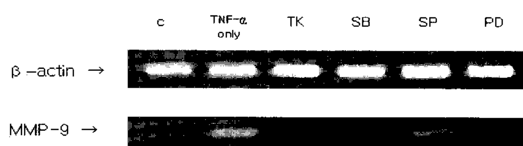

Fig. 10. Comparison of inhibitory effect of TS on MMP-9 activity with various MAPK inhibitors.

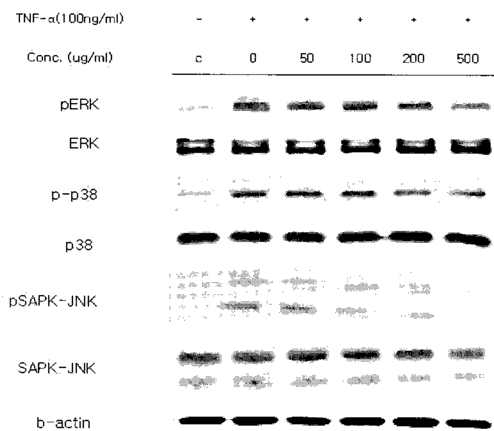

Fig. 11. Inhibitory effect of TS on various MAPKs activation

7. Comparison of inhibitory effect of TS with EGCG on the MMP-9 activity

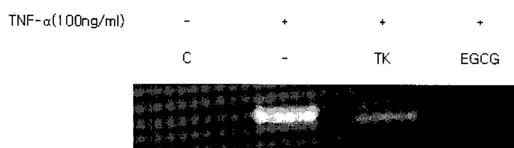

Fig. 12. Comparison of inhibitory effect of TS on the MMP-9 activity, with EGCG

Discussion

Trichosantehs kirilowii MAXIM. (*T. kirilowii*), Hanultari in Korean, is widely used in korean traditional medicine, but researches on its general composition are not abundant[17]. *T. kirilowii*, also known as Mongolian snakegourd, is widely used for treatment of pneumonia, pleurisy, intercostal neuralgia, tonsillar phayringitis, and sometimes for treatment of angina pectoris[18]. Root of *T. kirilowii* or Trichosantehs kirilowii MAXIM. var. japonicum KITAMURA is used for pyretolysis, diuresis, as an antitussive and an antiinflammatory drug[19]. The root of *T. kirilowii* has multiple pharmacological properties including abortifacient, anti-tumor and anti-HIV[20]. *T. kirilowii* contains triterpenoid saponin, organic acids, resins, saccharides, fatty oils etc., and its pharmacological actions include apophlegmatic, cathartic, anti-bacterial functions, as well as dilatation of coronary artery[21,22].

For instances, trichosanthin is reported to have inhibitory effect on retinoblatoma[23], choriocarcinoma cells, HIV-infected macrophages and lymphosites[24,25]. Also, Li suggested trichosanthin may be useful for modulation of inflammation through NO-mediated apoptosis[26]. Cytotoxic effects of solvent fraction of *T. kirilowii* have also been found to certain type of cells, mostly cancer cells such as L1210, S-180[27], P380[28], and HRT-18 which are human cancer cell[29].

Some components of *T. kirilowii* that have been studied are trichosanthin, karasurin, trichokirin, trichomislin, etc. Among these, trichosanthin has been found to induce apoptosis, enhance the action of chemokines and inhibit HIV-1 integrase[16]. Also, Wang et al. reported that trichosanthin-monoclonal antibody was a potent and quite specific antihepatoma agent and might have considerable potential in hepatoma therapy[30].

Trichosanthin was very recently reported that trichosanthin may be useful for the chemical treatment of retinoblastoma[17]. Trichosanthin is selectively toxic to some cells, such as choriocarcinoma cells, proximal tubule epithelial cells, antigen-specific T cells, and HIV-infected macrophages and lymphocytes[31,32]. Trichosanthin, a 27-kDa protein, has been found to have a function of immunoregulation, in addition to above mentioned functions. Trichosanthin is highly toxic to monocytes/macrophages, trophoblasts, choriocarcinoma cells, and melanoma cells, but has only slight effects on amniotic cells, fibroblast, and hepatoma cells, suggesting a specific mechanism for entry of trichosanthin into cells[33].

Trichokirin, a glycoprotein extracted from the seeds of *T. kirilowii*, was found to have selective toxicity to leukemia cells, when the protein was conjugated to a monoclonal antibody[34].

Bolognesi et al also showed that trichokirin modified to immunotoxin, had a cytotoxicity to human lymphocytes[35]. Mi et al. reported that trichomislin from *T. kirilowii* inhibited the growth of choriocarcinoma cells via induction of apoptosis. Its mechanism involves the release of cytochrome c and the increase of caspase-3 activities, that enables binding to and entering the choricarcinoma cells[25].

Solvent fractions of *T. kirilowii* showed considerably high cytoxicity to L1210, mouse leukemia cell, and S-180 tumor cells. In addition, injection administration into abdominal cavity prolonged the life span of the mice bearing L1210 and S-180[36]. Lim et al reported that hexane fraction of *T. kirilowii* showed a hypoglycemic effect on diabetic rats without acute toxicity, suggesting beneficial effect for insulin secretion[37]. Similarly, the inhibitory effects of *T. kirilowii* semen extracts on the growth of L1210 and P380 cells were increased, both in time and concentration dependent manner. Also Sarcoma 180 cell in ascite of mice were decreased by the administration of its extracts[27]. Even to human cancer cells, *T. kirilowii* is reported to have inhibitory effects. Shim et al found that *T. kirilowii* semen has inhibitory effect against the human rectal cancer cells, HRT-18[28]. The mechanism related seems to be through cell cycle arrest and proliferation. The methylene chloride fraction of *T. kirilowii* significantly inhibited the proliferation of human leukemic U937 cell, by inducing apoptosis[38]. Another inhibitory effect of *T. kirilowii* semen, which has been recently found is against human uterine cervical carcinoma cells[39]. Its results suggest that Trichosanthes semen extract induced cell apoptosis, in which the activation of caspase and mitochondiral pathway were involved. Fractions of Radix Trichosantis reduced NO production in mouse macrophages, via suppression of expression of iNOS[40].

A family of MMPs degrade extracellular matrix(ECM), among which MMP-9 is reported to be important in SMC proliferation and migration into the intima[41]. In this context, MMP-9 may be vital for the development of arterial lesions via regulation of both migration and proliferation of VSMC.

Various natural products, including green tea polyphenols, are known to have anti-atherosclerosis properties. EGCG, a catechin derivative, stronlgly inhibits gelatinolysis by MMP-9. Also, for the crude extracts, effectiveness in treating atherosclerosis by somel traditional herbal formulations have been reported[13,42]. *T. kirilowii* MAXIM., commonly known as "kwarugun", has long been used as a traditional Korean medicine for angina pectoris[14]. In this study, we describe the detailed molecular mechanism of action of TS extract. We found that the antimigration effect of TS is through MMP-9 inhibition and TS is effective in inhibiting the MMP-9 activity of VSMC. TS was remarkably effective against migration potential through MMP-9 inhibition, which is distinguished from other anti-atherosclerotic agents.

In short, the results suggest that TS has an inhibitory effect on TNF-α-induced HASMC migration through MMP-9 inhibition. Finding how TS inhibits MMP-9 activity and how it suppresses cell migration is helpful for using its properties for atherosclerotic treatment and prevention. These results implies that TS could be a potential anti-atherosclerotic agent with low cytotoxicity, and possibly a lead to a development of anti-atherosclerotic drugs.

Conclusion

The migration of vascular smooth muscle cells (VSMC) and the proliferation of matrix metalloproteinase-9 (MMP-9) may play a key role in the development of atherosclerosis. In this study, we have more extensively investigated the inhibitory effect of TS on MMP-9 activity and TNF-α-induced human aortic smooth muscle cells (HASMC) migration. The result from gelatin zymography showed that TS inhibited MMP-9 acitivty in a dose-dependent manner. Western blotting results show that TS suppresses MAPK phoshorylation. In addition, TS strongly inhibited the migration of HASMC induced by TNF-α treatment, although it has very low cytotoxic effect on HASMC. These results suggest that TS is a potential anti-atherosclerotic agent through inhibition of MMP-9 activity and VSMC migration.

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