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Full Length Research Paper

Effects of syringin from *Phellodendron chinensis* on monosodium urate crystal-induced inflammation and intercellular adhesion molecule-1 (ICAM-1) expression

Lian Yin[1]*, Yan-hua Yang[2], Ming-yan Wang[1], Xu Zhang[1] and Jin-ao Duan[3]

[1]Nanjing University of Traditional Chinese Medicine, Nanjing 210046, China.
[2]Apothecary of Changzhou Seventh People's Hospital of Jiangsu, Changzhou 213011, China.
[3]Key Laboratory for Modern Research of Traditional Chinese Medical Formula of Jiangsu, Nanjing 210046, China.

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Syringin is a phenylpropanoid glycoside isolated from the bark of *Phellodendron chinensis* Schneid. The present work was designed to examine the effects of syringin on the protection and expression levels of adhesion molecules in human umbilical vein endothelial cells (HUVECs) induced by monosodium urate (MSU). Syringin treatment increased the MSU-induced HUVEC viability as assessed by the 3-(4, 5-dimeth-ylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Syringin also suppressed the expression levels of MSU-induced endothelial cell intercellular adhesion molecule-1 as assessed by reverse transcription polymerase chain reaction (PCR). Syringin treatment decreased MSU-induced HUVEC apoptosis as well, as assessed by acridine orange/ethidium bromide staining and flow cytometry analysis. Overall, the present study suggests that syringin suppresses the expression levels of MSU-induced inflammation and is effective against acute gout.

Key words: Syringin, acute gout, human umbilical vein endothelial cells, cell viability, apoptosis, intercellular adhesion molecule-1(ICAM-1).

INTRODUCTION

Acute gouty arthritis is due to monosodium urate (MSU) in the joints, which stimulates acute inflammation. This disease manifests as intense pain, swelling, and skin reddening (Chia et al., 2008). In this disease, synovial cells, monocytes-macrophages, and neutrophils are stimulated to produce a variety of different cytokines as tumor necrosis factor-α (Tausche et al., 2004), interleukin (IL)-18, IL-1β, IL-6, and monocyte chemotactic factor (Inokuchi et al., 2006). These cytokines induce the increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Cianchetti et al., 2008). Essential for the occurrence of acute gout is enhanced adhesion between neutrophils and endothelial cells (Terkeltaub et al., 1998).

This adhesion is an important feature of the inflammatory injury process involving the interaction of adhesion molecules between the surfaces of neutrophils and endothelial cells (Fujiwara et al., 1998). ICAM-1 plays a key role in the trafficking of leukocytes across endothelial and epithelial barriers (Neeson et al., 2003). Using MSU crystals secreted by inflammatory cytokines as an *in vitro* model of acute gout, E-selectin expression is induced and the rolling and adhesion of neutrophils on human umbilical vein endothelial cells (HUVECs) are promoted (Landis et al., 2002). However, there is little information on the mechanisms of acute gout in protecting HUVECs and the expression levels of adhesion molecules.

Phellodendron cortex is a famous traditional Chinese medicine that has curative effects for acute gout, inflammatory diseases, etc., as recorded in the State Pharmacopoeia of People's Republic of China (2010). This drug has also been proven to reduce serum uric acid levels and inflammatory damage (Lu and Qiu, 1999; Yang et al., 2005). However, its active components

*Corresponding author. E-mail: lianyincn@hotmail.com. Tel/Fax: +86 25 85811524.

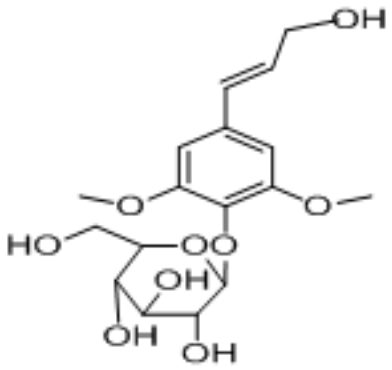

Figure 1. Chemical structure of syringin isolated from the bark of *P. chinense* Schneid.

against acute gout are not yet identified.

Syringin is a phenylpropanoid glycoside compound that protects HUVEC viability. Syringin has been isolated from the crude extract of the bark of *Phellodendron chinensis* while conducting an *in vitro* screening of the active components of the plant against acute gout in a rat swelling model induced by MSU. Several pharmacological actions of syringin include plasma glucose reduction, anti-oxidation, anti-cancer activity, antidepressant effect, immunomodulation, etc (Nakazawa et al., 2003; Eleni et al., 2004; Nour-EddineEs-Safi et al., 2007; Niu et al., 2008; Wan et al., 2010). However, there is limited information on the pharmacological action of syringin against inflammation in acute gout. Therefore, the present study was designed to examine the effects of syringin *in vitro* on the expression levels of MSU-induced inflammation and adhesion molecules. The findings can account for the beneficial effects of syringin on acute gout.

MATERIALS AND METHODS

Plant and solvents

Dried bark of *P. chinensis* Schneid was collected from Hunan province, China in November 2008, and was identified. Voucher specimens were deposited in the Nanjing University of Traditional Chinese Medicine, China. Uric acid (Sigma Chemical Co., USA) was dissolved in hot water (pH 7.4).

Extraction and isolation

Dried bark (1 kg) of *P. chinensis* was extracted two times with 70% EtOH (2 × 10 L) under reflux for 2 h each time. The solvent was removed under reduced pressure, and the residue was dissolved in hot water and was partitioned with EtOAc (5 × 1.5 L, 1 day each). The EtOAc extract (147.6 g) was concentrated and subjected to Si gel column (300 to 400 mesh, 5 × 100 cm) chromatography eluted with petroleum ether-EtOAc (100:1), followed by EtOAc. The extract (500 ml) was collected to yield 10 fractions. Fraction 4 (12.2 g) was subjected to Si gel column chromatography (petroleum ether-EtOAc, 100:6) and recrystallization by CH_2Cl_2 to afford a compound.

Compound identification

The structure of the compound was elucidated by spectroscopic methods and comparison of spectral properties (MS, ^{1}H-, ^{13}C-NMR) with previous reports. The compound was identified to be syringin (Figure 1) (Wu et al., 1999).

Cell culture

HUVECs were isolated as described previously (de Martin et al., 1993). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 75 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then serially passaged with 0.25% trypsin/1 mM (AERESCO, USA).

Cell viability

Cell viability was measured by the 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Welde, 1992). HUVECs were seeded onto a 96-well culture plate at a density of 1×10^4 cell/well and were incubated at 37°C for 24 h. The medium was replaced with 100 µl of serum-free DMEM containing MSU crystals (100 µg/ml) and various concentrations of syringin (2.5 to 250 µg/ml). The culture was incubated for 24 h. Each well was washed twice with phosphate-buffered saline (PBS) to remove the medium and crystals after 24 h. MTT was dissolved in serum-free DMEM at a concentration of 0.5 mg/ml, and 100 µl of this solution was added to the cell cultures. After 4 h, the 96-well culture plate was removed from the incubator and the formazan crystals were dissolved by adding 100 µl of dimethylsulfoxide, and the absorbance at 490 nm was read on a microplate reader (Bio-Rad, Hercules, CA, USA). The absorbance was used as a measurement of cell viability and normalized to cells incubated in control medium, which were considered 100% viable.

Morphological changes

HUVECs were seeded onto six-well plates (1×10^5 cells/well) before a cover slip was placed on the bottom of each well to make the cells growing on the surface form as a monolayer. The cells were incubated overnight before being treated with 25 µg/ml syringin and were stimulated with MSU (100 µg/ml) for 24 h. After 24 h of incubation, the cells were stained with 10 µl of aqueous acridine orange (AO)/ethidium bromide (EB) solution (100 µg/ml of AO in PBS; 100 µg/ml of EB in PBS) for 1 min. The changes in the nucleus of the cells after AO/EB staining were observed under a fluorescent microscope (Olympus, BX-60, Japan). Viable cells stained only by AO were bright green with an intact structure, and early apoptotic cells stained by AO had a bright green area in the nucleus. Late apoptotic cells stained by AO and EB were red-orange with visible chromatin condensation as dense orange areas, and had reduced sizes.

Reverse transcription PCR (RT-PCR)

HUVECs (1×10^5 cells/ml) were pretreated with syringin (2.5 to 25 µg/ml) and stimulated with MSU crystals for 24 h. Total RNA was extracted using an isolation kit according to the manufacturer's instructions. Reverse transcription was performed at 42°C for 30 min, followed by incubation at 95°C for 5 min and 0°C for 5 min.

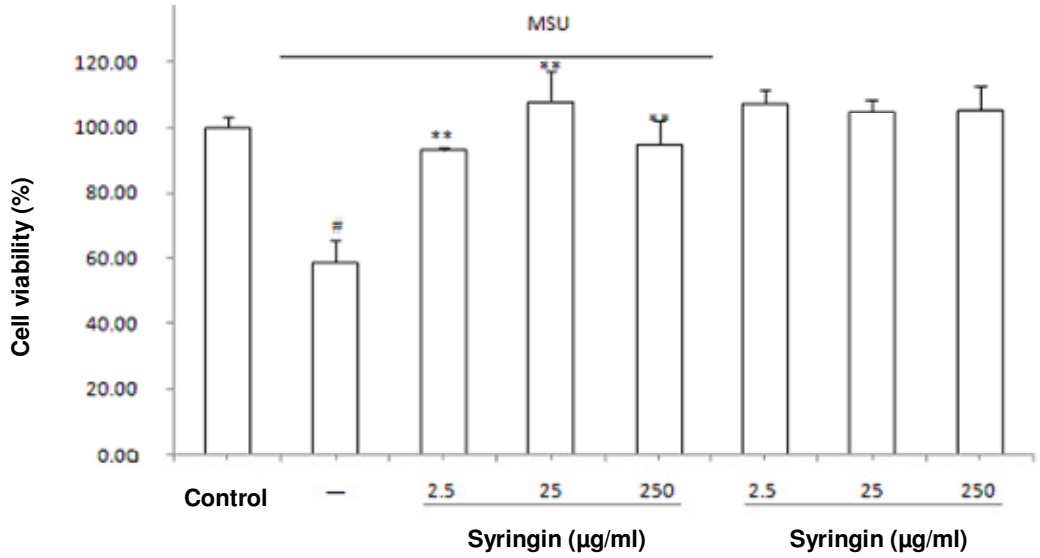

Figure 2. Effect of syringin itself on cell viability, the protective effect of syringin on MSU-mediated cytotoxicity. Cells were cultured in a medium containing MSU crystals (100 μg/ml) for 24 h and various concentrations of syringin (2.5 to 250 μg/ml). Cell viability was assessed by the MTT assay. Each value represents the mean ± standard deviation (SD) from triplicate cultures. MSU, monosodium urate. The symbols # and * indicate significant viability (**P < 0.01); #, Compared to control; *, Compared to MSU.

cDNA samples were analyzed for the specific cDNA of ICAM-1, and β-actin by PCR amplification using specific primers. About 4 μl of cDNA was added to 20 μl of PCR mixture containing 9.68 μl of H_2O, 1 μl of primer, 0.36 μl of dNTPs (2.5 mM), 1.96 μl of 10 × PCR buffer, and 0.5 μl of Taq DNA polymerase (5 U/ml). The following conditions were used for the PCR amplification: 10 min at 95°C, 40 cycles of 1 min 95°C, 1 min at 61°C, and 1 min at 72°C for ICAM-1; and 5 min at 95°C, 30 cycles of 50 s at 95°C, 50 s at 54.9°C, and 50 s at 72°C for β-actin. The primers for ICAM-1 were 5'-GGCAAGAACCTTACCCTA-3' (sense) and 5'-CATTCAGCG-TCACCTTGG (antisense) to amplify a 646 bp PCR product (primer annealed at 56°C) (Tan et al., 1998).

RESULTS

Cell viability

To determine whether syringin has a protective effect on the MSU-induced expression of inflammatory cytokine, the cell viability was assessed by the MTT assay. MTT cell viability assays have been extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. In the preliminary experiments, it had been confirmed that syringin did not show cytotoxicity at the concentration range of 2.5 to 250 μg/ml (Figure 2). When the HUVEC were exposed to 100 μg/ml MSU, the MTT reduction rate (cell viability) decreased to 58.6±6.5% over control, while syringin in 2.5, 25 and 250 μg/ml treated ones recovered cell viability up to 92.9±1.2, 108.0±9.5 and 94.8±7.3%, respectively, and cell viability on MSU-mediated cytotoxicity is enhanced with concentration of syringin increase in 2.5 to 25 μg/ml (Figure 2).

Nuclear morphology

To determine further whether the growth-inhibitory effects of MSU is related to the induction of apoptosis and protective effects of syringin, treated cells were analyzed by AO/EB staining under a fluorescence microscope. As shown in Figure 3a, the control cells were morphologically normal. The nuclei of the cells were similar in size, regularly shaped, and evenly stained. The more deeply colored parts of the nuclei represented heterochromatins, which do not participate in transcription under normal circumstances. However, with MSU treatment, the cells show marked morphological changes. In groups treated with MSU only (Figure 3b), orange cell fluorescence was significantly enhanced, representing late apoptotic cells. In groups treated with 25 μg/ml syringin (Figure 3c), the morphology of cells is normal, and the nuclei of cells were of similar sizes, regularly shaped, and evenly stained as the control.

Expression levels of MSU-induced ICAM-1

The expression level of ICAM-1 in the HUVECs is also up-regulated by treatment with MSU, as determined by RT-PCR. ICAM-1 was expressed at low levels on untreated HUVEC and up-regulated by stimulation with

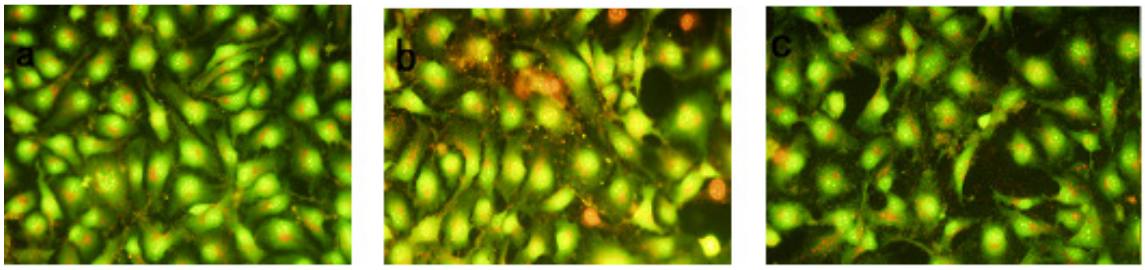

Figure 3. Effects of syringin on the MSU-induced protection of HUVECs in terms of the nuclear morphology. Cells (a) were stimulated with 100 µg/ml MSU; (b) treated with 25 µg/ml syringin, and (c) stimulated with 100 µg/ml MSU, for 24 h. Results are representative of three independent experiments. AO/EB staining × 200. MSU, monosodium urate; AO, acridine orange; EB, ethidium bromide.

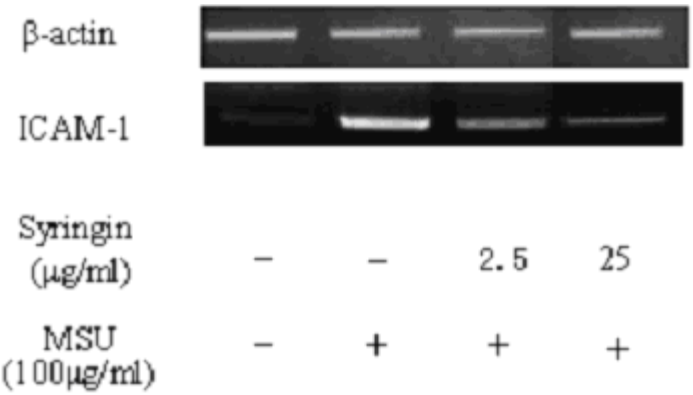

Figure 4. Effects of syringin on MSU-induced ICAM-1 mRNA expression in HUVECs. Cells were pretreated with syringin (2.5 to 25 µg/ml) and stimulated with 100 µg/ml MSU crystals for 24 h. The expression of mRNA was assessed by RT-PCR. MSU, monosodium urate.

MSU (100 µg/ml) for 24 h. Treatment with syringin (2.5 and 25 µg/ml) for 24 h attenuated MSU-induced ICAM-1 expression in HUVEC (Figure 4).

DISCUSSION

Gout is a common metabolic disorder in humans caused by MSU crystals in the joints, synovial fluid, etc., wherein acute inflammation is stimulated. Gout reportedly afflicts millions of people in the United States alone (Kramer and Curhan, 2002). The prevalence of this disease is also rapidly rising in China, probably due to recent changes in dietary habits. The therapeutic approaches for gout treatment include the use of anti-inflammatory agents for symptomatic relief, as well as xanthine dehydrogenase and xanthine oxidase, which block the synthesis of uric acid from purines.

The first clinical choice of anti-inflammatory drugs for acute gout is colchicine and nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin. Colchicine is an alkaloid that combines tubulin with neutrophils to inhibit chemotaxis and infiltration. However, it causes more severe gastrointestinal reactions and toxicity (Borstad et al., 2004). NSAIDs play an anti-inflammatory role by inhibiting cyclooxygenase (COX) activity, but they cause gastrointestinal bleeding and perforation risk significantly related to the drug dose (Garcia Rodriguez, 1998).

MSU damage HUVEC by the vascular intima is damaged directly so that there is increase in oxygen free radicals (Alderman, 2002; Feig et al., 2008), the apoptosis of HUVEC is induced and expression of adhesion molecules stimulate of VEC increase (Xia et al., 2006; Kleemann et al., 2008). The important feature of inflammatory injury process for the occurrence of acute gout is the interaction of adhesion molecules between surfaces of neutrophil-endothelial cell (Fujiwara et al., 1998).

ICAM-1 is a member of the immunoglobulin superfamily of proteins and plays a key role in the trafficking of leukocytes across endothelial and epithelial barriers

(Neeson et al., 2003). A variety of different cytokines can induce increased ICAM-1 expression. Previous study has proven that syringin inhibits the expression of inflammatory cytokines, such as IL-1β, IL-6, and COX-2, as well as matrix metalloproteinases-1 mRNA (Yamazaki et al., 2007). The present paper shows that syringin possesses anti-inflammatory mechanisms by inhibiting the expression of ICAM-1, protects HUVECs by reducing apoptosis and increasing cell viability in MSU-induced HUVECs, weakens adhesion between neutrophils and endothelial cells, as well as blocks the occurrence of acute gout.

Conclusively, syringin protects HUVECs by reducing apoptosis and increasing cell viability in MSU-induced HUVECs, suppresses *in vitro* MSU-induced inflammation and expression of ICAM-1 in HUVECs, suggesting that syringin can suppress the inflammatory process in acute gout. The results of the present study illustrate the potential of syringin as an anti-gout agent. The findings also provide foundation for further investigations on its biological mechanisms, and comparison with classical anti-inflammatory drugs *in vivo* and *in vitro* for the clinical treatment of acute gout.

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