Anti-inflammatory and anti-proliferative effects of *Rhus verniciflua* Stokes in RAW264.7 cells

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Abstract. Inflammatory response is a major defense mechanism against pathogens and chemical or mechanical injury. Rhus verniciflua Stokes (RVS) has traditionally been used as an ingredient in East Asian medicine for the treatment of gastritis, stomach cancer and atherosclerosis. The aim of the current study was to analyze the effect of RVS on LPS-induced inflammatory responses in the RAW264.7 mouse macrophage cell line. RAW264.7 cells were treated with various concentrations of RVS and LPS at specific time points. WST assay, trypan blue assay and quantification of activated cells revealed that RVS suppressed cell proliferation in a dose-dependent manner. RVS induced G1 cell cycle arrest, suppressed iNOS and COX-2 mRNA expression induced by LPS and decreased intracellular ROS levels induced by LPS. In addition, RVS induced PARP and caspase-3 cleavage suggesting that RVS causes cell death. Results of the present study indicate that RVS may be advantageous in treating inflammatory disease.

Introduction

Inflammatory response is a major defense mechanism against pathogens and chemical or mechanical injury. This mechanism is mediated by inflammatory cells, including macrophages. Activated macrophages produce reactive oxygen species (ROS) and nitric oxide (NO) and cause substantial oxidant injury to surrounding tissue (1). Chronic inflammation is known to contribute to cancer (2). Oxidative stress-induced neuron injury induces a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and cerebral ischemia (3). The bacterial peptidoglycan, recognized by toll-like receptor (TLR) 2 on monocytes/macrophages, induces inflammatory responses by activating MAPKs and NF- κ B (4). Macrophages are important in host defense mechanisms against tissue injury and microbial invasion and are also involved in various processes in autoimmune disease, infection and inflammatory disorders (5). Lipopolysaccharide (LPS) is a potent macrophage activator that binds to the TLR4 on the macrophage cell surface. LPS stimulation of macrophages produces various pro-inflammatory cytokines as well as prostaglandin E2 and nitric oxide (NO) (6).

Rhus verniciflua Stokes (RVS) has traditionally been used as an ingredient in East Asian medicine for the treatment of gastritis, stomach cancer and atherosclerosis. The compounds identified from RVS are as follows: gallic acid, protocatechuic acid, quercetin, fustin, fisetin, sulfuretin and butein (7). RVS protects against oxidative damage by scavenging ROS (8), causes antiproliferative activity and anticancer and anti-inflammatory effects (9).

The effect of RVS on LPS-induced inflammatory responses in the RAW264.7 mouse macrophage cell line was investigated in this study. We examined whether ethanol (EtOH) extract from RVS herbal medicine suppresses the LPS-induced inflammatory responses in RAW264.7. We also analyzed whether RVS exhibits anti-proliferative activity regulating intracellular molecules associated with cell survival and apoptosis.

Materials and methods

Cell culture. RAW264.7 mouse macrophage cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics at 37°C in a 5% CO₂ humidified incubator.

Extraction of RVS. RVS used in this study was purchased from Omniherb (Gyeongsangbuk-do, Korea). A 100 g ground powder was extracted twice from the wood and fruit with 80% v/v ethanol (Duksan Pharmaceutical Co. Ltd., Korea) using an Ultra-sonicator (Branson, Danbury, CT, USA) for 30 min at room temperature. Alcoholic extract was filtered through a 0.22 μ m filter, evaporated at 40°C and freeze-dried. The extract yield of RVS was 13.7% w/w.

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Cell proliferation assay. The cell proliferation rate was determined using the WST assay following RVS treatment. The WST assay is based on the cleavage of yellow tetrazolium salt to purple formazan crystals by metabolically active cells.

RAW264.7 cells (1x10⁴ cells/well) were seeded into 96-well plates, incubated overnight and treated with RVS for 24 h. WST solution (10 μ l) was added to 100 μ l cell culture medium and the plates were incubated for 2 h. Optical density was determined at 490 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Cell death assay. Cell death was determined using trypan blue assay following RVS treatment. Trypan blue selectively stains dead cells. RAW264.7 cells were treated with RVS for 12 and 24 h. Cells were suspended and stained with trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA). The cell number was quantified using a hemocytometer.

Cell surface observation. Cells were seeded into 60-mm culture dishes at a density of $3x10^5$ cells/dish. The following day, cells were treated with RVS for 12 h. The cell surface was imaged using a camera (Olympus Corporation, Tokyo, Japan) attached to a light microscope.

Mitochondrial membrane potential analysis. Loss of mitochondrial membrane potential is a specific characteristic of apoptosis. JC-1 is a membrane-permeable dye widely used for determining mitochondrial membrane potential in flow cytometry and fluorescent microscopy. Cells were seeded into 60-mm culture dishes at a density of $3x10^5$ cells/dish. The following day, the cells were treated with RVS for 24 h. The cells were harvested from each culture dish, washed with PBS, suspended in PBS containing 2 μ M JC-1 and incubated for 30 min at 37°C in the dark. The data were analyzed by FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Intracellular ROS level measurement. The molecule 2',7'-dichlorofluorescein diacetate (DCFH-DA) permeates cells where it is converted into fluorescent 2,7-dichlorofluorescein (DCF) by oxidative substances, revealing the intracellular production of redox-active substances. DCFH-DA has been widely used to investigate oxidative damage in intact cells. Cells were seeded into 35-mm culture dishes containing glass coverslips. Following various pretreatments, the cells were washed with PBS and incubated with 20 μ M DCFH-DA for 30 min at 37°C in the dark. Following washing with cold PBS, the fluorescence was captured by confocal laser scanning microscopy (LSM 510; Carl Zeiss, Thornwood, NY, USA) and FACSCalibur flow cytometry. DCF fluorescence was measured at an excitation wavelength of 488 nm and emission at 515-540 nm.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Cells were harvested by centrifugation (1,500 x g) and the pellet was washed with ice-cold PBS. RNA was isolated from the pellet using an Invitrogen Life Technologies kit (Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated RNA content was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Total cellular RNA (2 μ g) from each sample was reverse transcribed using cDNA synthesis kit (Takara, Japan). PCR was conducted in a 20 μ l reaction mixture consisting of DNA template, 10 pM of each gene-specific primer, 10X Taq buffer, 2.5 mM dNTP mixture and 1 unit of Taq DNA polymerase (Takara). PCR was performed using the specific primer. The following primers were used: COX-2 sense, 5'-GGAGAGACTATCAAGATAGT-3' and antisense, 5'-ATGGTCAGTAGACTTTTACA-3'; iNOS sense, 5'-AATGGCAA CATCAGGTCGGCCATCACT-3' and antisense, 5'-GCTGTGTGTCACAGAAGTCTCGAACTC-3'; and GAPDH sense, 5'-TGAAGGTCGGTCGGCCATGAGGAT TTGGC-3' and antisense, 5'-CATGTAGGCCATGAGGTC CACCAC-3'. The sequencing involved 30 cycles with denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec. The resulting PCR products were resolved on 1% agarose gels containing ethidium bromide.

Western blot analysis. Cells were lysed in modified RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na₃VO₄, and protease inhibitor mixture]. The lysates were cleared by centrifugation at 10,000 x g for 15 min and the supernatants were collected. The protein concentration was quantified using a Bio-Rad Bradford protein assay (Bio-rad, Hercules, CA, USA). Equal amounts of protein lysates were used for western blot analyses with the indicated antibodies (p-AKT, p-ERK, p-JNK, p-p38, p-NFkB, α -tubulin, PARP, Pro-caspase-9, cleaved caspase-3, Bcl-xL, Bcl-2, Bax, LC3). Immunoreactive protein bands were detected with an EZ-Western Detection kit (Daeillab service Co., Ltd., Seoul, Korea).

Statistical analysis. The experiments were performed in triplicate. The data are expressed as the means \pm standard deviation (SD). SDs for all measured biological parameters are displayed in the appropriate figures. Student's t-test was used for single variable comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of RVS on cell viability. Anti-proliferative effects of RVS were determined in RAW264.7 mouse macrophage cells using a WST assay (Fig. 1A). Cells were treated with RVS at concentrations between 0 and 1,000 μ g/ml and 1 μ g/ml LPS for 12 h. LPS alone did not show proliferative activity in RAW264.7 cells. However, RVS significantly inhibited cell proliferation at concentrations between 50 and 1,000 μ g/ml, indicating that RVS inhibits the growth of RAW264.7 cells. Cell death rate was determined using a trypan blue assay following RVS treatment (Fig. 1B). At 24 h, RVS significantly decreased the percentage of surviving cells. In additon, changes in cellular morphology under LPS and RVS treatment were observed (Fig. 1C). Untreated RAW264.7 cells are circular, however, under LPS-stimulated conditions, the cells presented as an irregular shape and became elongated. Microscopic examination of cell cultures showed a reversal of LPS-induced alteration in cell morphology when treated with RVS. Fig. 1D shows the number of cell surface changes under LPS and/or RVS treatment in RAW 264.7 cells. RVS significantly decreased the number of cell surface changes induced by LPS. These results indicate that RVS inhibits the



Figure 1. Effect of RVS on cell proliferation and death in RAW264.7 cells. (A) Cell proliferation rate was determined by WST assay. RAW264.7 cells were treated with RVS at concentrations between 0 and 1,000 μ g/ml in the absence or presence of 1 μ g/ml LPS for 24 h. RVS significantly inhibited cell proliferation at concentrations between 50 and 1,000 μ g/ml. Each value presents the mean \pm SD (*P<0.01, ***P<0.001 compared to 0 mg/ml RVS in the presence of LPS; Student's t-test). (B) Cell death rate was determined using trypan blue assay. RAW264.7 cells were treated with 15 μ g/ml RVS in the absence or presence of 1 μ g/ml LPS for 12 and 24 h. RVS caused cell death in a time-dependent manner. (C) LPS-induced morphological changes were reversed by RVS in RAW264.7 cells. Cells were treated with RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h. RVS in the absence or presence of 1 μ g/ml LPS for 12 h and observed by microscopy. RVS, *Rhus verniciflua* Stokes; LPS, lipopolysaccharide.



Figure 2. RVS decreased iNOX and COX-2 mRNA expression and suppressed intracellular ROS levels induced by LPS in RAW264.7 cells. (A) Expression of iNOS and COX-2 mRNA was assayed by RT-PCR. Cells were treated with $15 \mu g/ml$ RVS in the absence or presence of $1 \mu g/ml$ LPS for 12 h. RVS suppressed iNOS and COX-2 mRNA expression induced by LPS. Intracellular ROS was detected by (B) laser confocal scanning microscopy and (C) FACSCalibur with DCFH-DA. Cells were treated with $15 \mu g/ml$ RVS in the absence or presence of $1 \mu g/ml$ LPS for 30 min. DCF fluorescence was measured using a confocal laser-scanning microscopy and FACSCalibur. RVS inhibited ROS generation induced by LPS. RVS, *Rhus verniciflua* Stokes; RT-PCR, reverse transcription-polymerase chain reaction; LPS, lipopolysaccharide; ROS, reactive oxygen species; DCFH-DA, 2'7'-dichlorofluorescein.





Figure 4. RVS induces cell apoptosis via a mitochondrial-independent pathway. (A) The cells were treated with RVS ($15 \mu g/ml$) in the absence or presence of $1 \mu g/ml$ LPS for 3, 6 and 12 h. Following incubation with JC-1, the cells were analyzed using a FACSCalibur flow cytometer. RVS failed to decrease mitochondrial membrane potential. (B) Effect of RVS on caspase activity and apoptosis in RAW264.7 cells. The cells were treated with RVS ($15 \mu g/ml$) in the absence or presence of $1 \mu g/ml$ LPS for 3, 6 and 12 h. Cell extracts were subjected to western blot analysis with specific antibodies. RVS cleaved PARP and caspase-3. RVS, *Rhus verniciflua* Stokes; LPS, lipopolysaccharide.

proliferation of RAW264.7 cells and blocks the LPS-induced activation of RAW264.7 cells.

RVS decreases iNOS and COX-2 mRNA expression in RAW264.7 cells. Since NO and ROS are mediators in inflammatory reactions, iNOS mRNA and COX-2 mRNA expression in RAW264.7 cells was measured. RVS suppressed iNOS mRNA and COX-2 mRNA expression induced by LPS in RAW264.7 cells (Fig. 2A), suggesting that RVS suppresses inflammatory reactions.

RVS decreases ROS level in RAW264.7 cells. ROS levels were measured using confocal microscopy (Fig. 2B) and FACS analysis (Fig. 2C) stained with DCFH-DA. Following LPS treatment, cellular ROS levels were increased. However, RVS

Figure 3. RVS affects the cell cycle. (A) The cell cycle was analyzed using a FACSCalibur flow cytometer. Cells were treated with RVS (15 μ g/ml) in the absence or presence of 1 μ g/ml LPS for 3, 6 and 12 h. Cells were stained with propidium iodide staining solution and analyzed using a FACSCalibur. RVS caused G1 arrest. (B) G1, S and G2/M phase fractions were quantified from DNA histogram data in (A). (C) Effect of RVS on the expression of intracellular molecules in RAW264.7 cells. The cells were treated with RVS (15 μ g/ml) in the absence or presence of 1 μ g/ml LPS for 3, 6 and 12 h. Cell extracts were subjected to western blot analysis with specific antibodies. RVS did not have an inhibitory effect on AKT, ERK, JNK, p38 or NF- κ B phosphorylation. RVS, *Rhus verniciflua* Stokes; LPS, lipopolysaccharide.

co-treatment inhibited ROS generation induced by LPS in a time-dependent manner.

RVS affects the cell cycle. Cell cycle changes induced by RVS were analyzed by FACs analysis. RVS caused G1 arrest at 6 and 12 h in a time-dependent manner (Fig. 3A and B). The expression of intracellular molecules associated with cell proliferation was measured by western blot analysis (Fig. 3C). RVS failed to decrease the phosphorylation of AKT, ERK, JNK, p38 and NF- κ B.

RVS induces cell apoptosis via a mitochondrial-independent pathway. Loss of the mitochondrial membrane potential $(\Delta \Psi)$ is a hallmark for apoptosis. The mitochondrial permeability transition is an important step in the induction of cell apoptosis. During this process, several key events occur in the mitochondria, including the release of caspase activators such as cytochrome c, changes in electron transport and loss of mitochondrial transmembrane potential. JC-1 selectively enters the mitochondria and reversibly changes color from red to green as the membrane potential decreases. Thus, cells were stained with JC-1 and FACS analysis was used to determine whether mitochondrial membrane potential is decreased by RVS. It was observed that RVS did not decrease mitochondrial membrane potential in RAW264.7 cells (Fig. 4A). LPS alone showed a more stable mitochondrial membrane potential (green fluorescence, 9.61% at 12 h) than the control (green fluorescence, 31.72% at 12 h). RVS failed to alter this stability induced by LPS (green fluorescence, 13.34% at 12 h) indicating that RVS induces cell death via a mitochondrial-independent pathway.

In addition, RVS was confirmed to regulate the expression of apoptosis-related molecules. RVS induced cleavage of apoptotic products, PARP and caspase-3, indicating that RVS induces apoptosis (Fig 4B).

Discussion

In the current study, EtOH-extracted RVS was found to suppress LPS-induced inflammatory responses in the RAW264.7 mouse macrophage cell line. Inflammation is a host protection method against pathogens and is stimulated by diverse microbial products (10). Pro-inflammatory cytokines have been reported to aggravate the severity of multiple inflammatory diseases (11). Diverse inflammatory agents are known to activate NF- κ B and activation induces inflammation and increases cell survival and tumor cell transformation (12). MAPK pathways are associated with inflammation, for example, the ERK pathway is activated by inflammation (13).

Results of the present study indicate that RVS effectively inhibits growth stimulation and the activation of RAW264.7 cells induced by LPS. RVS significantly inhibited cell growth at concentrations between 50 and 1,000 μ g/ml and induced cell death at 15 μ g/ml (24 h). In addition, RVS negated morphological changes of RAW264.7 cells induced by LPS. RVS decreased intracellular ROS levels and suppressed iNOS and COX-2 mRNA expression induced by LPS. RVS failed to decrease mitochondrial membrane potential but cleaved caspase-3 and PARP indicating that RVS induces apoptosis via a mitochondrial-independent pathway. Since RVS has an anti-inflammatory effect it may be used for the treatment of inflammatory diseases, including rheumatoid arthritis and asthma (14). Transformation of a normal cell into a tumor cell is closely associated with chronic inflammation (15), therefore, RVS may represent a useful compound for cancer prevention.

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