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Inhibition of cell cycle progression via $p27^{Kip1}$ upregulation and apoptosis induction by an ethanol extract of *Rhus verniciflua* Stokes in AGS gastric cancer cells

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Abstract. Botanical preparations are widely used by patient with cancer in Korea, Japan and China. *Rhus verniciflua* Stokes (RVS) has traditionally been used as a medicinal ingredient for the therapy of stomach and uterine cancer. In this study, we showed that exposure to an ethanol extract of RVS (50 μg/ml) resulted in a synergistic inhibitory effect on cell growth in AGS cells. Growth inhibition was related with the inhibition of proliferation and induction of apoptosis. The extract induces G_1-cell cycle arrest through the regulation of cyclins, the induction of $p27^{Kip1}$, and decrease the CDK2 kinase activity. The upregulated $p27^{Kip1}$ level is caused by protein stability increment by the reduction of Skp2, a key molecule related with $p27^{Kip1}$ ubiquitination and degradation, and *de novo* protein synthesis. RVS extract induces apoptosis through the expression of Bax, poly(ADP-ribose) polymerase (PARP) and activation of caspase-3. RVS extract induces G_1-cell cycle arrest via accumulation of $p27^{Kip1}$ controlled by Skp2 reduction and apoptosis passing through an intrinsic pathway in human gastric cancer cells but not in normal cells, therefore we suggest that this extract could be a candidate medicine or compound for the development of novel class of anti-cancer drugs.

Introduction

The cooperative activation of cyclins and cyclin-dependent kinase (CDK) complexes has important regulatory roles during cell cycle progression. These complexes continuously act together in G_1 to S phase transition and in G_2 to mitosis transition (1). Since uncontrolled CDKs are often the cause of cancer, their function is tightly regulated by cell cycle inhibitors such as the $p21^{CIP/WAF}$ and $p27^{Kip1}$ Cip/Kip proteins (2,3). While preventing abnormal proliferation and DNA damage, $p21^{CIP/WAF}$ and $p27^{Kip1}$ bind to cyclin-CDK complexes to block their catalytic activity and induce cell cycle arrest (4,5). Especially, $p27^{Kip1}$ protein induces G_1-cell cycle arrest though binding of the cyclin E-CDK2 complexes leading to CDK inhibition (6). Many previous studies have mechanistically shown regulation of $p27^{Kip1}$ expression. The post-translationally ubiquitin-mediated proteolysis system is the principal regulatory machinery of $p27^{Kip1}$ protein level (7). The ubiquitin-target protein complex specified by the ubiquitinating enzymes E1, E2, and E3, recognizes the covalent adduction between ubiquitin and the target protein such as $p27^{Kip1}$, which then leads to degradation of the target protein (8). Several recent studies have shown that one mechanism implicated in $p27^{Kip1}$ degradation is the Skp1-Cullin-F-box protein (SCF)-type ubiquitin-proteasome pathway (9,10). Skp2, a component of F-box family subunit of SCF ubiquitin-protein ligase complexes, is shown to recognize $p27^{Kip1}$ and cyclin E (11-13). Therefore, Skp2 knock-out cells showed high levels of $p27^{Kip1}$ and free cyclin E (12,13).

Programmed cell death (apoptosis) is an evolutionarily conserved process of eliminating unwanted, damaged, aged and misplaced cells during embryonic development and tissue homeostasis (14-16). The caspases are central components of the apoptotic machinery in the proteolytic system. Two groups of caspases can be distinguished: upstream initiator caspases consisting of caspase-8 or -9, which cleave and activate other caspases, and downstream effector caspases including caspase-3, -6 and -7 to exert the proteolytic actions. Caspase-3 plays a pivotal role in execution of apoptosis and its activation occurs by proteolytic cleavage. Caspase-3 activation leads to cell demise via cleavage of cellular substrates, such as poly(ADP-ribose)

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polymerase (PARP), gelsolin, Rb, and ß-catenin (17,18). The Bcl-2 families of proteins as well as caspases play critical roles in apoptosis response. The family is subdivided into two classes: anti-apoptotic members such as Bcl-2 and Bcl-X_L, and pro-apoptotic members such as Bax and Bak. Bcl-2 family members act as checkpoints to determine the cell fate (19,20).

Rhus verniciflua Stokes (RVS) is traditionally used in Korea, Japan and China for the therapy of gastritis, stomach cancer and arteriosclerosis (21). Several earlier studies indicated that an ethanol extract of RVS is both anti-oxidant against hydroxyl radicals (22,23) and has anti-proliferative activity (24).

In this study, we investigated the molecular mechanism that underlies RVS extract-induced apoptosis and G_1-cell cycle arrest against AGS human gastric cancer cell lines. We show that in AGS cells, an ethanol extract of RVS induced G_1-cell cycle arrest via regulation of p27^{Kip1} accumulation. The p27^{Kip1} protein was increased via reduction of Skp2. Thus, CDK2 kinase activity was decreased leading to a growth arrest and caspase-3 involved apoptosis.

Materials and methods

Preparation of Rhus verniciflua Stokes (RVS) alcoholic extract. RVS was purchased from Omniherb (Yeongcheon, Korea) in Korea. Its ground powder of 200 g was twice extracted with 80% (v/v) ethanol (Duksan Pharmaceutical Co. Ltd., Korea) by using an ultra-sonicator (Branson, USA) for 30 min at room temperature. The alcoholic extracts were evaporated at 60˚C and then freeze-dried. The final yield was 14.87 g (7.41%). The high performance liquid chromatography (HPLC) analysis of the extract was performed using a standard material of 4-deoxyurushiol (Sigma-Aldrich Co. Ltd., St. Louis, MO). The powder form of the extract was dissolved in RPMI-1640 or DMEM media to 100 mg/ml, vortexed at room temperature for 1 min, and incubated at 37˚C overnight while rotating before use. This solution was then centrifuged at 12,000 rpm for 5 min to remove any insoluble ingredients. The supernatant was passed through a 0.22 μM membrane filter for sterilization and diluted with RPMI-1640 or DMEM culture media to final concentrations.

Cell culture and drug treatment. A human gastric cancer cell line AGS was obtained from American Type Culture Collection and a normal rat intestinal epithelial (RIE1) cell line was kindly gifted by Dr J.W. Lee at Seoul National University, Seoul, Korea. The cells were grown in RPMI-1640 or DMEM media (Gibco-BRL) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL) in a 37˚C humidified incubator with 5% CO_2. Subconfluent monolayers of cells were used in all experiments.

Cell viability assay. For cell viability assay, AGS cells ($1x10^4$ cells/100 μl) were seeded in a 96-well tissue culture plate and incubated for 24 h. After RVS treatment for the indicated periods, a mixture of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS) and an electron coupling reagent (phenazine methosulfate; PMS) solution (20 μl/100 μl) was added to each well. After incubation for 2 h at 37˚C the solubilized formazan product was spectrophotometrically quantified using an ELISA reader at 490 nm (Molecular Devices, Palo Alto, CA).

Flow cytometric cell cycle or DNA content analysis. A half million cells were seeded in 60 mm dishes and incubated for 24 h at 37˚C. RVS at the indicated concentrations was directly added to the culture media and incubated for an additional 24, 48, or 72 h. After the incubation, both detached (presumably apoptotic) and adherent cells were collected and combined, fixed by addition of 4 ml of 95% chilled ethanol with 0.5% Tween-20, and stored at -20˚C for at least 30 min. Cells were then pelleted, washed twice with ice-cold PBS, incubated in PBS containing 10 μg/ml of RNase A (Sigma) for 15 min at 37˚C, and stained with 10 mg/ml of propidium iodide (PI). The relative DNA content per cell of the samples was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA. The cell cycle was analyzed using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA) and a ModFit LT V2.0 software.

Western blot analysis. Whole cell lysates from cells under diverse conditions were prepared by washing with ice-cold PBS and lysis using a RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, leupeptin, pepstatin]. The protein concentration was determined using the Bio-Rad protein assay kit. An equal amount of proteins was loaded, and separated by SDS-PAGE and then transferred to nitrocellulose membrane. After blocking with PBS-0.1% Tween-20 (PBST) containing 1% skim milk and 1% BSA for 1 h, the membrane was incubated overnight at 4˚C with primary antibodies against PARP, procaspase-9, procaspase-3, cleaved caspase-3, Rb, cyclin A, cyclin B1, cyclin D1, cyclin E, Bcl-X_L, Bax, Bcl-2, p27^{Kip1}, p53, p21$^{CIP/WAF}$, p16^{INK4a} and Skp2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing in 1X PBST for 1 h (3 times x 20 min), membranes were incubated with HRP-conjugated secondary antibodies and the immunobands were visualized with the enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, UK). For the studies using inhibitors, AGS cells were pretreated with inhibitors including cycloheximide (CHX, 10 μg/ml, Sigma-Aldrich) or z-VAD-*fmk* (50 μM, Calbiochem) for 1 h, and treatment with RVS followed.

RNA extraction, Northern blot analysis. Whole cell lysates from cells under diverse conditions were prepared by washing with ice-cold PBS. Total RNA was extracted with TRIzol reagent (Invitrogen). The RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer, and the ratio of absorbance at 260 nm to that at 280 nm was 1.8 or higher. The integrity of RNA was checked by visual inspection of the two rRNAs 28S and 18S on an agrose gel. An equal amount of RNA was loaded, and separated by 1.2% agarose-6% formaldehyde gels and then transferred to nylon membrane (S&S). The membranes were then hybridized with radiolabeled probes for a p27^{Kip1} cDNA

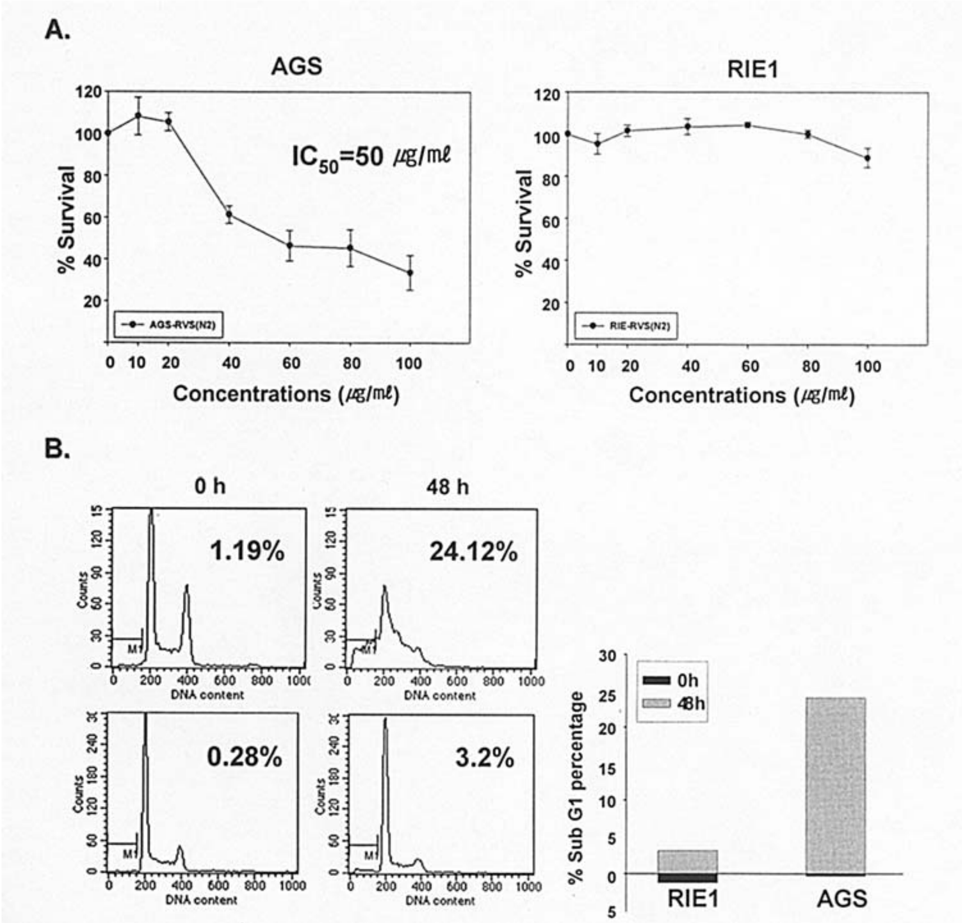

Figure 1. Effects on growth by treatment of *Rhus verniciflua* Stokes (RVS) of gastric cancer AGS cells. (A) Cell growth inhibition of AGS cells by RVS extract. AGS or RIE1 cells were treated with 10-100 μg/ml RVS extract for 72 h. Cell viability was measured by the MTS assay. Data are shown as the mean of three independent experiments [error bars are mean ± standard deviation (SD)]. (B) RVS extract induced sub-G_1 population. Cell cycle distribution was analyzed using a FACStar flow cytometer, percentages of sub-G_1 phase cells which were determined based on DNA content histogram.

fragment for RNA expression. The relative band intensities of $p27^{Kip1}$ mRNA at each time point were quantified by determining radioactivity using a Fuji FLA2000 (Fuji, Tokyo, Japan) and image analysis software (Image Gauge, version 3.12).

Immunoprecipitation and kinase assays. Cyclin E-associated CDK2 kinase activity was determined using histone H1 as substrate. Two hundred micrograms of cell extracts were used per immunoprecipitation with CDK2 antibody (Santa Cruz Biotechnology, Inc.) coupled protein A/G beads (Santa Cruz Biotechnology, Inc.). After being washed, CDK2 kinase assays on histone H1 was performed by incubating the immune complex beads with 30 μl of kinase reaction buffer [3 μl (3 μg) of histone H1, 0.6 μl (5 μCi) of [γ-^{32}P]ATP, 0.6 μl (20 μM ATP and 25.8 μl of kinase buffer] for 20 min at 30˚C. The reaction was stopped by boiling the samples in sample buffer for 5 min. Samples were then seperated by 12% SDS-PAGE, and the gels were dried and subjected to autoradiography.

Results

Effect of RVS extract on cell proliferation of cancer cells. To examine the molecular mechanism by which RVS extract induces cell cytotoxicity, we first checked the effect of RVS extract on cell proliferation of AGS human gastric cancer cells. As shown in Fig. 1A, treatment of RVS extract caused a dose-dependent decrease in the cell viability as determined by the MTS assay, with prominent inhibitory effect at a concentration of >50 μg/ml. The concentration required for 50% inhibition of growth (IC_{50}) by RVS extract was about 50 μg/ml. On the other hand, treatment of RIE1 rat intestinal epithelial cells with RVS extract showed no significant cell proliferation inhibition.

RVS extract induces apoptosis through activation of caspase-3 in AGS cells. We next determined whether RVS extract-induced decrease in cell proliferation was due to cytotoxic effects. AGS cells were treated with 50 μg/ml RVS extract for the indicated periods and then analyzed for DNA contents by flow cytometry. Treatment of AGS cells with RVS extract for 48 h increased apoptotic sub-G_1 fraction to 24.12% from 1.19% for no treatment (0 h) (Fig. 1B). On the other hand, in case of RIE1 cells, RVS extract did not significantly increase the apoptotic sub-G_1 fraction; treatment for 48 h led to only a 3.2% increase (Fig. 1B). These results indicate that prolonged exposure to RVS extract exerts cytotoxic effects on AGS cells in a dose- and time-dependent manner.

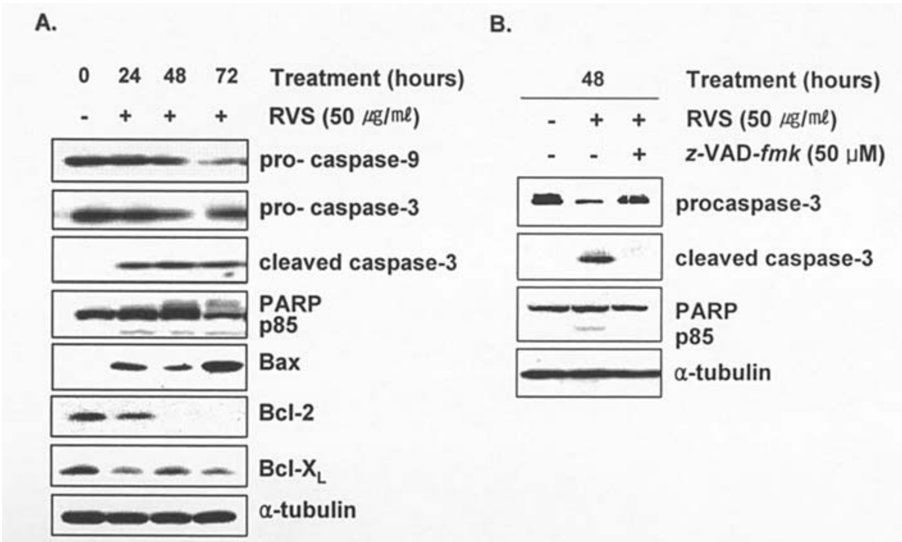

Figure 2. *Rhus verniciflua* Stokes (RVS) extract induces apoptosis in gastric cancer AGS cells. (A) Analysis of apoptosis-related molecules. AGS cells were treated with 50 μg/ml RVS extract for the indicated periods and then the cell lysates were prepared and analyzed by Western blots with anti-procaspase-9, -procaspase-3, -cleaved caspase-3, -PARP, -Bax, -Bcl-2, -Bcl-X_L, and -α-tubulin. (B) Involvement of caspase-3 in RVS extract-induced apoptosis. AGS cells were pretreated with z-VAD-*fmk* (50 μM) for 1 h and then exposed to 50 μg/ml RVS extract for 48 h. The cellular proteins were analyzed by Western blots with anti-procaspase-3, -cleaved caspase-3, -PARP, and -α-tubulin.

Next, we performed experiments to determine whether the RVS extract-induced inhibition of cell proliferation and cytotoxic effects induced apoptotic cell death in AGS cells. Since caspase-3 is a main executioner of the apoptotic response inside the cells (17,18), we examined by Western blotting for proteolytic processing of procaspase-3. Incubation of AGS cells with RVS extract for various periods (24-72 h) resulted in cleavage of procaspase-9 and -3 and subsequent cleavage of their substrate, poly(ADP-ribose) polymerase (PARP) (Fig. 2A). Prominent cleavage of PARP was observed 72 h after the treatment of 50 μg/ml RVS extract. We also checked the levels of Bcl-2 families of proteins (19). As shown in Fig. 2A, the level of proapoptotic molecules such as Bax was significantly increased in a time-dependent manner, with a maximal induction at 72 h after treatment with 50 μg/ml of RVS extract. The anti-apoptotic molecules including Bcl-X_L and Bcl-2 notably decreased. As shown in Fig. 2B, treatment of AGS cells with z-VAD-*fmk* (a pan-caspases inhibitor), completely suppressed PARP degradation, and caspase activation, indicating a critical role of caspase activation in the RVS extract-induced apoptosis. Thus, RVS extract induced apoptosis through the Bcl-X_L, and Bcl-2-mediated caspase-3 activation pathway.

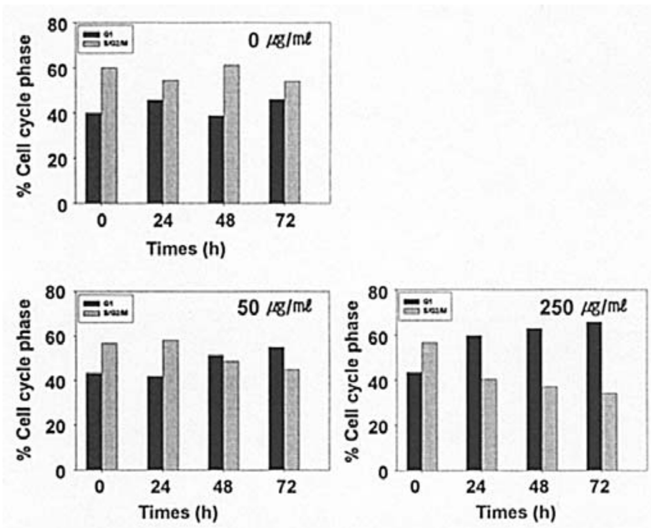

Figure 3. *Rhus verniciflua* Stokes (RVS) extract induces G_1-arrest in gastric cancer AGS cells. The population percentage of G_1 and S phase was determined by DNA contents analysis using flow cytometry.

RVS extract induces G_1-cell cycle arrest. Growth inhibition was associated with induction of apoptosis and inhibition of proliferation. We then analyzed the cell cycle phase through incubation of AGS cells with RVS extract for various periods (24-72 h) and concentrations (50-100 μg/ml). Exposure to RVS extract (50 μg/ml) resulted in a slow increase in G_1-cell cycle phase higher than untreated cells and concomitantly the S and G_2/M phase decreased (Fig. 3). RVS extract-induced G_1-cell cycle phase is significantly increased in a time- and dose-dependent manner. In order to confirm G_1-cell cycle arrest in RVS extract-treated AGS cells, we examined expression levels of cell cycle-related proteins by Western blots. As shown in Fig. 4A, AGS cells treated with 50 μg/ml RVS extract for indicated periods progressively decreased cyclin A and B1 proteins levels, with a maximal reduction at 72 h after treatment of RVS extract at 50 μg/ml. The levels of cyclin D1 and E did not change by the treatment with RVS extract. The cyclin D1 and E are associated with G_1-cell cycle phase and their expression account for CDK2, 4/6 activity (2). Accordingly, we tried to confirm G_1-cell cycle arrest in RVS extract-treated AGS cells by analyzing the levels of CDKIs, such as $p27^{Kip1}$, $p16^{INK4a}$, $p21^{CIP/WAF}$, and p53. The levels of $p27^{Kip1}$ proteins were significantly increased about 4

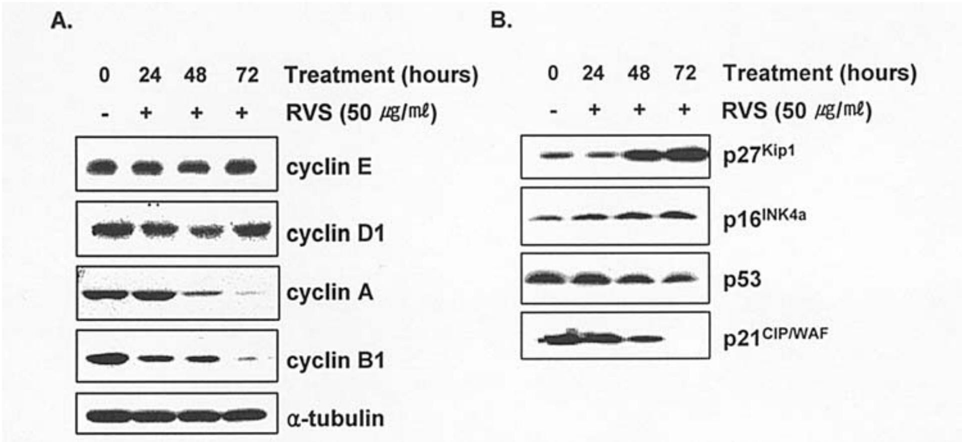

Figure 4. Expression of G_1 phase-associated regulatory proteins in gastric AGS cells treated with *Rhus verniciflua* Stokes (RVS) extract. (A) Effects of treatment with RVS extract on cell cycle regulatory proteins in AGS cells. AGS cells were treated with 50 μg/ml RVS extract for the indicated periods and then cell lysates were prepared and analyzed by Western blots with anti-cyclin D1, -cyclin E, -cyclin A, -cyclin B1, and -α-tubulin. (B) Anti-p27^{Kip1}, -p16^{INK4a}, -p53, and -p21$^{CIP/WAF}$.

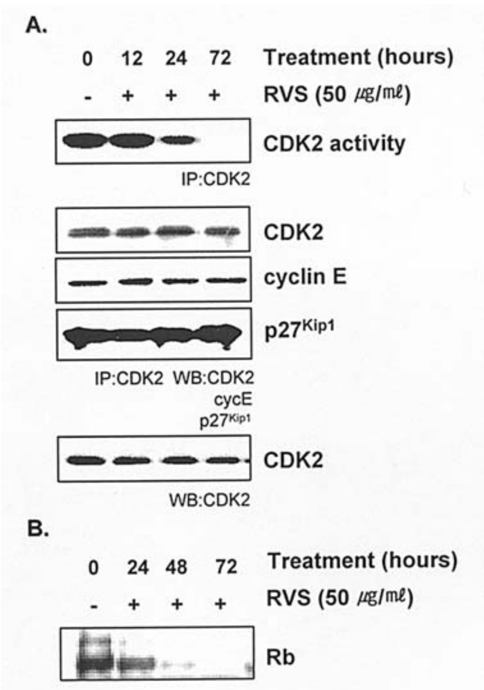

Figure 5. Effect of p27^{Kip1} induction by *Rhus verniciflua* Stokes (RVS) extract on kinase activity of CDK2 associated with cyclin E in AGS cells. (A) Treatment of RVS extract significantly decreased the kinase activity of CDK2 in a time-dependent manner. The complexes were immunoprecipitated with anti-CDK2 antibody and then CDK2 kinase assay was performed using histone H1 as a substrate. The anti-CDK2 immunoprecipitates were used in *in vitro* kinase reaction. Samples were analyzed by SDS-PAGE, and the gels were dried and then subjected to autoradiography. (B) RVS extract increased hypophosphorylated Rb. AGS cells were treated with 50 μg/ml of RVS extract. The cellular proteins were analyzed by Western blots with anti-Rb.

times more than untreated cells in a time-dependent manner, whereas p21$^{CIP/WAF}$ and p53 decreased (Fig. 4B). We also observed a slight induction of p16^{INK4a} protein expression.

Upregulation of p27^{Kip1} protein expression by RVS extract inhibits CDK2 kinase activity. In growing cells, p27^{Kip1} is primarily associated with CDK4/6-cyclin D complexes and free CDK2 remains active (4). In uncontrolled conditions, p27^{Kip1} protein induces G_1-cell cycle arrest especially through its binding to the CDK2-cyclin E complexes (5). Therefore, alteration of kinase activity of CDK2 associated with cyclin E was examined to see whether RVS extract altered the kinase activity of CDK2 associated with cyclin E. As shown in Fig. 5A, treatment of AGS cells with RVS extract strongly reduced the kinase activity of CDK2 on histone H1, especially with a maximal decrease at 72 h after treatment with RVS extract. We also confirmed the formation of more complexes immunoprecipitated with anti-CDK2 antibody bound to p27^{Kip1} protein and their volumes increased (Fig. 5A, lower panel). These data indicate an upregulation of p27^{Kip1} expression mediated by RVS extract-induced G_1-cell cycle arrest via reduction of CDK2-cyclin E complex kinase activity.

We checked the phosphorylation status of retinoblastoma (Rb) protein that regulates cell cycle progression from the G_1- to S-cell cycle phase through theirs phosphorylation. Incubation of AGS cells with RVS extract of various periods (24-72 h) resulted in increased levels of hypophosphorylated form of Rb and concomitantly its loss in a time-dependent manner (Fig. 5B). Hypophosphorylated Rb was recognized to inhibit proliferation through its association with E2F trans-activation domain (25-27). Therefore, these results indicate that RVS extract induced inhibition of cell cycle progression through actions on Rb as well.

Upregulated p27^{Kip1} level is caused by increased protein stability through blocking of p27^{Kip1} degradation system. Increased p27^{Kip1} by RVS extract induced G_1-cell cycle arrest in a time-dependent manner (Fig. 4B). Protein expression can be regulated at several different levels: the transcriptional level, the translational level (protein synthesis) or the post-translational level (protein modification such as phosphorylation). The expression of p27^{Kip1} in a cell is largely controlled post-transcriptionally by a number of pathways involved in human cancer. The SCF ubiquitin-proteasome proteolytic pathway mainly mediates the degradation of p27^{Kip1}, expression of Skp2, a component of the F-box

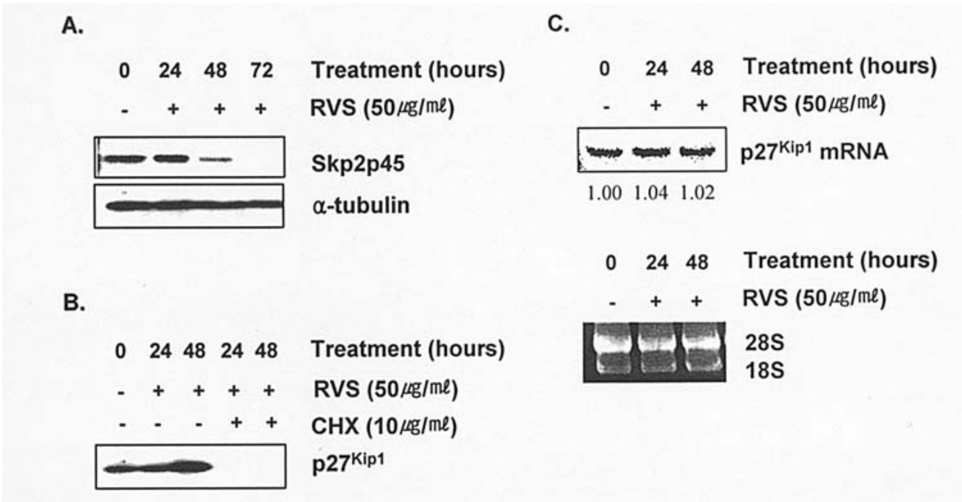

Figure 6. *Rhus verniciflua* Stokes (RVS) extract upregulates p27Kip1 protein expression in gastric AGS cells. (A) Treatment of RVS extract reduced Skp2 protein. AGS cells were treated with 50 μg/ml RVS extract for the indicated periods and cell lysates were prepared and analyzed by Western blots with anti-Skp2, and -α-tubulin. (B) p27Kip1 protein induction by RVS extract depends on the protein synthesis process. AGS cells were pretreated with 10 μg/ml cycloheximide (CHX) for 1 h and then exposed to 50 μg/ml RVS extract for 48 h. (C) RVS extract maintains the p27Kip1 mRNA expression. AGS cells were treated with 50 μg/ml RVS extract for the indicated periods and the total mRNA was prepared and analyzed by Northern blotting with radiolabeled probes for p27Kip1 cDNA fragment. The relative band intensities of p27Kip1 mRNA were quantified by determining radioactivity using Fuji FLA2000 and image analysis software (Image Gauge, version 2.12 and TINA, version 2.0).

families of proteins of the specific target-recognition subunit of SCF complexes (9), was required for the ubiquitination and degradation of p27Kip1 *in vitro* (10-12). We investigated alteration in the level of Skp2 protein. As shown in Fig. 6A, Skp2 markedly declined as p27Kip1 simultaneously enhanced. These results indicate that the expression of p27Kip1 is controlled post-translationally through RVS extract-decreased Skp2.

We also investigated another regulatory mechanism for the induction of p27Kip1 protein by examining the effects of cycloheximide (CHX, a protein synthesis inhibitor). Cells were pretreated 1 h with CHX before treatment with the RVS extract. As shown in Fig. 6B, CHX treatment decreased p27Kip1 induction. These results suggest that p27Kip1 enhancement was dependent on *de novo* protein synthesis. We also used another method to confirm the regulation of p27Kip1 expression. We measured p27Kip1 mRNA level by using Northern blot analysis. As shown in Fig. 6C, the levels of p27Kip1 mRNA were constant. Therefore, these data indicated that RVS extract-induced p27Kip1 increment may be regulated at multiple post-transcriptional mechanisms.

Discussion

Rhus verniciflua Stokes (RVS) is a traditional herbal medicine in East Asia to treat gastrointestinal trouble, stomach, ovarian and uterine cancer and arteriosclerosis (21). The mechanism which is responsible for the growth-inhibitory effects of RVS is still unknown. Cell cycle progression depends on an ordered sequence of cell division events, such as DNA replication, and nuclear division that cells tightly regulate. The elimination of unwanted, damaged, aged and misplaced cells repairs their DNA before progressing into cell division. Unless the damage is severe, apoptotic cell death may occur in most types of cells (15). In this study, it is certain that RVS extract-induced apoptosis is mediated by caspase activation, as we confirmed the pro-caspase-3 cleavage (i.e., activation) and a complete prevention of RVS extract-induced apoptosis via treatment with caspase inhibitor before RVS extract treatment (Fig. 2B). These results suggest an absolute and significant role for caspase activation in RVS extract-induced apoptosis. Interestingly, in rat intestinal epithelial RIE1 cells, RVS extract did not exert any significant cytotoxic effects, indicating cancer-specific effects of the RVS extract. In addition, 50 μg/ml RVS extract induced also G_1-cell cycle arrest. G_1-cell cycle phase gradually increased while G_2- and S-cell cycle phases concomitantly decreased. In uncontrolled cells, cell cycle is tightly regulated by cycle inhibitors such as the p21CIP/WAF and p27Kip1 Cip/Kip proteins via their interactive activation of cyclins and CDKs (3,4). It was shown that cyclin D1 and cyclin E levels remained even after the RVS extract treatment. Meanwhile, cyclin A and B1 were decreased. We also revealed that RVS extract induced p27Kip1, which is known to be important for G_1-cell cycle arrest (4). Following the anti-proliferation signal and DNA damage, p27Kip1 binds to CDK2-cyclin E complexes to inhibit their catalytic activity and induces cell cycle arrest. The p53/p21CIP/WAF related to G_1-cell cycle arrest was reduced by treatment with RVS extract (Fig. 4B), indicating that RVS extract-induced G_1-cell cycle arrest did not involve the p53/p21CIP/WAF pathway. This was consistent with the RVS extract-induced p27Kip1, kinase activities of CDK2 to phosphorylate histone H1-associated decreased (Fig. 5A). These results suggest that treatment with RVS extract causes G_1-cell cycle arrest via p27Kip1 induction and CDK2 kinase activity reduction. The Rb protein cooperated to arrest the cell cycle progression at G_1 prior to S phase transition. It has been reported that Rb and p27Kip1 are reciprocally implicated in the negative regulation of cellular hypophosphorylated Rb inhibiting proliferation through its association with the sactivation domain (28). Probably, RVS extract-d Rb protein hypophosphorylation inhibit E2F function as a transcription factor that regulates cell cycle progression-related protein expressions, such as for cyclin A, cdc2, c-Myc, and PCNA (29,30).

$p27^{Kip1}$ expression and/or function may be controlled by diverse post-transcriptional regulation mechanisms. The ubiquitin-proteasome system is required for the ubiquitination and degradation of $p27^{Kip1}$ (31-33). It was reported that human gastric carcinoma (34) and *in vitro* experiments (10-12) showed the expression of Skp2, a member of the ubiquitin-proteasome system, was required for the ubiquitination and subsequent degradation of $p27^{Kip1}$. We found in this study an inverse correlation between the expressions of $p27^{Kip1}$ and Skp2 protein (Fig. 6A). Furthermore, it was previously shown that $p27^{Kip1}$ degradation is regulated by phosphorylation; CDK2-cyclin E complexes phosphorylate $p27^{Kip1}$ and then lead to its turnover (35,36). Consistent with the previous results, we also found that RVS extract decreased CDK2-cyclin E kinase activity, and that, CDK2-cyclin E complexes could not lead to phosphorylation of $p27^{Kip1}$ and the eventual turnover. We also showed that the treatment of RVS extract regulated the increment of $p27^{Kip1}$ protein synthesis (Fig. 6B). Therefore, it is likely that upregulated $p27^{Kip1}$ level occurred via increased protein stability by blocking of $p27^{Kip1}$ degradation system and *de novo* protein synthesis.

The potential of $p27^{Kip1}$ as a prognostic and/or diagnostic marker or as a candidate of cancer therapy has been suggested. A number of studies revealed that $p27^{Kip1}$ protein level is declined in many human cancers such as breast, colon, gastric, lung non-small cell, and prostate carcinoma (37). $p27^{Kip1}$ protein has certain good characteristics as a prognostic and/or diagnostic marker. First, the abundance of the protein is maintained without mutation or loss at its gene level. Second, the amount of $p27^{Kip1}$ in a cell is controlled post-transcriptionally by a number of pathways implicated in human cancers (38). In addition, it was shown that the signaling pathway to control $p27^{Kip1}$ degradation system involves regulation of Skp2 (34).

Furthermore, these observations were valid in another gastric cancer cell line, SNU668 (data not shown). According to these observations, RVS extract inhibits gastric cancer cell growth, although it is not confirmed that these molecular mechanisms occur in other cancer cells. We analyzed the RVS ethanol extract used in this study and the compound urushiol, using HPLC. We observed a small amount of urushiol in the used RVS extract in the HPLC analysis (data not shown). This indicates that urushiol is not the main compound of RVS extract and the effect of anti-proliferation by $p27^{Kip1}$ upregulation and Skp2 reduction were caused by other compounds or synergic effects of compounds in the RVS extracts.

In summary, RVS extract induces G_1-cell cycle arrest via accumulation of $p27^{Kip1}$ controlled by Skp2 reduction and apoptosis passing through an intrinsic pathway in human gastric cancer cells but not in normal cells, therefore, we suggest that this extract could be a candidate medicine or compound for the development of novel class of anti-cancer drugs.

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