Proteomic Analysis of the Anti-Cancer Effect of 20S-Ginsenoside Rg3 in Human Colon Cancer Cell Lines

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Ginseng is a well known herbal medicine in Asia, and ginsenoside Rg3 has anti-cancer and various pharmacological effects. In particular, 20S-ginsenoside Rg3 may increase the anti-proliferative effects of chemotherapy. The authors investigated the mechanism of the anti-proliferative effect of 20S-Rg3 at the protein level in HT29 colon cancer cells. MTT, caspase-3 assays, and flow cytometry analysis were performed to determine cytotoxicity and apoptosis, and proteomic analysis was performed by two-dimensional gel electrophoresis and MALDI-TOF/TOF MS, and a database was used to identify protein changes in 20S-Rg3 treated HT29 cells. The proteins identified included down-regulated Rho GDP dissociation inhibitor, up-regulated tropomyosin1, and annexin5 and glutathione s-transferase p1, which are apoptosis associated proteins. The anti-proliferative mechanism of 20S-Rg3 was found to be involved in mitotic inhibition, DNA replication, and repair and growth factor signaling. The findings of this study suggest that the cytotoxicity of 20S-Rg3 in colon cancer is dependent on several mechanisms, including apoptosis.

Key words: ginsenoside; colon cancer; proteomics; anti-cancer activity

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), continues to be used as an herbal medicine in Asia. The pharmacologically active components of ginseng include ginsenosides, triterpene glycosides belonging to the protopanaxadiol and protopanaxatriol groups, and secondary metabolites. Ginsenoside Rg3 includes both optical isomers of protopanaxadiol, viz., 20R-Rg3 and 20S-Rg3, which results in different hydroxyl positions at carbon-20 (Fig. 1).

Several findings suggest that Rg3 may increase the efficacies of cancer chemotherapy. The anti-carcinogenic and anti-metastatic effects of ginsenoside Rg3 have been demonstrated in vitro and in vivo. Ginsenoside Rg3 was found to inhibit lung metastasis by inhibiting the adhesion and invasion of tumor cells and by inhibiting angiogenesis.5) In another study, Rg3 was suggested to inhibit tumor cell invasion by reducing intracellular calcium levels.2) Recently, the anti-tumor effect of Rg3 was suggested to be mediated by the down-regulation of the transcription factors of NF-κB (nuclear factor-kappa B) and activator protein-1(AP-1) and by its anti-oxidant activity.3) Another, in vitro study concluded that the anti-tumor property of 20R-Rg3 is due to its suppression of angiogenesis.4)

Colorectal cancer has high associated mortality, and better therapies are needed to reduce the side effects of the anti-cancer agents used today. Anti-cancer drugs reduce tumors by inhibiting the proliferation of tumor cells and by inducing apoptosis. Although most signaling pathways stimulated by anti-cancer agents ultimately activate caspases (cystein proteases), the mechanism underlying the anti-cancer effect of Rg3 has not been established in HT29 cells. Proteomic approaches involve the identification, characterization, and quantitation of proteins expressed in cells, tissues, and organisms under specific conditions. Global analyses of proteins offer valuable information, particularly in terms of understanding anti-cancer mechanisms. In the present study, we used a proteomic approach in HT-29 cells exposed to 20S-Rg3 at its IC50. 2D electrophoresis and MALDI TOF/TOF MS were used to separate and identify differentially expressed proteins.

Materials and Methods

Cell culture. HT29 cells (a human colorectal adenocarcinoma cell line) were provided by the Korean Cell Line Bank (Seoul, Korea). DMEM/F12 medium, fetal bovine serum and antibiotics were obtained from Gibco BRL (Grand Island, NY). HT29 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, and 100 µg/ml streptomycin antibiotics at 37 °C under 5% CO2 and 95% air for all experiments of cell culture.

MTT assays. The cytotoxic effect of Rg3 on HT29 cells was examined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assays. Briefly, HT29 cells (1 × 104/cm2) were incubated in 96-well assay plates for 24 h. Ginsenosides 20S-Rg3 and 20R-Rg3 were isolated and identified from ginseng, as previously reported.5) After treatment with various concentrations of 20S-Rg3 or 20R-Rg3 (10, 20, 40, 80, or 160 µM) for 48 h, plates were incubated with 40 µl of MTT solution (5 µg/ml in PBS) for 4 h at 37 °C. DMSO 100 µl was then added to solubilize the formazan crystals in viable cells, and 4 h later absorbances were read at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

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rehydration solution (8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% Brij 80) were performed using Bio-Rad IPGphor IEF and electrophoresis units. 

Briefly, 80 000 g supernatants were determined using the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA), and then stored at −80 °C until required for 2-DE.

Measurement of caspase-3 activation. Caspase-3 activity was measured using colorimetric caspase-3 assay kits (Molecular Probe, Eugene, OR). After treatment with ginsenoside, the cells were centrifuged, and the pellets were washed and lysed by repeated freeze-thawing in 50 μl of lysis buffer. The cell lysates were then centrifuged at 5,000 rpm for 5 min at 4 °C, and the supernatants were incubated with rhodamine 110 bis-(N-Cbz-t-aspartyl-t-glutamyl-t-vallyl-t-aspartic acid amide) (Z-DEV-D-R110) for 30 min at room temperature. The absorbance at 405 nm (reference wavelength, 490 nm) was determined using a microplate reader.

Cell treatment and protein extraction. To treat cells with ginsenoside, they were cultured in a T-75 flask in DMEM/F12 medium until 60–70% confluency. The medium was then removed and replaced with medium containing either 0.1% DMSO or 20S-ginsenoside Rg3 solubilized in 0.1% DMSO to a concentration of 100 μM. After 24 h, the cells were washed 3 times with PBS and scraped into 500-μl cell lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 65 mM DTT, protease inhibitor cocktail), transferred to a 1.5-ml Eppendorf tube, and then spun down at 16,000rcf for 30 min at 15 °C. The supernatant obtained was transferred to a new Eppendorf tube and centrifuged at 16,000rcf for 30 min at 15 °C. The protein concentrations in lysis supernatants were determined using the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA), and then stored at −80 °C until required for 2-DE.

Two-dimensional electrophoresis and image analysis. 2DE was performed using Bio-Rad IPGphor IEF and electrophoresis units. Briefly, 80 μg of whole cell protein was diluted to 300 μl with rehydration solution (8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% IPG buffer), and loaded onto an IPG strip holder fitted with 17-cm, pH 4–7 linear gradient IPG strips, and rehydrated for 12 h at 20 °C. IEF was performed using the following voltage sequence: step 1, 250 V for 15 min; step 2, 10 kV for 3 h; and step 3, 10,000 V for 60 kVh. After IEF separation, the strips were equilibrated with equilibration buffer (50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 1% SDS, 0.002% bromophenol blue) containing 5 mg/ml DTT for 15 min, and subsequently with equilibration buffer containing 25 mg/ml of iodoacetamide for 15 min. The equilibrated strips were applied to 12% polyacrylamide gels with a protein marker for molecular weight calibration, and were sealed in place with 5 mg/ml of agarose. The gels were run at constant current (26 mA/gel) and temperature (20 °C) for 8 h until the dye reached the end of the gel. Following electrophoresis, the gels were stained using a silver staining kit (Invitrogen, Carlsbad, CA). Images were analyzed using PDQuest Version 7.2 (Bio-Rad).

Three pairs of gels from control and 20S-Rg3 treatment cells were compared. The intensities of spots were measured and expressed as percentages of the total intensity of the spots detected in the gel. Spots with significantly different intensities (Student’s t-test, p < 0.05) were selected for protein identification.

**Tryptic in-gel digestion.** Total cell protein (300 μg) used for silver staining was electrophoresed using the above method and stained by Colloidal coomassie blue staining kit (Invitrogen). Spots sliced from gels were destained with 10 mM ammonium bicarbonate for 10 min, and then with DW and ACN until the color disappeared. After drying in a SpeedVac, the gels were incubated in 20 μl of 4 μl/ml trypsin in 10 mM ammonium bicarbonate at 37 °C overnight. The supernatants were collected, dried in a SpeedVac, and dissolved in 0.1% TFA for mass spectrometric analysis.

**Protein identification using MALDI-TOF TOF/MS.** For MS analysis, matrix solution containing 2 mg CHCA (tris-(hydroxymethyl)-aminomethane α-cyano-4 hydroxy cinnamic acid) in 60% ACN containing 0.1% TFA was mixed with an equal volume of the tryptic digest and loaded on an AnchorChip™ target plate. An Auto Flex II MALDI-TOF TOF/MS (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser, delayed extraction, and reflectron was utilized in positive ion mode. Proteins were identified using the MASCOT peptide mass fingerprint search program using the NCBI database specifying a maximum of two miss cleavages.

**Statistical analysis.** MTT and caspase-3 assay data were expressed as means ± SD of triplicate determinations. Data were subjected to one-way analysis of variance (ANOVA), followed by Duncan’s multiple-range test. Student’s t-test was applied to proteomic data obtained in triplicate. In all cases, significance was accepted for p values of <0.05.

**Results**

**Cytotoxic effect of 20S-Rg3 on HT29 cells**

We compared the cytotoxic activities of 20S-Rg3 and 20R-Rg3 in HT29 cells. According to our MTT assay data, treatment with 20S-Rg3 also reduced proliferation in a dose-dependent manner. The IC50 of 20S-Rg3 was 600 μM for a 24-h treatment and 100 μM for a 48-h treatment (Fig. 2). The anti-proliferation rate of 20R-Rg3 treated HT29 cells decreased in proportion to concentration. After 48 h of treatment, the anti-proliferation rate for 600 μM 20R-Rg3 was 2.1%, and the anti-proliferation rate for 25 μM 20R-Rg3 was 18.2%. Similarly, reductions in anti-proliferation were observed after treatment for 24 h (Fig. 2). These data suggest that 20R-Rg3 has little effect on HT29.

**Increased apoptosis in 20S-Rg3 treated HT29 cells**

Morphological changes in the cells revealed cell shrinkage, rounding, poor adherence, and floating round cells by light microscope at 100 μM 20S-Rg3 for 24 and 48 h (Fig. 3). We investigated 20S-Rg3 induced apoptosis using a flow cytometer to exclude cell death as a result of necrosis. The percentages of early apoptosis cells (lower-right, annexinV positive) were 27.11% for 20S-Rg3 and 47.42% at 100 μM 20S-Rg3 for 48 h. At 12 h of treatment, there were few induced apoptotic cells. Moreover, 12.44% for 24-h treatment and 28.99% for 48-h treatment were detected as necrotic cells (upper-right, annexin V and PI positive) (Fig. 4). Though we observed that 20S-Rg3 induced early apoptosis and necrosis, 20S-Rg3 was more effective in inducing early apoptosis of HT29 cells than necrosis.
In caspase-3 activity assays of 20S-Rg3 treated HT29 cells, 20S-Rg3 showed only marginal caspase-3 activity at 50µM for 24h. Treatment with 20S-Rg3 at 100µM increased caspase-3 activity compared to non-treated cells. The activity was 140%. 5-FU at 50µM was used as a positive control, and its caspase-3 activity was 169%, which was a little higher than the activity of 100µM 20S-Rg3 (Fig. 5).

2DE and image analysis

In preliminary 2-D experiments using pH 3–10 non-linear IPG strips, most of the differentially expressed proteins were located in the pH 4–7 range. Therefore, 17-cm pH 4–7 linear IPG strips were used to analyze proteins differentially expressed in HT29. All spots detected by PDQuest were normalized and calculated as mean intensities over 3 runs for 20S-Rg3 treated and non-treated cells. Spot intensities were normalized versus the sums of spot intensities in the gel. After detecting and normalizing protein spots, 20 spots showed different expression in 20S-Rg3 treated and untreated 2D images using PDQuest software, and the expression levels of eight of these 20 spots were significantly different (p < 0.05) by Student’s t-test. All eight proteins were successfully identified. They showed increased or decreased spot intensities by more than 2-fold between the control and 100µM 20S-Rg3 treated cells (Figs. 6, 7).

Differential expression of 20S-Rg3 treated HT29 cells

Using MALDI-TOF/TOF-MS and the NCBI database, five proteins were up-regulated and three proteins were down-regulated (by 2.5- to 6.1-fold) by 100µM of 20S-Rg3. Clathrin and retinoblastoma binding protein 4 (both related to inhibition of mitosis) were up-regulated. Rho GDP dissociation inhibitor alpha was down-regulated and tropomyosin1 and annexin5 (three proteins involved in apoptosis) were up-regulated. Furthermore, we observed proteome changes related to ROS expression, DNA replication and repair, and growth factor signaling. 20S-ginsenoside Rg3, which is known to have anti-oxidative activity, increased glutathione s-transferase p1 (GSTP1) expression in HT29 cells. The down-regulation of proliferating cell nuclear antigen (PCNA) in 20S-Rg3 induced HT29 cells might reduce DNA replication and repair process. Furthermore, serine/threonine kinase receptor associated protein (STRAP), which is associated with tumor progression, was down-regulated by 20S-Rg3 (Table 1).

Discussion

Several studies have found that ginsenoside Rg3 inhibited tumor cell proliferation, invasion, and metastasis and induced differentiation and apoptosis.1-3,6 Liu et al. examined Rg3 inhibited prostate cancer proliferation with an IC50 after 48 h of incubation by suppressed cell cycle progression genes (PCNA and cyclin kinase D1) and apoptosis-related genes (Bcl-2 and caspase-3).6 In the present study, we used a proteomic approach to identify cellular proteins differentially expressed by 20S-ginsenoside Rg3 in HT29 colorectal cancer cells, because 20S-Rg3, in contrast to 20R-Rg3, was found to have an anti-proliferative effect by MTT assay. And we
confirmed induced apoptosis at 100 µM 20S-Rg3, and increased necrotic cells were observed for 48 h treatment compared to 24 h treatment using a flow cytometer. Therefore we applied 24 h incubation of 20S-ginsenoside Rg3 at IC30 as determined by MTT assay to minimize the effect of necrosis for proteomic analysis.

Caspase-3 is most commonly involved in apoptosis signaling.7) Our caspase-3 assay indicated that 20S-Rg3 induced HT29 apoptosis at 100 µM. In proteomic data, we found down-regulation of Rho GDP dissociation inhibitor (GDI) alpha. This may be related to reduced GTPase and induced apoptosis with increased caspase-3 after 20S-ginsenoside Rg3 treatment. Rho GDP dissociation inhibitor (GDI) is a major regulator that controls cellular distribution and the activities of Rho GTPases. Rho GDI alpha (also known as Rho GDI and Rho GDI 1-1) is a member of the GDI family. Rho GTPases are commonly found in human cancers. They play essential roles in tumor cell proliferation, angiogenesis, and metastasis.8) GDI is known to regulate Rho GTPase positively and to protect cancer cells against drug-induced apoptosis by inhibiting the caspase-mediated cleavage of GTPase.9)

Increased levels of ANXA5 involved in 20S-Rg3-induced apoptosis were confirmed by flow cytometer and proteomic analysis. Annexin5 (ANXA5) is one of most abundant annexins. It is found in a wide variety of tissues. Annexins are a family of calcium-regulated membrane-binding proteins. They function in many membrane domains, e.g., in the regulation of phagocytosis, cell signaling, and proliferation.10) Though ANXA5 is not firmly associated with human cancer and metastasis, annexin levels were found to be reduced in colon cancer,11) and ANXA5 binds with high affinity to external cell membrane phosphatidylserine on apoptotic tumor cells.

In addition, up-regulation of tropomyosin1 (TM1) and glutathione S-transferase p1 (GSTP1) may contribute to the apoptotic effect of HT29 based on the proteomic data. Tropomyosin1 (TM1) is essentially required for normal mammalian development. TM1 forms continuous polymers along the major groove of actin filaments, and is a known tumor suppressor because its expression in breast cancer cell lines suppresses the malignant phenotype and inhibits anchorage independent growth, which induces resistance to anoikis, a special type of apoptosis that restricts abnormal proliferation of non-transformed cells by
promoting the cell suicide program when attachment to the extra-cellular matrix is breached.\textsuperscript{12} Glutathione s-transferase p1 (GSTP1) is a member of the glutathione s-transferase superfamily, which is composed of phase II detoxification enzymes that conjugate electrophilic compounds with reduced glutathione. Moreover, overexpression of GSTP1 has been detected at all stages of colorectal cancer, from aberrant crypt foci to advanced carcinomas. Furthermore, most studies have shown that overexpression of GSTP1 in cancer cells is related to increased resistance to anticancer agents.\textsuperscript{13} In addition, it was also reported that enhanced GSTP1 expression induced by protective agents leads to the differentiation and apoptosis of HT29 cells.\textsuperscript{14} Therefore, we suggest that 20\textsuperscript{S}-Rg\textsubscript{3} up-regulates GSTP1 and that this has a dual effect by promoting apoptosis and protecting HT29 cells from oxidative stress.

Other proteome changes induced by 20\textsuperscript{S}-Rg\textsubscript{3} in HT29 cells can be classified according to some functions. Proteins related to the regulation of mitosis are composed of clathrin and retinoblastoma binding protein 7. Clathrin is essential for clathrin-mediated endocytosis (CME) in mammalian cells. Endocytosis is used to control the densities of cell-surface receptors and for nutrient take-up. Clathrin knockouts in many organisms have resulted in reduced endocytosis and multiple mitotic defects. Furthermore, mis-segregation of chromosomes during mitosis leads to genetic instability that may induce cancer.\textsuperscript{15} Retinoblastoma binding protein 4 (Rbp4) was initially identified as an Rb binding. It represses transcriptional activity in response to mitogen stimulation. E2F transcription factor controls progression into the S phase of the cell cycle, and Rb represses the transcription of E2F-regulated genes with histone deacetylase complex.\textsuperscript{16} We suggest that upregulation of clathrin and Rbp4 is associated with reduced proliferation, and that 20\textsuperscript{S}-Rg\textsubscript{3} may interfere with mitosis and inhibition cancer cell growth in HT29 cells.

In addition, two of the three down-regulated proteins were found to be related to DNA replication and repair and growth factor signaling. Proliferating cell nuclear antigen (PCNA) is essentially required for both DNA replication and repair, which includes nucleotide excision repair (NER), a major means of removing DNA-damaged cells. PCNA forms a sliding clamp that bind proteins, such as polymerases, to DNA.\textsuperscript{17} PCNA has been reported to be a suitable biomarker of more aggressive colorectal adenomas that have the ability to...
undergo malignant transformation.\(^{18}\) Furthermore, PCNA suppression in Rg\(_3\)-treated human prostate cancer inhibits cancer cell proliferation.\(^{6}\) Serine/threonine kinase receptor associated protein (STRAP) is a new member of WD40 domain-containing proteins. It is up-regulated in 60% of colon carcinomas, and while STRAP overexpression promotes tumorigenicity in athymic nude mice, knockdown of endogenous STRAP by si-RNA promotes transforming growth factor (TGF-\(\beta\)) signaling, reduces ERK activity, upregulates p21\(^{16}\), and downregulates tumorigenicity.\(^{19}\) The above results suggest that the two forms of down-regulation observed may inhibit cancer cell growth.

In conclusion, our experimental results verify that 20\(S\)-ginsenoside Rg\(_3\) inhibits the proliferation by MTT assay and promotes the apoptosis of HT29 cells (a colon cancer cell-line), as revealed by apoptotic morphology, flow cytometric analysis, and caspase-3 assay. Using proteomic analysis after treatment with 20\(S\)-Rg\(_3\), we identified four up/down-regulated proteins related to apoptosis. Moreover, we observed proteome changes related to inhibited mitosis, DNA replication and repair, and growth factor signaling in HT29 cells. We suggest that these pathways act complementarily and explain the cytotoxicity of 20\(S\)-Rg\(_3\) in colon cancer cells. Though the proteomic data presented suggest various mechanisms for the anti-cancer effect of 20\(S\)-Rg\(_3\), the mode of action of 20\(S\)-Rg\(_3\) in colon cancer calls for further study.

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**References**


**Fig. 7.** Enlarged View of (A) the Down-Regulation and (B) the Up-Regulation of Protein Spots in 2D Gel.