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Proteome changes related to the anti-cancer activity of HT29 cells by the treatment of ginsenoside R_d

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Ginseng is a representative herbal medicine in Asia and various pharmacological activities of ginsenoside R_d isolated from ginseng have been reported. However, anti-cancer activity and mechanism of ginsenoside R_d in HT29 colon cancer cell lines were not studied yet. We performed proteomic analysis through two-dimensional gel electrophoresis, MALDI-TOF/TOF-MS and database to identify altered protein induced by ginsenoside R_d treatment in HT29. We can identify fourteen proteins contributed to cell growth inhibition induced by R_d. Proteins involved in the inhibition of mitosis (Stathmin1, Microtubule-associated protein RP/EB family and Stratifin) were significantly up- and down-regulated. And proteins associated with apoptosis (Rho GDP dissociation inhibitor, Tropomyosin1 and Annexin5) were significantly changed. Furthermore, ginsenoside R_d in HT29 was involved in cytoprotection, DNA replication and repair, protein synthesis and degradation, metastasis and mutagenesis. It was supposed that ginsenoside R_d contributed to induce anti-cancer activity by complementary functions of these proteins in colon cancer cells.

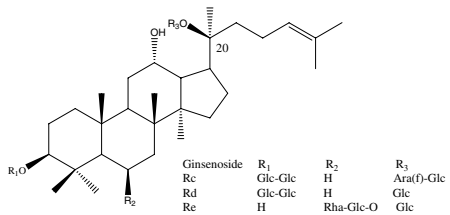
1. Introduction

Ginsenoside-R_d is a diol-type saponin belonging to the damaran group and is a major ingredient in ginseng. R_d inhibited proliferation and induced apoptosis in an human cervical cancer cell line with an IC_{50} value of 150.5 μg/ml after 48 h of incubation. These strong anti-cancer activity related to apoptosis was demonstrated through down-regulated *Bcl-2* expression, up-regulated *Bax* expression, reduced mitochondrial transmembrane potential and activated caspase-3 pathway (Yang et al. 2006). Ginsenoside-R_d restored cisplatin-induced renal injury with significant suppression of the DNA fragmentation (Yokozawa and Dong 2001). R_d activated the immune system by the induction of Th1 and Th2 immune responses (Yang et al. 2007). In addition, ginsenoside R_d decreased vascular contractility by blocking receptor-operated calcium channels (Guan et al. 2006).

We investigated the anti-proliferative effects of R_c, R_e and R_d and the anti-cancer mechanism of ginsenoside R_d in order to evaluate their suitability as drug candidates in chemotherapy of colon cancer. Proteomic analysis can give useful information to understand various anti-cancer mechanisms working simultaneously in a cellular system. We used a proteomic analysis to identify altered proteins in HT-29 cell line after exposure at the IC_{50} of R_d, more cytotoxic than other ginsenosides, using 2D electrophoresis and MALDI TOF/TOF MS.

2. Investigations and results

R_c and R_d are 20(S)-protopanaxadiol types and R_e is a 20(S)-protopanaxatriol type according to the structure of the non-sugar (aglycon) part of ginsenoside. The cytotoxicity of ginsenosides R_c, R_e and R_d was evaluated using MTT assay. The results showed that ginsenoside R_d had a significant antiproliferation activity in HT29 colon cancer cells. On the contrary, R_c and R_e showed a low inhibition rate of cell growth after 48 h of treatment. Ginsenoside R_d had stronger cytotoxicity than others in the concentration range of 200 μg/ml to 500 μg/ml. The IC_{50} of R_d was 277 μg/ml for a 48 h treatment in HT29 cells (Fig. 1).



Ginsenoside	R_1	R_2	R_3
Rc	Glc-Glc	H	Ara(f)-Glc
Rd	Glc-Glc	H	Glc
Re	H	Rha-Glc-O	Glc

Glc = Glucopyranosyl Ara(f) = Arabinofuranosyl
Rha = Rhamnopyranosyl

In the caspase 3 acitivity assay, HT29 cell showed low acitivity at 200 μg/ml R_d treatment for 24 and 48 h. At 400 μg/ml, the caspase 3 activity of 24 h treatment was 220% and the activity of 48 h treatment was 242%. 400 μg/ml R_d treatment showed an over twofold increase of caspase 3 activity compared to non-treated cells (Fig. 2). In the DNA fragmentation assay, the enrichment

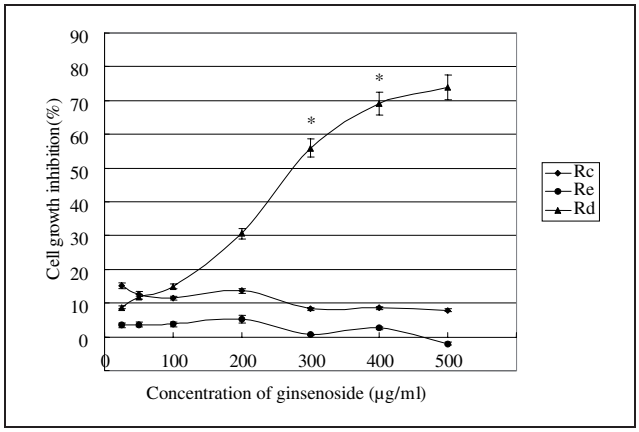


Fig. 1: HT29 cell growth inhibition of ginsenoside (Rc, Re, Rd) treatment during 48 h. Anti-proliferation was assessed by MTT assay following ginsenoside concentration (0–500 μg/ml). The data shown are the means (percentage of controls) ± SEM of three independent experiments. * Significantly is different from the group with untreated cells ($p < 0.05$)

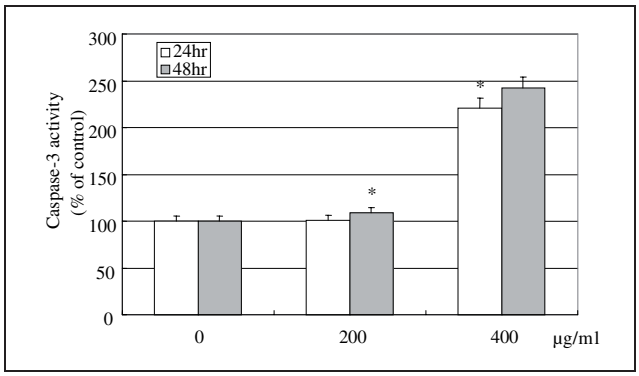


Fig. 2: Caspase-3 activity of Rd-treated HT29 cells. HT29 cells were treated with 0, 200, 400 μg/ml Rd during 24 h and 48 h. Caspase-3 activity was measured using colorimetric caspase-3 assay kit. Data are means ± S.E. ($n = 3$). * Significantly is different from the group with untreated cells ($p < 0.05$)

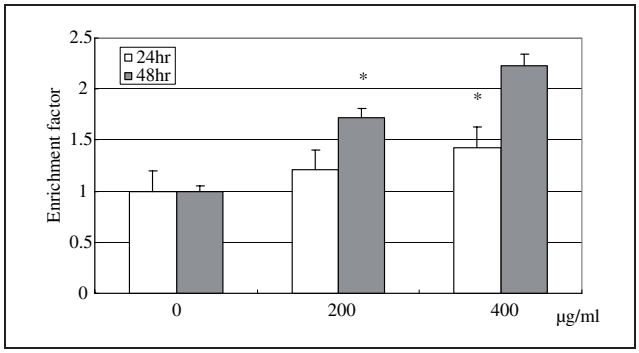


Fig. 3: DNA fragmentation of Rd-treated HT29 cells. HT29 cells were treated with 0, 200, 400 μg/ml Rd for 24 h and 48 h. DNA fragmentation was quantified by the Cell Death Detection ELISAplus kit and expressed as enrichment factors. Data are means ± S.E. ($n = 3$). * Significantly is different from the group with untreated cells ($p < 0.05$)

factor of R_d treated HT29 cell was increased in proportion to the concentration. After 24 h treatment, enrichment factors were 1.2 and 1.4 at 200 and 400 μg/ml, respectively (Fig. 3).

All the spots detected by PDQuest were normalized and calculated as mean intensities of three runs for non-treated and R_d treated cells (Fig. 4). We found 23 spots with different expression between control and R_d treatment by PDQuest. Fourteen spots of 23 spots showed a significant difference ($p < 0.05$) in the student's t-test and were identified successfully using MALDI-TOF/TOF-MS and NCBI database (Table).

Eight proteins in R_d-treated HT29 cells were down-regulated in the range of −1.4 fold to −4.6 fold and identified as stathmin1, PCNA, rho GDP dissociation inhibitor (GDI) alpha, reticulocalbin1 precursor, nudix hydrolase NUDT5, microtubule-associated protein RP/EB family, proteasome beta 6 subunit and tyrosine3/tryptophan 5-monooxygenase activation protein epilson. Stathmin1 showed distinct suppression over fourfold. Other proteins in R_d-treated HT29 cells were up-regulated in range of 1.3 fold to 2.9 fold and identified as tropomyosin1 (alpha), glu-

Table: Identification of differentially expressed proteins in Rd-treated cells

Spot No.	Protein identified	Fold change[a]	Accession. No.[b]	Mr/pI[c]	Score	Sequence coverage (%)[d]	Peptides matched
S1	Stathmin1	−4.6	gi|5031851	17/5.76	58	36	6
S2	PCNA (Proliferation cell nuclear antigen)	−1.4	gi|49456555	29/4.57	66	31	11
S3	Rho GDP dissociation inhibitor (GDI) alpha	−3.2	gi|4757768	23/5.02	86	36	8
S4	Reticulocalbin 1 precursor	−1.9	gi|4506455	39/4.86	84	37	10
S5	Tropomyosin 1 (alpha), isoform CRA_m	+2.3	gi|119598039	29/4.78	189	63	27
S6	Glutathione S-transferase-P1c	+1.3	gi|119595056	57/4.84	57	51	9
S7	Annexin 5	+2.9	gi|4502107	36/4.94	176	67	21
S8	Nudix hydrolase NUDT5	−1.9	gi|6694937	24/4.74	72	32	7
S9	Microtubule-associated protein, RP/EB family, member 1	−2.9	gi|6912494	30/5.02	78	30	7
S10	Proteasome beta 6 subunit	−3.4	gi|23110925	25/4.80	74	21	6
S11	Tyrosine 3/tryptophan 5-monooxygenase activation protein, epilson	−1.6	gi|5803225	29/4.63	153	56	21
S12	Nm23 protein	+1.5	gi|35068	20/7.07	103	51	12
S13	Tropomodulin 3 (Ubiquitous)	+1.7	gi|7657649	40/5.08	91	33	14
S14	Stratifin	+1.5	gi|5454052	28/4.68	91	43	16

Proteins identified using MALDI-TOF/TOF-MS through PMF

[a] Positive value means up-regulation against control and negative value means down-regulation in terms of fold changes. All the ratio are statistically significant with $p < 0.05$ of student's t-test

[b] Entry number from NCBI database

[c] Mr; Theoretical molecular mass of the identified protein in KDa, pI; Theoretical isoelectric point of the identified protein

[d] Percentage of matched sequence to the whole sequence of known protein

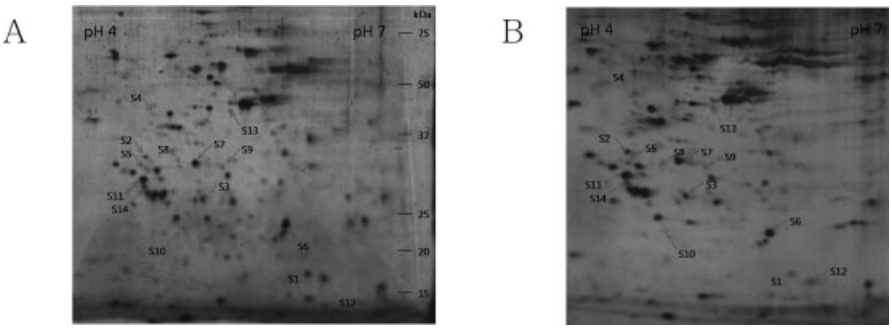


Fig. 4:
Comparative 2-DE gel analyses of untreated (A) and 277 μg/ml Rd-treated (B) HT29 cells for 48 h. The protein extracts (80 μg/300 μl) were displayed across a 17 cm IPG strip (pH 4–7) in the first dimension and a 12% SDS-PAGE gel in the second dimension, stained with silver stain. The gel pair was the representative gel from three independent experiments. Differentially expressed protein spots are shown by the arrows

tathione S-transferase-P1, annexin5, nm23 protein, tropomodulin3 (ubiquitous) and stratifin. Tropomyosin1 (alpha) and annexin5 of these proteins showed up-regulations over twofold (Fig. 5).

3. Discussion

In our study we used a proteomic approach to identify differential expressions of cellular proteins in ginsenoside R_d induced HT29 colorectal cancer cells since we found the anti-proliferative activity of R_d by MTT assay and the induction of apoptosis by caspase3 and DNA fragment assay.

Proteome change of R_d treated HT29 cells can be classified into several mechanisms according to their known functions. Stathmin1, microtubule-associated protein, RP/EB family, member1 and stratifin are involved in the inhibition of mitosis through the interference of cell cycle progression. Stathmin1 is known as an oncoprotein and microtubule-destabilizing protein. Suppression of stathmin causes cell accumulation in the G2/M phases and interrupts the formation of a normal mitotic spindle (Rubin and Atweh 2004). Therefore, stathmin1 may be decreased when HT29 cells ceased to proliferate upon exposure to ginsenoside R_d. RP/EB family, microtubule-associated protein (MAP), binds to the C-terminal region of APC protein concerned in colorectal tumorigenesis. RP/EB family influences microtubule cytoskeleton organization and mitosis progress (Juwana et al. 1999). Stratifin, called 14-3-3 sigma as a molecular chaperone, is a tumor suppressor protein. Stratifin is involved in cell cycle progression, proliferation, differentiation, apoptosis, and regulation of signal transduction pathways. Stratifin negatively regulates G2 cell cycle progression and reduces cell growth (Medina et al. 2007). R_d treatment in HT29 cells may induce the interference of mitosis process resulting in the inhibition of cancer cell growth.

Glutathione s-transferase p1 (GSTP1) is a phase II detoxification enzyme that conjugates electrophilic compounds with reduced glutathione. Most studies have focused on the overexpression of GSTP1 following increased resistance to anticancer agents in cancer cells (Townsend and Tew 2003). On the contrary, the enhanced expression of GSTP1 induced by protective agents contributes to differentiation and apoptosis in HT29 cells (Ebert et al. 2001). *In vivo*, ginsenoside-R_d increased glutathione (GSH) in the administration of ginsenoside-R_d at a dose of 1 or 5 mg kg^{-1} to young rats and promoted the defense system through the increased activity of glutathione peroxidase and reductase (Yokozawa et al. 2004). Therefore, we suggest that R_d may induce the up-regulation of GSTP1 and lead to both apoptosis and protection from oxidative stress in HT29 cell.

Five altered proteins were related to DNA replication and repair, protein synthesis and degradation, metastasis and mutagenesis. Proliferating cell nuclear antigen (PCNA) is a ring shaped trimeric complex that forms a sliding platform needed to process the DNA polymerase complex and to coordinate correctly leading and lagging strand DNA synthesis (Warbrick 2000). PCNA is an effective biomarker that may diagnose more aggressive colorectal adenomas with the high ability of malignant transformation (Lavezzi et al 2002). PCNA suppressed in R_d treated HT29 cells inhibits cancer cell proliferation through the regulation of DNA replication and repair. Recticulocalbin1 precursor (RCN1) is a calcium-binding protein with multiple elongation factor (EF)-hand motifs in the endoplasmic reticulum and is required for Ca^{2+} dependent cell adhesion (Ozawa and Muramatsu 1993). High expression of RCN1 was found in the SW480 colorectal carcinoma cell line that is more invasive than other colorectal carcinoma cell lines (Hiller et al. 1996). Therefore, the reduced expression of RCN1 in HT29 cells exposed by R_d may negatively regulate Ca^{2+} dependent cell adhesion and lead to inhibit the growth of HT29 cells. Proteasomes eliminate ubiquitin-tagged proteins in an ATP-dependent manner such as misfolded proteins or harmful metabolic enzymes (Ciechanover 1994). The degradation of unnecessary proteins regulates cell homeostasis, growth and development (Ouatas et al. 2003). The down-regulation of proteasome beta 6 subunit induced by R_d treatment may result in decreased growth rates following lower degradation rates. Metastasis, the major cause of mortality in cancer, enhances the survival or invasiveness of the tumor cells. During the metastatic process, tumor cells interact with various host cells, extracellular matrices and basement membrane components. Nm23 protein (nucleoside diphosphate kinase A: NDKA or nm23) is a metastasis suppressor that interrupts metastasis *in vivo* by reducing signal transduction involved in a histidine protein kinase activity. It has been reported that the low expression of nm23 is related with poor prognosis and the low survival rate of cancer patients in several epidemiological cohort studies (Hartsough and Steeg 2000). The up-regulation of nm23 protein induced by R_d may cause the suppression of metastasis in colon cancer cells. Nudix hydrolase NUDT5 is an anti-mutator candidate and belongs to the nudix hydrolase, ubiquitous protein family. NUDT5 in cooperation with NUDT1 may contribute to mutagenesis inhibition and to prevent transcriptional errors and mistranslation by the elimination of 8-OH-GTP and 8-OH-GDP (McLennan 2006). The decreased expression of NUDT5 in colon cancer cells may promote mutagenesis and lead to the failure of transcriptional process.

In addition, 14-3-3ε as a member of the 14-3-3 protein family mediates interaction between heat shock transcription factor1 (HSF1) and extracellular signal regulated protein kinase (ERK) during heat shock and promote the activities of HSF1 and ERK (Wang et al. 2004). The down-

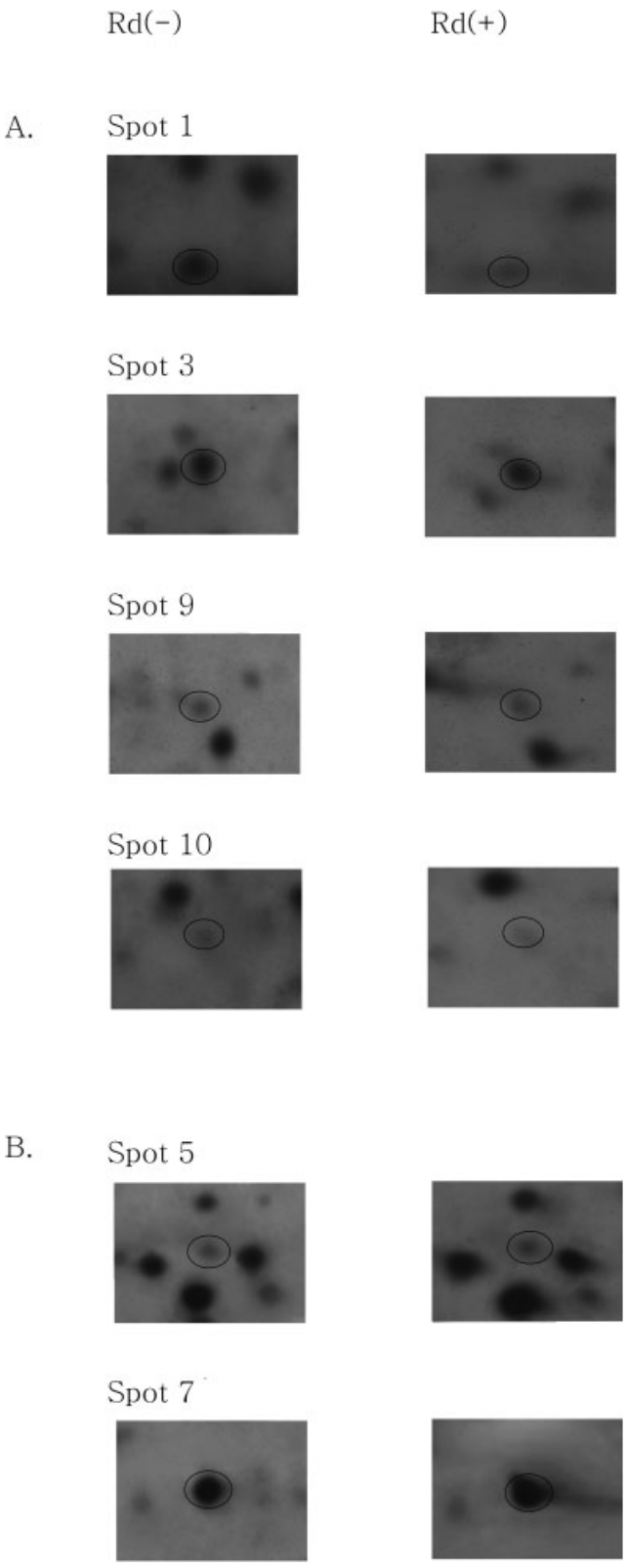


Fig. 5: Enlarged view of the down-regulations (A) and the up-regulations (B) of the representative protein spots in 2D gel

regulation of 14-3-3ε may be involved in reduced cytoprotection against Rd treatment. Tropomodulin, classified into cytoskeleton, stabilizes tropomyosin-actin filaments in many types of actin networks and structures. Tropomoulin3 (ubiquitous) to maintain the cell shape was up-regulated in Rd-induced colon cancer cells (Kostyukova 2008).

We demonstrated R_d-induced apoptosis of HT29 through capase-3 and DNA fragment assay. In our proteomic data, Rho GDP dissociation inhibitor (GDI) alpha, tropomyosin1 (TM1) and annexin5 (ANXA5) led to apoptosis in HT29 cells by R_d treatment. GDI is thought to inhibit Rho GTPase function. Rho GTPases are commonly found in human cancers and regulate tumor cell proliferation, angiogenesis and metastasis (Zhang 2006). However, recent studies have demonstrated that GDI is an antiapoptotic molecule to protect cancer cells against drug-induced toxicity. It was suggested that Rho GDI inhibits caspase-mediated cleavage of GTPase (Zhang et al. 2005). We speculate that the down-regulation of Rho GDI may induce apoptosis from terminating the downstream signal pathways of GTPase. Tropomyosin 1 (TM1) is an essential protein for normal mammalian development. TM1 was described as a tumor suppressor since its increased expression in breast cancer cell lines suppresses the malignant phenotype and inhibits anchorage independent growth (O'Neill et al. 2008). Hence the up-regulation of TM1 may contribute to the apoptotic response. Annexins are a family of calcium-regulated membrane-binding proteins. These proteins are involved in many membrane domains, regulation of phagocytosis, cell signaling, and proliferation (Raynal and Pollard 1994). ANXA5 binds with high affinity to the external cell membrane phosphatidylserine excreted to apoptotic tumor cells. Therefore, the increased level of ANXA 5 may indicate R_d-induced cell apoptosis in HT29.

In conclusion, we demonstrated in MTT, DNA fragment and capase-3 assays that ginsenoside R_d leads to the anti-proliferation and apoptosis of colon cancer cells. Using proteomic analysis we found various mechanisms involved in the anti-cancer activity. The exposure of colon cancer cells to ginsenoside R_d resulted in inhibited mitosis, induced apoptosis and reduced oxidative stress. Furthermore, the anti-proliferation effect of R_d was associated with DNA replication and repair, protein synthesis and degradation, mutagenesis and metastasis in HT29 cells. These pathways may act complementarily and enhance the anti-proliferation activity of ginsenoside R_d in colon cancer cells. R_d-induced proteins can be developed as biomarkers if ginsenoside R_d is applied to colon cancer chemotherapy.

4. Experimental

4.1. Reagents and materials

Ginsenosides were supplied by Korea Ginseng and Tobacco Research Institute (Purity; Rd 99%, Rc 98%, Re 99.5%). The human colorectal adenocarcinoma cell, HT29 cell line, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). DMEM/F12, fetal bovine serum and 100 μg/ml streptomycin antibiotics were purchased from Gibco BRL. The electrophoresis reagents including acrylamide solution (40%), urea, thiourea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), dithioreitol (DTT), Tris base, sodium dodecyl sulfate (SDS), ammonium persulfate (AP), N,N,N′,N′-tetramethylene diamine (TEMED), glycine, glycerol, agarose, iodoacetamide (IAA) and silver stain were purchased from Amresco. Immobilized pH gradient (IPG) strips (pH 4–7, linear), IPG cover mineral oil, protein marker and protein assay kit were purchased from Biorad. Colloidal coomassie blue staining kit and silver staining kit were purchased from Invitrogen. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), protease inhibitor cocktail, bovine serum albumin (BSA) and tris-(hydroxymethyl)-aminomethane α-cyano-4 hydroxy cinnamic acid (CHCA) were purchased from Sigma. Trypsin (modified) was purchased from Promega. Cell Death Detection ELISA^{plus} kit was purchased from Roche Molecular Biochemicals, Germany. Colorimetric capase-3 assay kit was purchased from Molecular probe, Eugene, OR, U.S.A.

4.2. Cell culture and protein extraction

The human colorectal adenocarcinoma cells (HT29 cell line), were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin antibiotics at 37 °C under 5% CO_2 and 95% air. For treatment with ginsenoside, cells were cultured in T-75 flask with medium until 60–70% confluent. Medium was removed and replaced by a medium containing either 0.1% DMSO or ginsenosides solubilized in 0.1% DMSO according to the half-maximal inhibitory concentration (IC_{50}). After 48 h, cells were washed by PBS three times and gathered by 500 μl cell lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 65 mM DTT, protease inhibitor cocktail) and centrifuged at 16000 rcf for 30 min at 15 °C. Supernatants were taken and centrifuged at 16000 rcf at 15 °C for 30 min. Protein concentration of the lysis supernatant was determined by the Bradford method and stored at −80 °C for 2-DE.

4.3. MTT assay

The cytotoxic activities of ginsenosides against HT29 were evaluated by the MTT assay based on the reduction of the MTT by viable cells. Briefly, HT29 cells ($1 \times 10^5/cm^2$) were incubated in 96 well assay plates for 24 h. After treatment with various concentrations of ginsenosides (0–500 μg/ml) for 48 h, the plates were incubated with 40 μL of MTT solution (5 mg/ml in PBS) at 37 °C for 4 h. DMSO 100 μL was replaced to solubilize MTT formazan crystals formed in viable cells. A Versamax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) was used for MTT assay. After 4 h, the absorbance was read at 550nm on a microplate reader.

4.4. Measurement of Capase-3 activation

Caspase-3 activity was performed with the colorimetric caspase-3 assay kit following the manual supplied by the manufacturer. After treatment with ginsenoside, the cells were centrifuged and the pellets were washed and lysed in 50 μl lysis buffer and repeated a freeze-thaw cycle. The cell lysate was centrifuged at 5000 rpm for 5 min at 4 °C. Supernatants were incubated with the substrate, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) for 30 min at room temperature. The absorbance at 405 nm (reference wavelength 490 nm) was read by microplate reader.

4.5. Quantification of DNA fragmentation

Quantification of DNA Fragmentation was performed with the Cell Death Detection ELISA^{plus} kit. After incubation with ginsenoside, the cells were lysed for 30 min at room temperature. Cell lysate was centrifuged at 16000 rcf for 10 min and 20 μl aliquots of the supernatants were loaded into the streptavidin-coated plate. Eighty microliters of fresh immunoreagent was added to each well and incubated for 15 min until the color is appeared clearly. The absorbance at 405 nm (reference wavelength 490 nm) was read by microplate reader. DNA fragmentation was calculated using the following equation:

Enrichment factor = absorbance of cells treated with Rd/absorbance of untreated cells

4.6. Two-dimensional electrophoresis and image analysis

2DE was performed with Biorad IPGphor IEF and electrophoresis units. Briefly, 80 μg of total cell protein was mixed to 300 μL of rehydration solution (7 M urea, 2 M thiourea, 2% w/v CHAPS, 20 mM DTT, 0.5% IPG buffer). It was loaded onto an IPG strip holder with 17 cm, pH 4–7 linear gradient IPG strips and incubated for 12 h at 20 °C. IEF was run following a step-wise voltage condition: step 1, 250 V for 15 min; step 2, 10 kV for 3 h; step 3, 10000 V for 60 kVh. After IEF separation, the strips were incubated 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, and subsequently 25 mg/ml iodoacetamide 15 min for each equilibration buffer. The equilibrated strip was inserted to a 12% polyacrylamide gel with protein marker for molecular weight calibration and sealed in place with 5 mg/ml agarose. Gels were run at constant current (26 mA/gel) and temperature (20 °C) for 8 h until the dye reached the bottom of the gel. After electrophoresis, the gels were stained with silver staining kit. The image was analyzed with PDQuest Version 7.2 (Bio-Rad). Three pairs of gels from non-treatment and Rd-treatment cells were matched. The intensities of spots were calculated and normalized as percentages of the total intensity of detected spots in a gel and applied in Student's t-test. Spots with significantly different intensities (Student's t-test; $p < 0.05$) were used for protein identification.

4.7. Tryptic in-gel digestion

300 μg of total cell protein used for silver staining was electrophoresed with the above method and stained using colloidal coomassie blue staining kit to excise identical spots by a comparison for in-gel digestion. Spots excised from the gel were destained with 10 mM ammonium bicarbonate for 10 min, and then with DW and acetonitrile 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. Supernatants were taken after digestion and dried in SpeedVac and dissolved in 0.1% TFA for mass spectrometric analysis.

4.8. Protein identification using MALDI-TOF TOF/MS

For MS analysis, the matrix solution containing 2 mg CHCA in 60% ACN with 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) equipped with a 337 nm nitrogen laser, delayed extraction, and reflectron was performed in the positive ion mode. Proteins were identified in MASCOT peptide mass fingerprint search program using NCBI database and maximum of 2 miss cleavages.

4.9. Statistical analysis

MTT, DNA fragmentation and caspase-3 assay data were expressed as the means ± SD of triplicate samples. 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 analysis in three separate experiments for reproducibility. In all cases, a p value of < 0.05 was used to determine the significance.

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