Synergistic antitumor effect of ginsenoside Rg3 and cisplatin in cisplatin-resistant bladder tumor cell line

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Abstract. Cisplatin-based chemotherapy is the first-line treatment for metastatic urothelial cell carcinoma. However, for cisplatin-resistant cases, no chemotherapeutic agent has been established as a standard of treatment. This study aimed to investigate the synergistic antitumor effect of ginsenoside Rg3 on cisplatin in cisplatin-resistant bladder cancer cells (T24R2). T24R2 cells were treated with cisplatin and/or ginsenoside Rg3. Cell viability was assessed by the Cell Counting Kit-8 and clonogenic assays. Synergism between ginsenoside Rg3 and cisplatin was determined when the combination index was <1.0. Flow cytometry was used to evaluate the cell cycle distribution. To estimate the changes of proteins associated with the cell cycle and apoptosis following the treatment of ginsenoside Rg3, western blotting and densitometric assays were performed for caspase-3, -8 and -9, cyclin B1, Bcl-2, Bad, p21 and cytochrome c. The Cell Counting Kit-8 and clonogenic assays showed the synergistic antitumor effect of ginsenoside Rg3 on cisplatin, while the combination index was <1.0, confirming the synergism. Cell cycle alterations at the G2/M phase caused by cisplatin were greater after the combination with ginsenoside Rg3. Western blotting and densitometric assay showed that the expression of Bcl-2 was decreased after the combined treatment of ginsenoside Rg3 and cisplatin, whereas the expression of cytochrome c and caspase-3 were increased, suggesting the activation of the intrinsic apoptotic pathway. In conclusion, ginsenoside Rg3 inhibited the proliferation of cisplatin-resistant bladder cancer cells in a synergistic manner with cisplatin. Activation of the intrinsic apoptotic pathway and the enhancement of cell cycle alterations are possible explanations for this result.

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Introduction

Cisplatin-based chemotherapy remains the first-line treatment of metastatic urothelial or transitional cell carcinoma. However, advanced age, poor performance status and renal dysfunction often preclude it. As no standard regimen has been established for patients who relapse after first-line cisplatin-based chemotherapy, it is essential to establish an effective chemotherapy regimen for patients with cisplatin-resistant bladder cancer. Taxane-based salvage chemotherapy and novel chemotherapeutic agents may be used for patients with cisplatin-resistant diseases. Numerous agents are under investigation or being tested in clinical trials to identify a novel tolerable target agent (1). However, the outcomes thus far are unsatisfactory.

Ginseng has been used in Asia as a medicinal plant to treat various diseases. Ginsenoside is an active compound of ginseng. Ginsenoside Rg3 is a natural product isolated from Panax ginseng. It induces apoptosis in human glioblastoma cell lines (2) and has an antitumor effect on hepatocellular carcinoma cells via the intrinsic apoptotic pathway (3-5).

In the present study, we investigated the antitumor effect of ginsenoside Rg3 and its synergism with cisplatin in cisplatin-resistant bladder tumor cells.

Materials and methods

Cell lines and chemicals. Bladder cancer cells (HTB5, J82, JON, UMUC14 and T24) were obtained from ATCC (Manassas, VA, USA). T24R2 cisplatin-resistant bladder cancer cells were generated by serial desensitization (6). The cells were maintained in RPMI (T24, T24R2), Dulbecco's modified Eagle's medium (J82, UMUC14) and modified Eagles' medium (HTB5) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA, USA) and 100 U/ml penicillin/100 mg/l of streptomycin (Invitrogen, Carlsbad, CA, USA). Cisplatin was obtained from Pfizer Korea Ltd., Seoul, Korea. Ginsenoside Rg3 was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Cell proliferation assay. Bladder cancer cells were plated in 96-well plates and treated with cisplatin (0-20 μg/ml) and ginsenoside Rg3 (0-50 μM) for 48 h. At the end of drug exposure, 10 μl of the Cell Counting Kit-8 solution (Dojindo
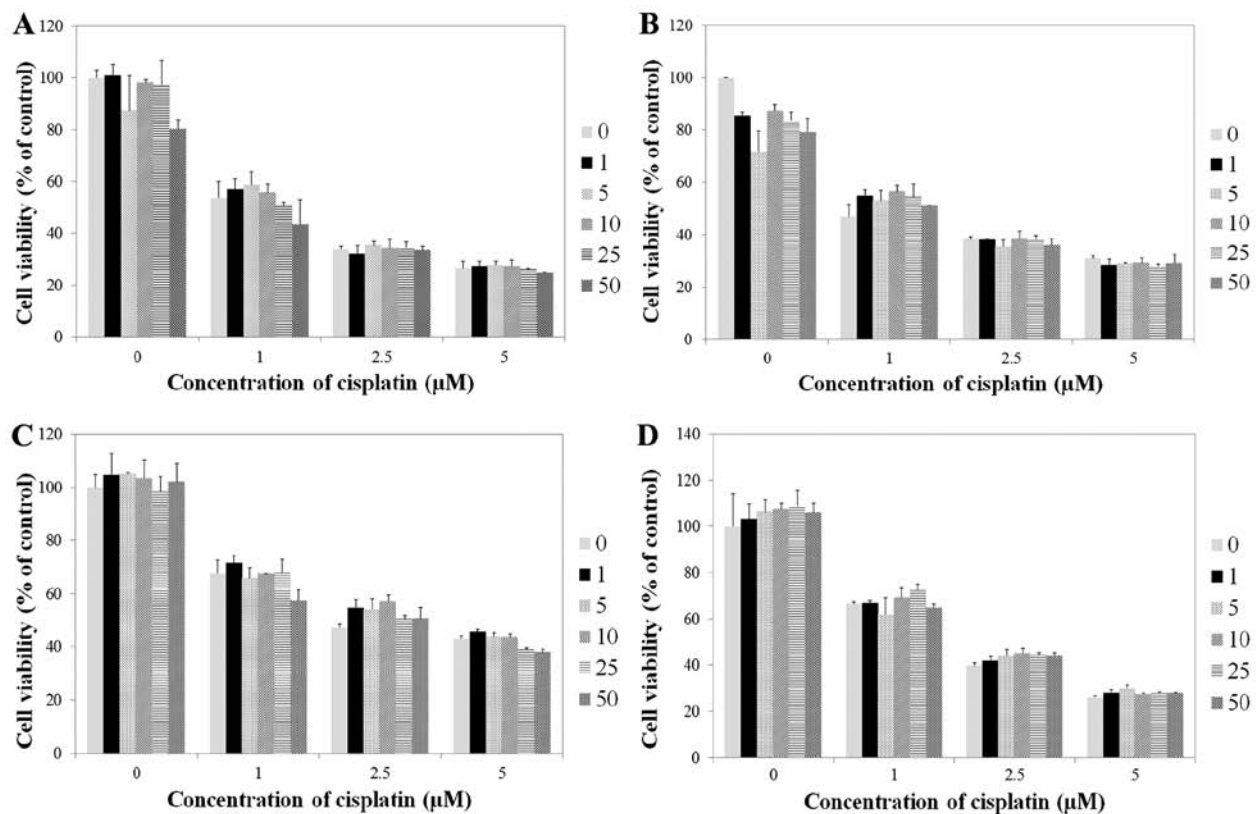
Figure 1. Cell Counting Kit-8 assay results after 48 h of treatment with cisplatin and ginsenoside Rg3 in bladder tumor cells. (A) HTB5, (B) J82, (C) JON and (D) UMUC14 cells.

Molecular Technologies, Gaithersburg, MD, USA) was added to each well containing 100 μl of medium. After 4 h of incubation, absorbance at 450 nm was measured.

Determination of synergism. The synergism between the two drugs was determined according to the combination index using CalcuSyn, version 2.1 (Biosoft, Cambridge, UK) (7). CI values of <1.0, 1.0 and >1.0 indicated synergism, additive effect and antagonism, respectively (8).

Clonogenic assay. For the colony-forming assay, T24R2 cells were treated with cisplatin (5.0 μg/ml) and/or ginsenoside Rg3 (50 μM) for 48 h and maintained in a drug-free medium for an additional 2 weeks. After staining with the 0.4% crystal violet, the number of colonies >0.2 mm in diameter was counted.

Cell cycle analysis by flow cytometry. T24R2 cells were treated with cisplatin (5 μg/ml) and/or ginsenoside Rg3 (50 μM) for 48 h, and flow cytometric analyses were conducted as previously described (9). The cell cycle distribution was determined by FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. After the 48-h of treatment of T24R2 cells with cisplatin (5.0 μg/ml) and/or ginsenoside Rg3 (50 μM), proteins were extracted using RIPA lysis buffer and Mitochondrial Isolation kit (Thermo Scientific, Rockford, IL, USA). Electrophoresis was performed using sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred with

polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and blocked for 1 h with Tris-buffered saline-Tween containing 5% milk. Primary antibodies to caspase-3, -8 and -9, full length PARP, fragmented PARP, cyclin A, B1 and D1, cytochrome *c*, p21, Bcl-2 and Bad were incubated overnight at 4°C.

Statistical analysis. Unless otherwise specified, the results are expressed as the means ± SD after a minimum of three repeated experiments. Tukey's multiple range tests were used to assess the statistical significance. P<0.05 was considered to indicate a statistically significant result. All the analyses were conducted using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Antitumor effect of cisplatin and ginsenoside Rg3 on bladder cancer cell lines. Cisplatin exerted an antitumor effect on bladder tumor cells. The viability of HTB5, J82, JON and UMUC14 cells was decreased after cisplatin treatment in a dose-dependent manner. However, ginsenoside Rg3 did not affect cell viability at any dose, regardless of the presence of cisplatin (Fig. 1).

In contrast to that of the HTB5, J82, JON and UMUC14 bladder tumor cells, the viability of T24R2 cells was not affected by the cisplatin dose up to the concentration of 2.0 μg/ml, as shown in a previous study (10). However, at a dose of >2.0 μg/ml of cisplatin, T24R2 cell viability was reduced according
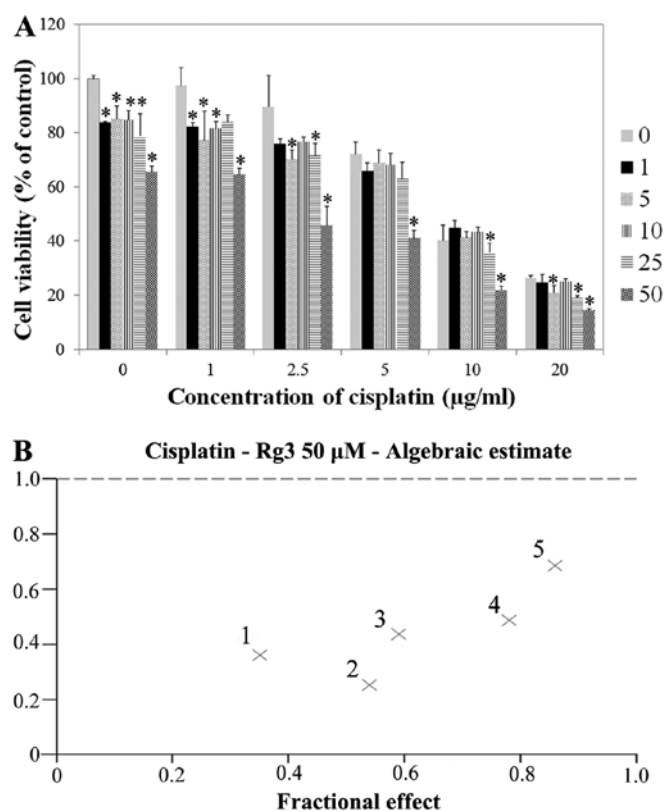
Figure 2. CCK-8, Cell Counting Kit-8 assay and combination index in T24R2 cells. (A) CCK-8 assay after the combined treatment of ginsenoside Rg3 and cisplatin in T24R2 cells for 48 h showed the synergistic inhibition of ginsenoside Rg3 on cisplatin. *Significant differences compared to the control. (B) Combination index of cisplatin and 50 μM of ginsenoside Rg3 was <1.0, revealing the synergism.

Figure 3. Clonogenic assay performed in T24R2 cells. (A) Clonogenic assay performed in T24R2 cells treated with cisplatin (5 μg/ml) and/or ginsenoside Rg3 (50 μM) after 48 h. a, control; b, ginsenoside Rg3; c, cisplatin; d, combination treatment. (B) The number of colonies counted are shown. *Significant difference compared to the control.

to the incremental dose of cisplatin (Fig. 2). The addition of ginsenoside Rg3 reduced T24R2 cell viability at any cisplatin dose. The antitumor effect was most prominent at the dose of 50 μM of ginsenoside Rg3. Synergism was evident when concentrations of cisplatin were <2.0 μg/ml, where T24R2 cell viability was significantly reduced according to the increase of the cisplatin dose. The combination index of cisplatin and 50 μM of ginsenoside Rg3 after 48 h of exposure was <1.0, showing the synergism between the two agents (Fig. 2B). The IC$_{50}$ value of ginsenoside Rg3 in T24R2 cells after 48 h of exposure was 207.7 μg/ml.

Ginsenoside Rg3 mediated T24R2 cell resensitization. A clonogenic assay was performed to evaluate the cytotoxic effect of cisplatin and ginsenoside Rg3. T24R2 cells were treated with cisplatin (5 μg/ml) and/or ginsenoside Rg3 (50 μM) for 48 h and the results of subsequent staining are shown in Fig. 3. The treatment of cisplatin reduced the number of colonies significantly and the inhibitory effect was greatest after the combined treatment of cisplatin and ginsenoside Rg3.

Ginsenoside Rg3 potentiates cisplatin-induced cell cycle alterations. A flow cytometric analysis was performed to investigate the effect of ginsenoside Rg3 on cell cycle progression. Cell cycle arrest was observed at the G2/M phase after the treatment of cisplatin (Fig. 4). The combined treatment
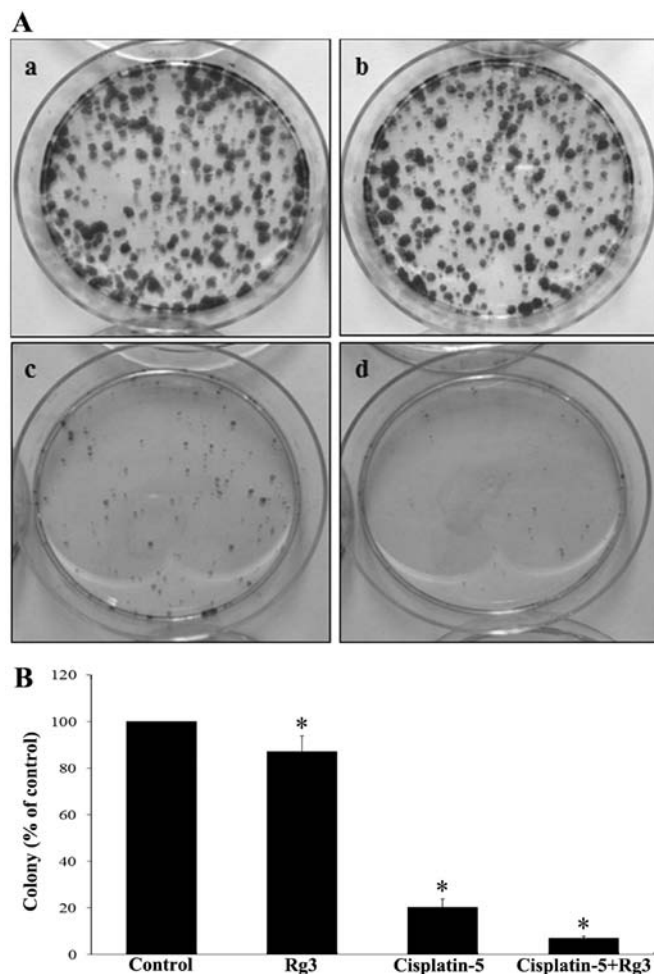
of cisplatin and ginsenoside Rg3 enhanced cisplatin-induced cell cycle alterations, although the result was not statistically significant. When T24R2 cells were treated with 10 μg/ml of cisplatin with ginsenoside Rg3, the distribution of the cell cycle was shifted towards the S phase, showing the cell cycle alteration by ginsenoside Rg3 (data not shown).

Ginsenoside Rg3 and changes of apoptosis and cell cycle regulator expression. Western blotting was performed to investigate the underlying mechanism of cell cycle regulation. Cleaved PARP, caspase-3, -8 and -9 (Fig. 5) and cytochrome *c* (Fig. 6) were increased significantly after the treatment of cisplatin. The increase was enhanced following the combined treatment. The expression of full-length PARP was decreased following the treatment of cisplatin and the combined treatment. Bcl-2 expression was decreased, whereas the activity of Bad was increased following the combined treatment of cisplatin and ginsenoside Rg3 (Fig. 6). Thus, activation of the intrinsic apoptotic pathway was associated with the synergism of these agents.

Cyclin B1 expression was significantly increased after the treatment with cisplatin and it was further enhanced following
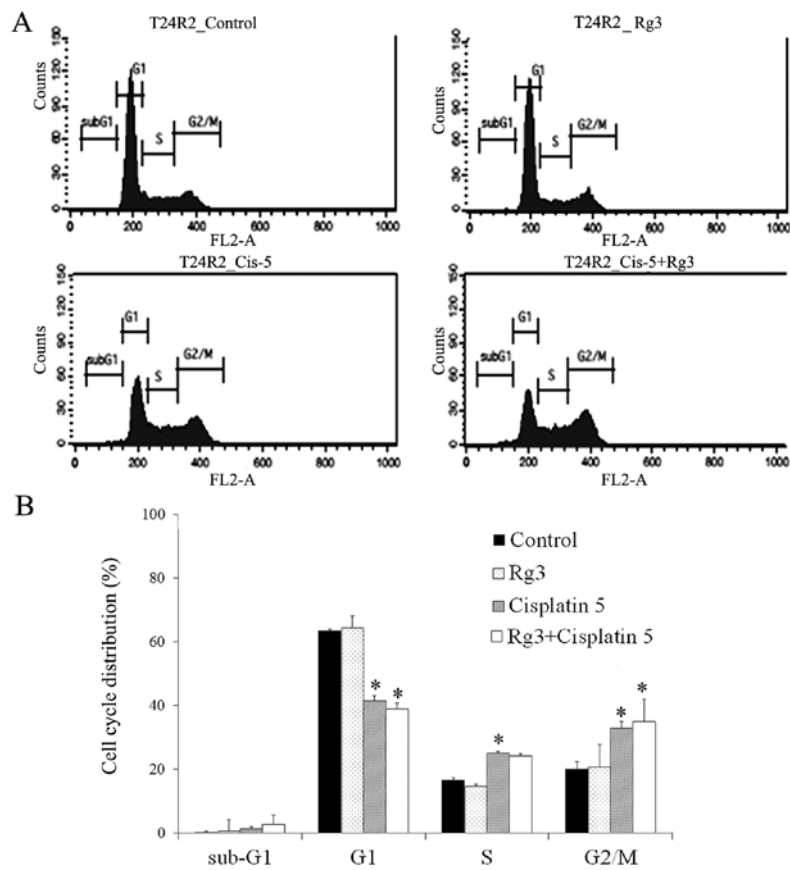
Figure 4. Flow cytometric analysis in T24R2 cells. (A) Flow cytometric DNA content histogram of T24R2 cells after 48 h of treatment with 5 µg/ml cisplatin and/ or 50 µM of ginsenoside Rg3. (B) Cell cycle distribution was measured quantitatively and plotted according to the type of treatments and the phase of cell cycle.
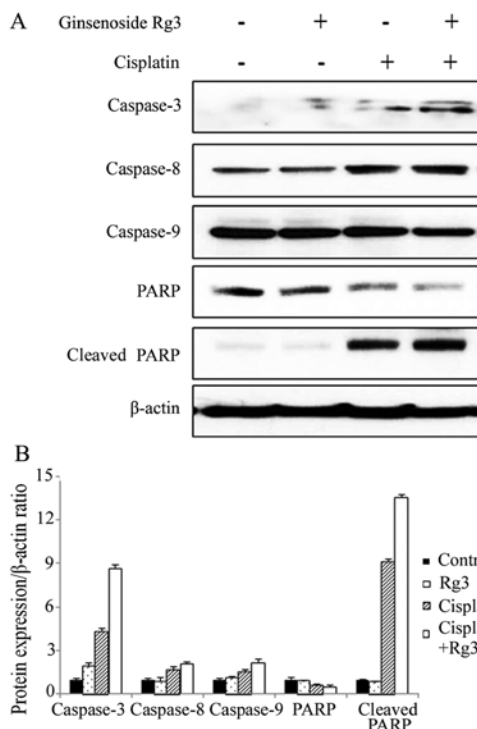

Figure 5. Western blotting and densitometric analysis used to compare the caspase expression. (A) T24R2 cells were treated with 5.0 µg/ml of cisplatin and/or 50 µM of ginsenoside Rg3 for 48 h. Expression of caspase-3, -8 and -9, and PARP were assessed by western blot assay. β-actin was the loading control. (B) Densitometric measurement of caspase-3, -8 and -9 and PARP expression ratios are in percentiles.
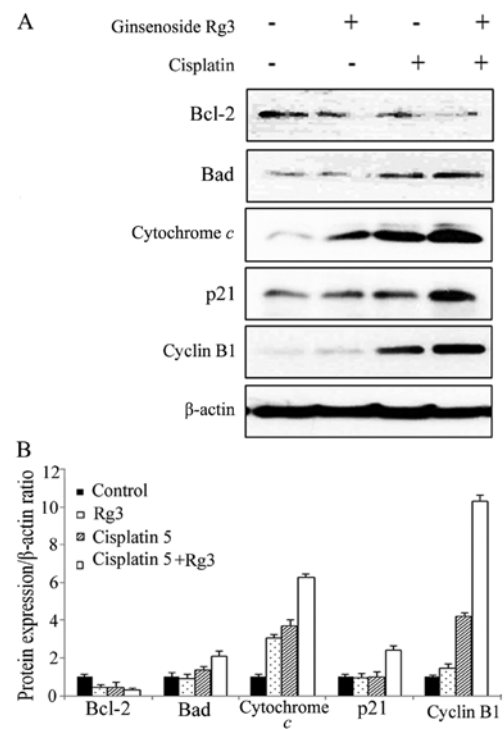
Figure 6. Western blotting and densitometric analysis of proteins associated with the apoptotic pathway and cell cycle regulation. (A) Expression of cell cycle and apoptotic-associated proteins by western blotting in T24R2 cells after the treatment of 5.0 µg/ml of cisplatin and/or 50 µM of ginsenoside Rg3 for 48 h. β-actin was the loading control. (B) Densitometric measurement of the same proteins in percentiles.

the combined treatment. The expression of other cyclins such as cyclin A and D1 was not identified by western blotting (data not shown). The increase of cyclin B1 after the combined treatment was correlated with the result of cell cycle alterations, suggesting the cell cycle arrest at the G2/M phase.

Discussion

Cisplatin is a platinum-based DNA alkylating agent that induces DNA damage, cell cycle arrest at G2/M phase, and apoptosis (11). Cisplatin-based combination chemotherapy has been the standard treatment method of advanced bladder cancer since the late 1980s (12-15). For patients with cisplatin-resistant bladder cancer, second-line treatment remains to be determined and many drugs are under investigation or being tested in clinical trials. The results of the present study have shown that ginsenoside Rg3 inhibits the growth of T24R2 cells in a synergistic manner with cisplatin. No synergism was observed between cisplatin and ginsenoside Rg3 when other cisplatin sensitive bladder cancer cell lines were treated alone or in combination. The survival of those cells was affected by cisplatin.

The chemosensitization of ginsenoside Rg3 has been previously reported. Kim *et al* reported that for prostate cancer, ginsenoside Rg3 enhanced the chemosensitivity of prostate cancer cells for docetaxel by the inhibition of NF-κB (16). They also demonstrated that the combined treatment of ginsenoside Rg3 with cisplatin inhibited the growth of prostate cancer cells more than either of the single regimen treatments. Lee *et al* reported that for colon cancer, ginsenoside Rg3 enhanced the chemosensitivity of tumors for cisplatin *in vivo*, using a murine xenograft model with CT-26 colon carcinoma cells (17). The results of the present study are in concordance with the results of those studies.

There are two possible explanations for the abovementioned results. First, the result suggests that the impact of ginsenoside Rg3 was not sufficiently strong to surpass the effect of cisplatin in cisplatin-sensitive cells. By contrast, when it was combined with cisplatin, the cell cycle arrest of the G2/M phase was potentiated with ginsenoside Rg3, which may contribute to the cell cycle arrest at the G1/S phase, consolidating the cell cycle alterations. The optimum concentration at which the antitumor effect was evident was <2.0 µg/ml of cisplatin. Second, since cisplatin-resistant bladder cancer cells have an increased expression of Bcl-2 (10), the inhibition of Bcl-2 following the treatment of ginsenoside Rg3 may lead to the more potent inhibition of viability in those cells.

Although the underlying mechanism of cell cycle regulation was not examined in the present study, the change of p21, which occurred only after the combined treatment, may be important in the synergism between the two agents. The combined treatment of ginsenoside Rg3 enhanced the cell cycle arrest at the G1/S checkpoint with the induction of p21, leading to a synergistic antitumor effect. However, irrespective of whether ginsenoside Rg3 was treated alone, alterations in the cell cycle may not have been sufficiently strong to induce the antitumor effect.

Cyclin B1 is expressed predominantly during the G2/M phase of the cell cycle (18), and its increased expression is correlated with the result of flow cytometry. Chen *et al* have shown that ginsenoside Rg3 inhibited the proliferation of EJ cells in bladder cancer cells by inducing apoptosis (19). The proportion of the S and G2/M phase was increased, while that of the G0-G1 transition was decreased in the cell cycle analysis, which was in agreement with our results. In addition, the increase of p21 only after the combined treatment may contribute to the cell cycle alteration, resulting in synergism.

Bcl-2 protein is an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells. Our results showed that the combined treatment of ginsenoside Rg3 and cisplatin was associated with the reduced level of expression in Bcl-2. Duggan *et al* showed that the downregulation of Bcl-2 protein enhanced mitomycin C induced apoptotic cell death in bladder cancer (20), although the prognostic significance of those findings was not consistent in the literature (21-24). Bolenz *et al* also demonstrated that the addition of Bcl-2 antisense oligodeoxynucleotides enhanced the cytotoxic potential of cisplatin as well as other chemotherapeutic agents such as mitomycin C in transitional carcinoma cell lines (25). Hong *et al* demonstrated that the expression of Bcl-2 mRNA and protein was increased in cisplatin-resistant bladder cancer cells and treatment with antisense Bcl-2 oligonucleotide enhanced the cytotoxicity of cisplatin significantly (10). Those results may have an impact on results of this study.

In addition to the suppression of Bcl-2, western blotting showed increased activity of proteins including cytochrome *c* and caspase-3, which was known to be involved in the intrinsic apoptotic pathway. Jiang *et al* demonstrated that ginsenoside Rg3 alone or the combined treatment with chemotherapeutic agents inhibited tumor growth in hepatocellular carcinoma via the activation of an intrinsic apoptotic pathway through alterations in the expression of Bcl-2 family proteins (4), which was in concordance with our result.

Bad is a pro-apoptotic protein and known to interfere the anti-apoptotic function of Bcl-2 and Bcl-xL in mitochondria (26). The increase of Bad and the decrease of Bcl-2 with the simultaneous increase of cytochrome *c* after the combined treatment of ginsenoside Rg3 and cisplatin suggests that the activation of the intrinsic apoptotic pathway is involved in the underlying mechanism.

In conclusion, ginsenoside Rg3, when combined with cisplatin, synergistically inhibits the growth of cisplatin-resistant bladder cancer cells. The enhancement of cell cycle alterations and activation of the intrinsic apoptotic pathway may be a possible underlying mechanism.

Acknowledgements

References

1. Ortmann CA and Mazhar D: Second-line systemic therapy for metastatic urothelial carcinoma of the bladder. Future Oncol 9: 1637-1651, 2013.
2. Choi YJ, Lee HJ, Kang DW, Han IH, Choi BK and Cho WH: Ginsenoside Rg3 induces apoptosis in the U87MG human glioblastoma cell line through the MEK signaling pathway and reactive oxygen species. Oncol Rep 30: 1362-1370, 2013.

3. Park HM, Kim SJ, Kim JS and Kang HS: Reactive oxygen species mediated ginsenoside Rg3- and Rh2-induced apoptosis in hepatoma cells through mitochondrial signaling pathways. Food Chem Toxicol 50: 2736-2741, 2012.

4. Jiang JW, Chen XM, Chen XH and Zheng SS: Ginsenoside Rg3 inhibit hepatocellular carcinoma growth *via* intrinsic apoptotic pathway. World J Gastroenterol 17: 3605-3613, 2011.

5. Zhang C, Liu L, Yu Y, Chen B, Tang C and Li X: Antitumor effects of ginsenoside Rg3 on human hepatocellular carcinoma cells. Mol Med Rep 5: 1295-1298, 2012.

6. Byun SS, Kim SW, Choi H, Lee C and Lee E: Augmentation of cisplatin sensitivity in cisplatin-resistant human bladder cancer cells by modulating glutathione concentrations and glutathione-related enzyme activities. BJU Int 95: 1086-1090, 2005.

7. Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27-55, 1984.

8. Chou TC, Motzer RJ, Tong Y and Bosl GJ: Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst 86: 1517-1524, 1994.

9. Yoon CY, Park MJ, Lee JS, *et al*: The histone deacetylase inhibitor trichostatin A synergistically resensitizes a cisplatin resistant human bladder cancer cell line. J Urol 185: 1102-1111, 2011.

10. Hong JH, Lee E, Hong J, Shin YJ and Ahn H: Antisense Bcl2 oligonucleotide in cisplatin-resistant bladder cancer cell lines. BJU Int 90: 113-117, 2002.

11. Sorenson CM, Barry MA and Eastman A: Analysis of events associated with cell cycle arrest at G_2 phase and cell death induced by cisplatin. J Natl Cancer Inst 82: 749-755, 1990.

12. von der Maase H, Hansen SW, Roberts JT, *et al*: Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of a large, randomized, multinational, multicenter, phase III study. J Clin Oncol 18: 3068-3077, 2000.

13. Loehrer PJ Sr, Einhorn LH, Elson PJ, *et al*: A randomized comparison of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma: a cooperative group study. J Clin Oncol 10: 1066-1073, 1992.

14. Logothetis CJ, Dexeus FH, Finn L, *et al*: A prospective randomized trial comparing MVAC and CISCA chemotherapy for patients with metastatic urothelial tumors. J Clin Oncol 8: 1050-1055, 1990.

15. Sternberg CN, Yagoda A, Scher HI, *et al*: Preliminary results of M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) for transitional cell carcinoma of the urothelium. J Urol 133: 403-407, 1985.

16. Kim SM, Lee SY, Cho JS, *et al*: Combination of ginsenoside Rg3 with docetaxel enhances the susceptibility of prostate cancer cells via inhibition of NF-κB. Eur J Pharmacol 631: 1-9, 2010.

17. Lee CK, Park KK, Chung AS and Chung WY: Ginsenoside Rg3 enhances the chemosensitivity of tumors to cisplatin by reducing the basal level of nuclear factor erythroid 2-related factor 2-mediated heme oxygenase-1/NAD(P)H quinone oxido-reductase-1 and prevents normal tissue damage by scavenging cisplatin-induced intracellular reactive oxygen species. Food Chem Toxicol 50: 2565-2574, 2012.

18. Entrez Gene: CCNB1 cyclin B1. Accessed January 15, 2014.

19. Chen JX, Peng HM, Pu SP and Guo YP: Inducement effect of ginsenoside Rg3 on apoptosis of human bladder transitional cell carcinoma cell line EJ. Zhongguo Zhong Yao Za Zhi 32: 1680-1684, 2007 (In Chinese).

20. Duggan BJ, Maxwell P, Kelly JD, *et al*: The effect of antisense Bcl-2 oligonucleotides on Bcl-2 protein expression and apoptosis in human bladder transitional cell carcinoma. J Urol 166: 1098-1105, 2001.

21. Touloupidis S, Fatles G, Kalaitzis C, *et al*: The significance of p53 and bcl-2 overexpression and other prognostic factors in transitional cell carcinoma of the bladder. Int Urol Nephrol 38: 231-236, 2006.

22. Glick SH, Howell LP and White RW: Relationship of p53 and bcl-2 to prognosis in muscle-invasive transitional cell carcinoma of the bladder. J Urol 155: 1754-1757, 1996.

23. Wu TT, Chen JH, Lee YH and Huang JK: The role of bcl-2, p53, and ki-67 index in predicting tumor recurrence for low grade superficial transitional cell bladder carcinoma. J Urol 163: 758-760, 2000.

24. Uchida T, Minei S, Gao JP, Wang C, Satoh T and Baba S: Clinical significance of p53, MDM2 and bcl-2 expression in transitional cell carcinoma of the bladder. Oncol Rep 9: 253-259, 2002.

25. Bolenz C, Becker A, Trojan L, *et al*: Optimizing chemotherapy for transitional cell carcinoma by application of bcl-2 and bcl-xL antisense oligodeoxynucleotides. Urol Oncol 25: 476-482, 2007.

26. Le Bras M, Rouy I and Brenner C: The modulation of inter-organelle cross-talk to control apoptosis. Med Chem 2: 1-12, 2006.