Solamargine induces apoptosis and sensitizes breast cancer cells to cisplatin

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Abstract

Solamargine (SM), a major steroidal alkaloid glycoside, was purified from Solanum incanum plant. SM exhibited the most cytotoxic effect comparing with that of cisplatin (cDDP), methotrexate (MTX), 5-fluorouracil (5-FU), epirubicin (EPI) and cyclophosphamide (CP) against human breast cancer cells. In this study, SM induces apoptosis of the breast cancer cells and the mechanism was characterized. SM up-regulated the expressions of external death receptors, such as tumor necrosis factor receptor I (TNFR-I), Fas receptor (Fas), TNFR-I-associated death domain (TRADD), and Fas-associated death domain (FADD). SM also enhanced the intrinsic ratio of Bax to Bcl-2 by up-regulating Bax and down-regulating Bcl-2 and Bcl-xL expressions. These effects resulted in the release of mitochondrial cytochrome c and activation of caspase-8, -9 and -3 in the cells, indicating that SM triggered extrinsic and intrinsic apoptotic pathways of breast cancer cells. Similar to function way of SM, cDDP causes cancer cell apoptosis though caspase-8/caspase-3 and Bax/cytochrome c pathways, but the resistance to cDDP is correlated with Bcl-2 and Bcl-xL overexpression. However, the overexpression of Bcl-2 and Bcl-xL can be broken through by SM. The combined treatment of SM and cDDP significantly reduced Bcl-2 and Bcl-xL expressions, and enhanced Bax, cytochrome c, caspase-9 and -3 expressions in breast cancer cells. Thus, the combined use of SM and cDDP may be effective in cDDP-resistant breast cancer.

Keywords: Solamargine; Breast cancers; TNF; Fas; Cisplatin; Apoptosis

1. Introduction

Apoptosis has a key role in the regulation of growth of normal and neoplastic tissues (Ghobrial et al., 2005). Extrinsic and intrinsic-mediated pathways lead to apoptosis. The extrinsic-mediated pathway is also called the receptor-mediated pathway. It is characterized by the activation of cell surface ligand-gated death receptors, including those of the superfamily of tumor necrosis factor receptors, such as TNFR, the Fas and TRAIL receptors (Ashkenazi and Dixit, 1998). When specific ligands bind to the extracellular domain of death receptors, receptor trimerization will be triggered. The TRADD and the FADD adaptor molecules
then bind to the cytoplasmic domain of the receptor. Initiator caspase-8 then causes activation and further cleaves and activates effector caspase-3, inducing irreversible cell death (Thorburn, 2004). The intrinsic or mitochondria-mediated pathway can be activated directly without being triggered by a death receptor. In this process, mitochondria will be disrupted by cell stress, releasing of cytochrome c into the cytoplasm. This is a known means of converting procaspase-9 into its activated form, caspase-9, which can active caspase-3, and trigger the irreversible apoptotic program (Li et al., 1997; Thomas et al., 2003). The Bcl-2 protein family is the most important regulator of the intrinsic and apoptotic processes, and this family includes a pro-apoptotic member, such as Bax and such anti-apoptotic members as Bcl-2 and Bcl-xL. Bcl-2 and Bcl-xL act as repressors of apoptosis by blocking the release of cytochrome c and also are important in resistance to chemotherapy and radiotherapy (Tortora et al., 2003; Tsujimoto, 2003). However, Bax has been demonstrated to play a key role in initiating mitochondrial dysfunction (Wei et al., 2001). Mitochondria dysfunction leads to the release of cytochrome c, which activates downstream effectors and develops a DNA damage-initiated apoptotic pathway (Plas and Thompson, 2002). In fact, the apoptotic process is regulated by the ratio between Bax and Bcl-2, and the Bax:Bcl-2 ratio is enhanced by anti-cancer drugs, before apoptosis (Del Bello et al., 2001).

Many chemotherapeutic drugs cause the cytotoxicity of cancer cells by apoptosis. cis-Dichlorodiammineplatinum(II) is commonly used as a chemotherapeutic agent in the treatment of human breast cancers. The cellular responses to cis-Dichlorodiammineplatinum(II) include the suppression of DNA replication, cell cycle arrest, transcriptional inhibition, and apoptosis. Two of the signaling pathways that are involved in cis-Dichlorodiammineplatinum(II)-induced DNA damage to irreversible program cell death are the caspase-8/caspase-3 pathway and the Bax/cytochrome c pathway (Siddik, 2003). However, the resistance of Bcl-2 and Bcl-xL has also been reported in breast cancer chemotherapy (Del Bufo et al., 2002; Mercatante et al., 2002). Bcl-2 and Bcl-xL molecules can prevent permeability transition and stabilize the outer-membrane of mitochondria (Shimizu et al., 1998). A recent study has shown that the Bcl-2-mediated cDDP resistance in breast cancer cells depends on the up-regulation of glutathione production (Rudin et al., 2003).

SM is a major glycoalkaloid that is found in at least 100 Solanum species (Lorey et al., 1996). Structurally, SM has a steroidal aglycone and the trisaccharide (bis-z-L-rhamnopyranosyl-β-glucopyranose) chain attaches to the 3-hydroxy group of steroidal aglycone (Alzerrerca and Hart, 1982). Our previous studies demonstrated that SM-caused apoptosis of human hepatoma and lung cancer cells though extrinsic pathway (Kuo et al., 2000; Liang et al., 2004; Liu et al., 2004). This study determined the extrinsic and intrinsic apoptosis pathways of SM on human breast cancer cells. Besides, SM and cis-Dichlorodiammineplatinum(II) synergistically up-regulated the Bax, cytochrome c, caspase-9 and -3 expressions, and down regulated the Bcl-2 and Bcl-xL expressions. That may increase the susceptibility of breast cancer cells to cis-Dichlorodiammineplatinum(II).

2. Materials and methods

2.1. Drugs

Drugs were provided as pure substances. SM was purified from Solanum incanum according to the previous procedures (Lin et al., 1990). SM (CAS number: 20311-51-7) and 5-FU (CAS number: 51-21-8; Sigma Chemical Co., St Louis, MO) were diluted in dimethyl sulfoxide (DMSO). cDDP (CAS number: 15663-27-1; Bristol-Myers Squibb, Woerden, The Netherlands), CP (CAS number: 6055-19-2; Sigma Chemical Co., St Louis, MO), MTX (CAS number: 59-05-2; Lederle Arzneimittel GMBH & Co.) and EPI (CAS number: 56420-45-2; FARMITALIA CARLO ERBA, Italy) were diluted in phosphate buffered saline (PBS). The drugs were stored at 4 °C before the experiments and freshly diluted to the final concentrations in culture medium.

2.2. Cell culture

The breast cancer cell lines HBL-100, ZR-75-1 and SK-BR-3 were obtained from American Type Culture Collection (Rockville, Maryland, USA). All cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazelton Products, Denver, PA), 100 μg/ml streptomycin and 100 unit/ml penicillin. Cells were grown in a humidified incubator at 37 °C under 5% CO₂ in air.

2.3. Measurement of cell death

For the determination of cell death, 1 x 10⁶ cells per well were seeded in each 100 μl of 96-well multi-dishes (Corning, Elmlira, NY) and separately treated with serial concentrations of SM, cis-Dichlorodiammineplatinum(II), MTX, 5-FU, EPI and CP for 16 h. Cell death was assessed by MTS assay (CellTiter 96™ AQ, Promega, Madison). The absorbance at 490 nm (A₄₉₀) was measured with an automated plate reader (Dydatech, Alexandria, VA). Cell viability was expressed as percentage of A₄₉₀ of treated and untreated values. The ICₕ₀ was calculated from the drug concentration that induced a 50% of cell death.

2.4. Measurement of apoptosis

Breast cancer cells (1 x 10⁶/well) were seeded in 24-well multi-dishes (Corning, Elmlira, NY), and treated with SM (ICₕ₀) for 0, 1, 3 and 16 h. After SM treatment, cells were fixed by ice-cold 4% para-formaldehyde at 4 °C for 30 min, and stained with haematoxylin. The morphological changes were observed by light-microscopy (Olympus CK40, ×400). In flow cytometric analysis, cells were incubated with SM (IC₂₅ and ICₕ₀) for 0, 1, 3 and 16 h. For detection of cell cycle distribution by flow cytometry, the cells were fixed by ice-cold 4% para-formaldehyde in 1 × PBS (pH 7.4) at 4 °C for 30 min. After centrifugation at 1500 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4 °C for 2 min. PI in 1 × PBS (10 μg/ml) was added to stain the cells at 4 °C for 30 min. The intensity of red fluorescence was measured with a FACScan flow cytometer (Becton Dickinson). A minimum of 5000 cells was collected for analysis by WinMDI software.

2.5. Determination of caspase activity

Breast cancer cells (at a density of 2 x 10⁶ cells/ml) were treated with IC₂₅ of SM at 37 °C for 16 h. Cells were harvested and lysed. The protein content in the cell lysate was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). The activities of caspase-8, -9 and -3 were detected by the Caspase-8, Caspase-9 and Caspase-3 Colorimetric Assay (R&D Systems, Minneapolis, MN). After SM treatment, cells were fixed by ice-cold 4% para-formaldehyde in 1 × PBS (pH 7.4) at 4 °C for 30 min, and stained with haematoxylin. The morphological changes were observed by light-microscopy (Olympus CK40, ×400). In flow cytometric analysis, cells were incubated with SM (IC₂₅ and ICₕ₀) for 0, 1, 3 and 16 h. For detection of cell cycle distribution by flow cytometry, the cells were fixed by ice-cold 4% para-formaldehyde in 1 × PBS (pH 7.4) at 4 °C for 30 min. After centrifugation at 1500 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4 °C for 2 min. PI in 1 × PBS (10 μg/ml) was added to stain the cells at 4 °C for 30 min. The intensity of red fluorescence was measured with a FACScan flow cytometer (Becton Dickinson). A minimum of 5000 cells was collected for analysis by WinMDI software.

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Colorimetric substrate (Ac-DEVD-pNA) was mixed for caspase-3 activity test. Samples were incubated at 37 °C, and the absorbance at 405 nm was measured in an enzyme-linked immunosorbent assay reader (Multisken Ascent, Thermo Labsystems). Caspase-8 activity was measured in breast cancer cells using Ac-IETD-pNA as substrate. Substrate LEHD-pNA was used for caspase-9 activity test.

2.6. Immunoblots

Cells (1 × 10^5) were lysed in NP40 lysis buffer (50 mM HEPES (pH 7.25), 150 mM NaCl, 50 μM ZnCl₂, 50 μM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1.0% NP40, and 2 mM PMSF). The total cell lysate was centrifuged at 12,000 rpm for 5 min, and protein concentration was determined by an ESL protein assay (Boehinger–Mannheim) with BSA as standard. Cell lysate protein (100 μl) was subjected to 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in 5% skim milk. Blots were incubated with the antibodies against TNFR-I (R&D Systems, Inc.), Fas, TRADD, FADD (Santa Cruz Biotechnology), and cytochrome c (PharMingen). The membranes were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Blots were visualized on X-ray film with enhanced ECL Western blotting detection reagents (Amersham, Piscataway, NJ).

2.7. Flow cytometric analysis of Bcl-2, Bcl-xL and Bax expressions in breast cancer cells

Breast cancer cells (2 × 10^5) were treated with SM (IC₂₅), cDDP (150 μM) and SM plus cDDP for 16 h at 37 °C. After drug treatment, cells were washed twice with ice-cold 1 × PBS, and fixed by ice-cold 4% paraformaldehyde at 4 °C for 30 min. After centrifugation at 1500 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4 °C for 2 min. The cells were washed two times with 1 × PBS, and resuspended at 2 × 10^5 cells/100 μl in 1 × PBS containing 3% BSA. The breast cancer cells were incubated with monoclonal antibodies against Bcl-2, Bcl-xL and Bax (BD Transduction Laboratories). Anti-mouse IgG conjugated FITC for Bcl-2, Bcl-xL and Bax antibodies (DAKO Diagnostic, Hamburg, Germany) were used as the secondary antibody in the dilution of 1:100. For negative controls, the primary antibodies were replaced with 1 × PBS under the same condition. The FITC-stained cells were analyzed with FACScan flow cytometer (Becton Dickinson) and 5000 events were acquired and analyzed with the LYSIS II software (Lysis, Mountainview, CA) and WinMDI software.

3. Results

3.1. The cytotoxic effect of SM is stronger than that of chemotherapeutic agents against human breast cancer cells

The cytotoxicities of SM, cDDP, MTX, 5-FU, EPI and CP in human breast cancer cells (HBL-100, SK-BR-3 and ZR-75-1) were compared by the MTS assay. SM exhibited greatest cytotoxicity among the chemotherapeutic drugs against breast cancer cells, and the action was dose-dependent (Fig. 1a). The concentrations (IC50) of the SM that caused 50% cell death were approximately 2.07, 3.00 and 2.15 μM for HBL-100, SK-BR-3 and ZR-75-1 cells, while those of most of the chemotherapeutic drugs exceeded 250 μM for all of the cells. A time-course of cell death after SM (IC₂₅, IC₅₀ and IC₇₅) treatment was monitored (Fig. 1b). The maximum number of dead breast cancer cells was detected within 3 h of incubation with constant...
concentrations of SM, and no further cell death was observed after an extended incubation with SM for 16 h.

SM(IC50)-induced morphological signs of a reduction in size, membrane bleeding and chromatin condensation in the nuclei of the breast cancer cells indicated apoptosis of the cells (Fig. 2). After SM (IC25 and IC50) treatment, the cells were stained with PI and the apoptotic cells were counted with reference to the sub-G1 peak by a flow cytometer. The changes of cell cycle distribution were showed in Table 1, the increases in the SM(IC25)-induced sub-G1 peaks at 3 h were 16.2 ± 0.7, 23.2 ± 1.2 and 27.2 ± 0.6%, and those for the SM(IC50)-induced sub-G1 of HBL-100, SK-BR-3 and ZR-75-1 cells were 20.6 ± 0.4, 32.0 ± 0.1 and 36.0 ± 1.2%, respectively. Thereafter, no significant increase of sub-G1 (%) can be observed after extended exposure for 16 h (data not shown). In the other hand, the changes of other cell cycle phases could be observed. The cell population of the G2/M phase was reduced concurrently with the increase of the sub-G1 population after 3 h of incubation with SM; no significant changes (<10%) of cell population in the G0/G1 phase were observed. This implies that SM might predominantly stimulate the cells of the G2/M phases to apoptosis. These results reveal that SM has greater cytotoxicity than conventional chemotherapeutic agents, and that SM-induced cell death is by apoptosis.

3.2. SM induces apoptotic signal cascades in breast cancer cells

The expressions of apoptosis-related protein molecules such as TNFR-I, Fas, TRADD and FADD were examined to elucidate the mechanism of SM in human breast cancer cells. As shown in Fig. 3a, SM (IC25) significantly up-regulated the protein expressions of TNFR-I, Fas, the downstream signal proteins, including TRADD and FADD. The relative intensities of TNFR-I, Fas, TRADD

![Fig. 2. Morphological changes of the nuclear chromatin of breast cancer cells after SM (IC50) treatment. The cells were stain with haematoxylin, and the morphological changes were observed by light-microscopy (Olympus CK40, ×200).](image-url)
and FADD expressions were determined by densitometric analysis. The intensities of TNFR-I, Fas, TRADD and FADD expression increased by folds of 1.74, 2.75, 2.37 and 1.67 respectively, for HBL-100; 5.59, 3.45, 2.78 and 5.59 for SK-BR-3, and 3.95, 6.46, 5.08 and 6.48 for ZR-75-1 cells compared with the control cells. Since the TNFR superfamily has an important role in triggering apoptosis, these data suggest that TNFR and Fas may mediate SM-induced apoptosis in breast cancer cells.

The intrinsic pathway of apoptosis is associated with Bax, cytochrome c and caspase cascade, and regulated by Bcl-2 and Bcl-xL. Flow cytometric analysis is the forceful method for detection of Bcl-2, Bcl-xL and Bax expressions (Aiello et al., 1992; Van Stijn et al., 2003; Hartung and Bahler, 2004). In this work, the expressions of Bax, Bcl-2 and Bcl-xL were detected by flow cytometric analysis, and the expression of cytosolic cytochrome c was determined by Western blotting, to clarify the intrinsic mechanism of SM in breast cancer cells. After SM (IC\textsubscript{25}) treatment for 16 h, SM reduced the expressions of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and promoted the expression of pro-apoptotic protein Bax in human breast cancer cells (Fig. 3b). Besides, SM induced the release of large amounts of cytochrome c into the cytosol (Fig. 3c), and increased the downstream apoptotic caspase cascades, such as those associated with caspase-8, -9 and -3 activities in the cells (Fig. 3d). These results demonstrate that SM may mediate apoptosis though not only extrinsic but also intrinsic apoptotic pathways.

### 3.3. Synergistic effect of SM and cDDP in breast cancer cells

CDDP is one of the most commonly used anticancer drugs in clinics, and its Bax-dependent apoptotic effect can be blocked by Bcl-2 and Bcl-xL (Del Bufalo et al., 2002; Mercatante et al., 2002). Since SM can inhibit the expressions of Bcl-2 and Bcl-xL, the synergistic effect of CDDP and SM treatment in breast cancer cells was evaluated by MTS assay and flow cytometric analysis. Originally, most of the breast cancer cells were resistant to CDDP at high (250 \( \mu \)M) concentration. However, a synergistic cytotoxic effect was observed after the co-treatment of CDDP with SM in all breast cancer cells, and this effect depended on the SM dose (Fig. 4a). The apoptotic sub-G\textsubscript{1} fractions were measured by flow cytometry after CDDP (150 \( \mu \)M), SM (IC\textsubscript{25}) and their combinations were applied in all breast cancer cells. As shown in Fig. 4b, the combination of CDDP and SM significantly increased the cytotoxicity of the cells, which could be blocked by the caspase-3 inhibitor Z-DEVD-FMK (100 \( \mu \)M).

Similar to the function of SM in breast cancer cells, CDDP-mediated apoptosis also through the increase of Bax: Bcl-2 ratio, cytochrome c expression, and caspase-9 and -3 activities. Furthermore, CDDP can also trigger apoptosis by caspase-8/caspase-3 pathway. Therefore, these two pathways were studied to investigate further

| Cell cycle distribution of breast cancer cells after SM (IC25 and IC50) treatments |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Time (h) | IC\textsubscript{25} | IC\textsubscript{50} | IC\textsubscript{25} | IC\textsubscript{50} | IC\textsubscript{25} | IC\textsubscript{50} | IC\textsubscript{25} | IC\textsubscript{50} | IC\textsubscript{25} | IC\textsubscript{50} |
| HBL-100 | 13.0 ± 0.2 | 19.7 ± 0.5 | 23.2 ± 1.2 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 |
| SK-BR-3 | 13.0 ± 0.2 | 19.7 ± 0.5 | 23.2 ± 1.2 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 |
| ZR-75-1 | 13.0 ± 0.2 | 19.7 ± 0.5 | 23.2 ± 1.2 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 | 54.0 ± 0.6 |

No significant increase of sub-G\textsubscript{1} change after extended exposure for 16 h.
the synergistic mechanism of SM (IC_{25}) and cDDP (150 μM). As shown in Fig. 5a, cDDP promoted pro-apoptotic Bax expression only in HBL-100 cells, and SM markedly increased Bax expression in all breast cancer cells. In addition, SM decreased the expressions of Bcl-2 and Bcl-xL. Co-treatment of breast cancer cells with SM and cDDP resulted in the synergistic elevation of Bax expression and the down-regulation of anti-apoptotic Bcl-2 and Bcl-xL expressions. Additionally, cDDP does not affect the release of cytochrome c in ZR-75-1 and SK-BR-3 cells. However, combinational treatment with SM and cDDP synergistically increased cytochrome c release in all of the cells (Fig. 5b). Caspase activity in cancer cells can be triggered by two main pathways – transmembrane receptors/caspase-8 and Bax/cytochrome c pathways. As shown in Fig. 5c, SM markedly increased caspase-8 activity in all breast cancer cells. However, the SM-induced caspase-8 activity was not synergistically increased when used in a combined treatment with cDDP. Furthermore, SM greatly enhanced the activities of cDDP-induced caspase-9 and -3 in all breast cancer cells. These results imply that increasing the ratio of Bax to Bcl-2 and Bcl-xL may overcome the suppression of cDDP by Bcl-2 and Bcl-xL which results in the increase of susceptibility of the breast cancer cells to cDDP. The synergistic effect of SM and cDDP on the breast cancer cells may be mediated through the Bax/cytochrome c pathway.

4. Discussion

Apoptosis is an essential physiological process for the normal development and maintenance of tissue homeostasis. During apoptosis, morphological changes can be observed, including cytoplasm shrinking, chromatin condensation, plasma membrane blebs, DNA fragmentation and apoptotic body formation. In our present study, SM-caused cell death more rapidly than conventional chemotherapeutic agents and the maximum of SM-caused cytotoxicity was at 3 h (Fig. 1b). The apoptotic features can be visualized after SM treatment for 1 h. The changes in the cell cycle of breast cancer cells after SM treatment were determined by flow cytometry. The G2/M populations were shifted drastically to the sub-G1 population in all the cell lines, and no significant changes (<10%) in the G0/G1 phase (Table 1). This result indicates that SM might arrest breast cancer cells at the G2/M phase, and primarily stimulates the cells from the G2/M phase to the sub-G1 phase of apoptosis. In the other words, cells in the G2/M phase are relatively susceptible to SM-induced apoptosis. The similar result is consistent with our previous report which SM arrested Hep3B cell in G2/M and promoted hepatoma cells to apoptosis (Kuo et al., 2000).

Death receptors, including TNFR, Fas and TRAIL receptors trigger the extrinsic pathway of apoptosis (De Vries et al., 2003). Down-regulation of the signal from
death receptors appears to be a strategy that is commonly used by tumor cells to escape from the host immune system (Chouaib et al., 2002). Fas belongs to the TNFR superfamily and can cause apoptosis in a manner similar to that of TNFR-I. In breast cancer cells, the loss of Fas or function is an important regulation associated with tumor development (Song and Santen, 2003). Apoptotic signaling through the TNFR superfamilies require their interaction with the intracellular adaptor molecule TRADD and FADD, which in turn promotes activation of procaspase-8 and its recruitment into the death-inducing signaling complex. Procaspase-8 after proteolytic activation induces activation of caspase-3 along the common final pathway of apoptosis (Schneider and Tschopp, 2000). SM up-regulated TNFR-I, Fas, TRADD and FADD expressions and activated caspase-8 and -3 suggesting that the extrinsic pathway of apoptosis might be involved in SM-induced cell death. TNF receptor superfamily, including TNFR-I, Fas and TRIAL receptors are cell membrane receptors that trigger apoptosis by binding of specific ligands. Recombinant TNFα, a FDA approved limb perfusion ligand, binds to TNFR-I and triggers apoptosis in many cancer cells (Lejeune et al., 2006; Ricci and Zong, 2006). However, cancer cells escape TNFα binding by the absence of TNFR-I. Furthermore, the expression of Fas is important in mediating the cytotoxicity of the chemotherapeutic drugs, such as cisplatin. However, the loss of Fas expression during cancer chemotherapy is one of the mechanisms by which cancers become drug-resistance (Sharma et al., 2000). Thus, modulations of TNFR-I and Fas levels have provided a novel effective means to treat cancers.

The release of cytochrome c from mitochondria initiates the intrinsic pathway of apoptosis. The pathway is regulated mainly by pro-apoptotic member Bax, and anti-apoptotic members Bcl-2 and Bcl-XL. Bax is typically removed from the cytosol to mitochondria during apoptosis, and
targets the mitochondrial outer-membrane contact sites that are associated with an apparent oligomerization. Both Bcl-2 and Bcl-xL seem to exert their influences by interacting directly with Bax and preventing caspase activations (Deming and Rathmell, 2006). Overexpression of Bcl-2 and Bcl-xL in many cancer cell types can block the effects of Bax, prevents cytochrome c release from mitochondria, caspase activation and cell death (De Giorgi et al., 2002).

In the present study, down-regulation of Bcl-2 and Bcl-xL, up-regulation of Bax, cytochrome c release, and activation of caspase-9 and -3 were determined in breast cancer cells after SM treatment. Taken together, these results indicated that SM may induce apoptosis by extrinsic and intrinsic pathways.

cDDP is a widely used anticancer agent for breast cancer treatment and has been shown to induce apoptosis in vitro and in vivo. cDDP toxicity was associated with an increase in caspase-8 activity, followed by translocation of Bax, release of cytochrome c, and activation of caspase-9 and -3, suggesting that death receptor and mitochondrial pathways are involved in cDDP-induced apoptosis of cancer cells (Gonzalez et al., 2001). However, overexpression of Bcl-2 and Bcl-xL leads to cDDP resistance (Hanahan and Weinberg, 2000). Overexpression of Bcl-2 is an important mechanism of resistance in treating cancers with chemotherapy and radiotherapy. Down-regulation of Bcl-2 and Bcl-xL can potentially reduce tumor resistance to anticancer drugs. Antisense oligodeoxynucleotides for Bcl-2 and Bcl-xL sensitize cancer cells to cytotoxic therapies (Manion et al., 2006). Many preclinical and clinical studies suggest that the combination of cytotoxic therapy with antisense oligonucleotide of Bcl-2 results in synergistic anticancer efficacy in many hematologic and solid tumors, such as breast cancer (Gutierrez-Puente et al., 2002; Buchele, 2003; Piro, 2004). Therefore, down-regulation of Bcl-2 may be a principal strategy for breast cancer therapy. Additionally, natural extracts have potential effects on Bcl-2 regulation. Petrotetrayndiol A, a polyacetylene from the sponge Petrosia sp., induced apoptosis in human melanoma cells was associated with a dose-dependent up-regulation of cytosolic factors, such as Bax, release of cytochrome c and caspases activities, and down-regulation of Bcl-2 (Choi et al., 2006). Our previous studies indicated that SM enhanced susceptibility of cDDP in human lung cancer cells was depended on up-regulation of TNFR, TRADD, FADD, Bax, cytochrome c and caspases, and down-regulation of Bcl-2 and Bcl-xL (Liang et al., 2004; Liu et al., 2004). Overexpression of Bcl-2 and Bcl-xL can enhance resistance against many chemotherapeutic drugs, including cDDP. SM down-regulated Bcl-2

Fig. 5. Changes of the expressions of Bax, Bcl-2, Bcl-xL and cytochrome c, and activities of caspase-8, -9 and -3 in the breast cancer cells after the treatments with cDDP (150 μM), SM (IC_{25}) and SM plus cDDP. (a) The proteins were determined by Bax, Bcl-2 and Bcl-xL specific antibodies and flow cytometry. The percentage indicated fluorescence intensity of the proteins detected by the specific antibodies. (b) The synergistic effects of SM and cDDP in cytochrome c release. Immunoblotting of cytochrome c release in the cells after SM, cDDP and SM plus cDDP treatments. β-actin was measured as the quantity control. (c) Changes of the caspases’ activities after SM, cDDP and SM plus cDDP treatments. The other descriptions were the same as in Fig. 3.
and Bcl-xL expressions, and enhanced the susceptibility of human lung cancer cells to cDDP (Liang et al., 2004). HBL-100 cells express low levels of Bcl-2 and Bcl-xL, while ZR-75-1 and SK-BR-3 cells express high levels of Bcl-2 and Bcl-xL (Figs. 3b and 5a). Thus, the variant effects of cancer cells to cDDP might be resulted from different levels of Bcl-2 and Bcl-xL expressions. SM can down-regulate Bcl-2 and Bcl-xL expressions. Combined treatment of SM and cDDP synergistically reduced Bcl-2 and Bcl-xL expressions, and enhanced Bax, cytochrome c and caspase-9 and -3 expressions (Fig. 5). Therefore, combination of SM with cDDP caused significant reduce cell survival of breast cancer cells, and the effects were dose dependent. Moreover, SM is a steroidial glycoalkaloid that diffuses rapidly into cells (Chang et al., 1998), and may cause cancer cell death more rapidly than other anticancer agents do. The maximum of SM-induced cell death was at 3 h, and no further cell death was detected after 16 h (Fig. 1b). The level of SM-induced cell death was dose-dependent rather than time-dependent. Thus, pre-treatment of cells with constant amount of SM for 2 or 4 h might not be significantly different from the co-treatment with SM and cDDP.

In conclusion, SM not only activated the apoptotic proteins, such as TNFR, Fas, TRADD, FADD, Bax, cytochrome c, caspase-8, -9 and -3, but also inhibited the anti-apoptotic Bcl-2 and Bcl-xL proteins in human breast cancer cells. Since the combination of SM and cDDP increased the susceptibility of breast cancer cells to cDDP, SM might be a candidate supplementary treatment agent with cDDP against human breast cancers.

References


