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# Anticancer Activity Evaluation of the *Solanum* Glycoalkaloid Solamargine

## TRIGGERING APOPTOSIS IN HUMAN HEPATOMA CELLS

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**ABSTRACT.** Solamargine, an herbal and molluscicidal medicine derived from *Solanum incanum,* is a steroidal alkaloid glycoside. To characterize the anticancer mechanism of solamargine on human hepatoma cells (Hep3B), changes of cell morphology, DNA content, and gene expression of cells after solamargine treatment were studied. The appearance in solamargine-treated cells of chromatin condensation, DNA fragmentation, and a sub-$G_1$ peak in a DNA histogram suggests that solamargine induces cell death by apoptosis. The maximum number of dead Hep3B cells was detected within 2 hr of incubation with constant concentrations of solamargine, and no further cell death was observed after an extended incubation with solamargine, indicating that the action of solamargine was irreversible. To determine the susceptibility of cell phases to solamargine-mediated apoptosis, Hep3B cells were synchronized at defined cell cycles by cyclosporin A, colchicine, and genistein, followed by solamargine treatment. The $IC_{50}$ values of solamargine for control, $G_0/G_1$-, M-, and $G_2$/M-synchronized Hep3B cells were 5.0, > 10, 3.7, and 3.1 μg/mL, implying that cells in the $G_2$/M phases are relatively susceptible to solamargine-mediated apoptosis. In addition, a parallel up-regulation of tumor necrosis factor receptor (TNFR)-I and -II on Hep3B cells was detected after solamargine treatment, and the solamargine-mediated cytotoxicity could be neutralized with either TNFR-I or -II specific antibody. Therefore, these results reveal that the actions of TNFR-I and -II on Hep3B cells may be independent, and both are involved in the mechanism of solamargine-mediated apoptosis. BIOCHEM PHARMACOL **60**;12:1865–1873, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** solamargine; hepatoma; apoptosis; cell cycle; TNF receptors; drug discovery

Apoptosis is considered to be the major process responsible for cell death in various physiological events. It acts as a regulating mechanism of tissue growth where it balances cell proliferation [1]. During apoptosis, the dead cells or their fragments are rapidly phagocytosed by neighboring cells or macrophages before there is any leakage of the contents of the cells, and thus they do not induce an inflammatory response [2]. Apoptosis became a focus of interest in oncology because the disregulation of the apoptotic process may prompt malignancy [3, 4]. In the past few years, there has been spectacular progress in understanding the intracellular mechanism of apoptosis and its control [5, 6].

Solamargine, a steroidal glycoalkaloid, has been isolated from the fruits of *Solanum incanum,* and its structure has been determined [7]. Like other steroidal molecules, solamargine can exert its action by penetrating cell membranes by simple diffusion. Originally, solamargine mixed with solasonine, isolated from the fruit of *S. mammosum* for agricultural purposes [8], as the extract was shown to display a strong molluscicidal property on *Lymnaea cubensis* snails [9, 10]. Consequent studies on the activities of solamargine demonstrated that it inhibits the growth of the larvae of red flour beetles, *Tribolium castaneum,* tobacco hornworms, *Trypanosoma cruzi,* and frog embryos [11–13], and more recently, it was shown to inhibit the growth of human tumor cells, e.g. colon (HT-29, HCT-15), prostate (LNCaP, PC-3), breast (T47D, MDA-MB-231), and human hepatoma (PLC/PRF/5) cells [14]. In addition, it is hepatoprotective against $CCl_4$-induced toxicity in mice [15]. However, a detailed study of the mechanism of solamargine on human cancer cells has not been conducted.

TNFRs‖ have been reported as important regulators in triggering apoptosis [16]. TNFR-I functions in almost every cell type, and can independently transmit most biological activities of TNF-α [17]. TNFR-II seems to be largely

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‖ *Abbreviations*: DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PI, propidium iodide; TNFR, tumor necrosis factor receptor; RT–PCR, reverse transcriptase–polymerase chain reaction; and TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

restricted to cells of lymphoid origin, where it induces cell proliferation and cytokine production [18]. In the present study, the expression of TNFR-I and -II on human hepatoma cells (Hep3B) in the presence and absence of solamargine was analyzed. Specific TNFR-I and -II antibodies were utilized to verify the role of TNFRs in the apoptosis of Hep3B cells. Morphological changes, susceptibility of the phases of the cell to apoptosis, and the DNA content of Hep3B cells after solamargine treatment were studied. Our results demonstrate the anticancer activity of solamargine against human hepatoma cells and the relative mechanisms of action of solamargine.

## MATERIALS AND METHODS

### *Cell Line and Culture*

Hep3B cells were cultured at 37° in a humidified atmosphere of carbon dioxide:air (5:95). The culture medium consisted of DMEM (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazelton Products), 100 μg/mL of streptomycin, and 100 U/mL of penicillin. The cells ($1 \times 10^5$ cells/mL) were seeded in each 100 μL of 96-well multi-dishes with DMEM–10% fetal bovine serum for at least 24 hr prior to use.

### *Cell Viability Assay*

Hep3B cells were treated with a constant concentration of solamargine (5 μg/mL) at 37° for 16 hr. A colorimetric tetrazolium MTS method was used for the determination of cell viability according to the manufacturer's procedures (CellTiter 96$^{TM}$ AQ, Promega).

### *TUNEL Assay*

The cells after solamargine (5 μg/mL) treatment were fixed using 4% para-formaldehyde in PBS (pH 7.4) for 30 min, and incubated continuously in permeability solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. The intensities of fluorescence in solamargine-treated and untreated cells were analyzed by the TUNEL fluorescent dye method, in accordance with the manufacturer's protocol (Boehringer Mannheim). The stained cells were viewed and photographed with a fluorescent microscope (Olympus BX-50 with a 3CCD color camera).

### *Flow Cytometry*

Hep3B cells were incubated with solamargine (5 μg/mL). In serial time intervals, incubations were terminated by rinsing the cells with PBS. The cells were fixed with 4% para-formaldehyde in PBS (pH 7.4) at room temperature for 30 min. After centrifugation at 200 *g* for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4° for 2 min. PI in PBS (10 μg/mL) was added to stain the cells at 37° 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 LYSIS II software.

### *Determination of the Expression of TNFR-I and -II*

Cellular RNAs were isolated from solamargine (5 μg/mL)-treated and untreated Hep3B cells by the guanidinium thiocyanate/cesium chloride gradient ultracentrifugation method. All RNA preparations were treated with DNase, and stored in liquid nitrogen prior to use. The gene expressions of TNFR-I and -II were carried out by a single-tube RT-PCR followed by the Southern hybridization technique. The primer sequences used for detecting TNFR-I and -II are 5′-AGTGCTGTTGCCCCTGGTCATTTTCTT-3′, 5′-ATTGTTTGTGGGAAATCGACACCTGAAA-3′ [19] and 5′-TGGAAACTCAAGCCTGCACTCGGGAA-3′, 5′-GGGCTTGCACACCACGTCTATGTTT-3′, respectively. The changes of surface receptors of TNFR-I and -II on Hep3B cells after incubation with solamargine (5 μg/mL) for 16 hr were determined by fluorescent immunohistochemistry and $^{125}$I-labeled TNFR-I and -II antibodies according to standard procedures [20]. $^{125}$I-Labeled antibodies were prepared using the chloramine-T method [21].

### *Neutralization of the Cytotoxicity of Solamargine by TNFR-Specific Antibodies*

Neutralization capacity ($ND_{50}$) is defined as the concentration of antibody required to yield one-half maximal inhibition of the receptor activity on a responsive cell line. The $ND_{50}$ values of TNFR-I and -II antibodies were approximately 3–6 and 0.5–1.5 μg/mL in the presence of 300 ng/mL of recombinant human TNFR-I and -II, respectively (R&D Systems). Constant amounts of TNFR-I and -II antibodies (5 and 10 μg/mL) were preincubated individually with Hep3B cells for 3 hr. Then serial concentrations of solamargine were added to the cells, and the cells were incubated continuously for 16 hr. A colorimetric tetrazolium MTS method was used to determine cell viability in accordance with the description above.

### *Synchronization of Hep3B Cells at Defined Cell Cycles*

Hep3B cells in serum-free medium were synchronized at $G_0/G_1$, M, and $G_2$/M phases by cyclosporin A (10 μg/mL), colchicine (8 μg/mL), and genistein (20 μg/mL) (Calbiochem) for 24 hr. Serial concentrations of solamargine were added to cells, and the cells were incubated continuously for 5 hr. The MTS method was used to measure cell viability in accordance with the description above.

## RESULTS

### *Cell Death Induced by Solamargine*

Hep3B cells were incubated with various concentrations of solamargine (1–20 μg/mL) for 16 hr. The cytotoxicity of

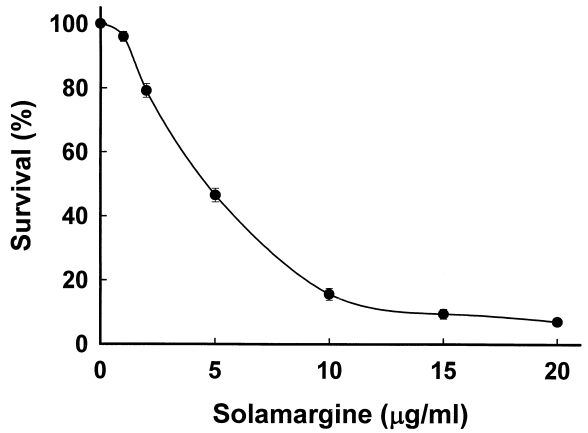


**FIG. 1. Inhibition of the proliferation of Hep3B cells by solamargine. Hep3B cells ($1 \times 10^4$) were treated with various concentrations of solamargine for 16 hr. An MTS assay was performed according to the procedure described in Materials and Methods. Data represent means ± SD of the proliferation (O.D. 490 nm) of solamargine-treated and untreated Hep3B cells from quaternary determinations.**

solamargine against Hep3B cells was determined by the MTS method. As shown in Fig. 1, solamargine inhibited cell growth in a concentration-dependent manner. The concentration of solamargine causing 50% cell death ($IC_{50}$) was approximately 5.0 μg/mL. A time-course of cell death after solamargine treatment was monitored (Fig. 2). The maximum number of dead Hep3B cells was detected within 2 hr of incubation with constant concentrations of solamargine (2, 5, and 10 μg/mL), and no further cell death was observed after an extented incubation with solamargine. Since the steroid molecule exerts its effect by binding to intracellular receptors, these results implied that the binding of solamargine to the receptor was irreversible.

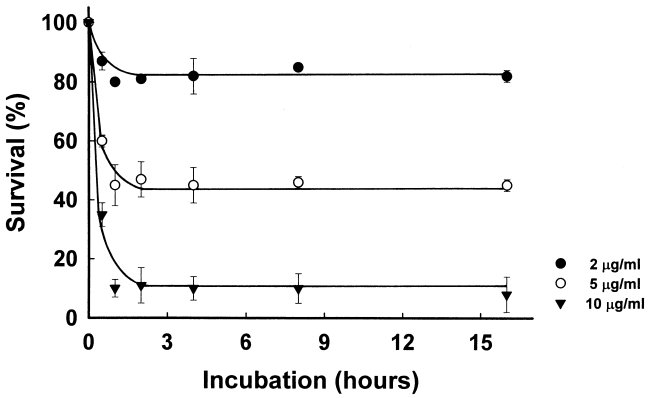


**FIG. 2. Time-courses of cell death after treatment with constant amounts of solamargine. Hep3B cells ($1 \times 10^4$) were incubated with solamargine (2, 5, and 10 μg/mL) for serial time intervals. Cell viability was determined by the MTS assay. Data represent means ± SD of the percentage of proliferation of the solamargine-treated/untreated cells from quaternary determinations.**

### *Morphological Changes of Hep3B after Solamargine Treatment*

The changes of cell morphology after solamargine (5 μg/mL) treatment were analyzed. In contrast to cell necrosis, characterized by an early loss of membrane function and structural integrity, the membranes of solamargine-treated Hep3B cells retained their integrity. Chromatin condensation in the nucleus of Hep3B cells was increased with the incubation time of solamargine (Fig. 3). The percentages of chromatin condensation calculated from 1000 individual cells treated for 0, 3, 7, and 12 hr with solamargine were approximately 1, 8, 29, and 40%, respectively. DNA fragmentation is one of the hallmarks of cell apoptosis. In this experiment, terminal deoxynucleotidyl transferase was applied to label fluorescein-dUTP to the 3′-end of DNA fragments in individual cells after apoptosis. The fluorescence in Hep3B cells intensified significantly after solamargine treatment (Fig. 4), suggesting that solamargine induced cell death by apoptosis.

### *Analysis of DNA content of Hep3B Cells by Flow Cytometry*

Cell death by apoptosis displays a sub-$G_1$ peak as seen when analyzing a DNA histogram by flow cytometry. A constant amount of solamargine (5 μg/mL) was incubated with Hep3B cells for serial time intervals. The cells were stained with PI (10 μg/mL) for 30 min. As shown in Fig. 5, the sub-$G_1$ peak started to increase drastically after 5 hr of incubation with solamargine. To eliminate the cell clusters that might influence the DNA histogram, the cells were sorted according to size by means of a singlet population plot (FL2-Width vs FL2-Area). As shown in Fig. 6, the cell population of the $G_2$/M phase was reduced concurrently with the increase of the sub-$G_1$ population after 5 hr of incubation with solamargine; no significant change of cell population in the $G_0/G_1$ phase was observed. This implies that solamargine might predominantly stimulate the cells of the $G_2$/M phases to apoptosis.

### *Susceptibility of Hep3B Cells at Defined Cell Cycles to Apoptosis*

To verify the susceptibility of Hep3B cells at defined cell cycles to solamargine-mediated apoptosis, the cells ($1 \times 10^6$) were synchronized in the presence of serial concentrations of cyclosporin A, colchicine, and genistein for 24 hr. The cytotoxicity of synchronization agents on Hep3B cells was determined by the MTS assay. As shown in Fig. 7A, the concentrations of cyclosporin A (10 μg/mL), cochicine (8 μg/mL), and genistein (20 μg/mL) did not reduce the viability of Hep3B cells significantly. The cell phases of synchronized Hep3B cells were analyzed by flow cytometry (Fig. 7B). Next the cells were synchronized by the concentration of agents to define cell cycles, followed by incubation with increasing amounts of solamargine. As shown in

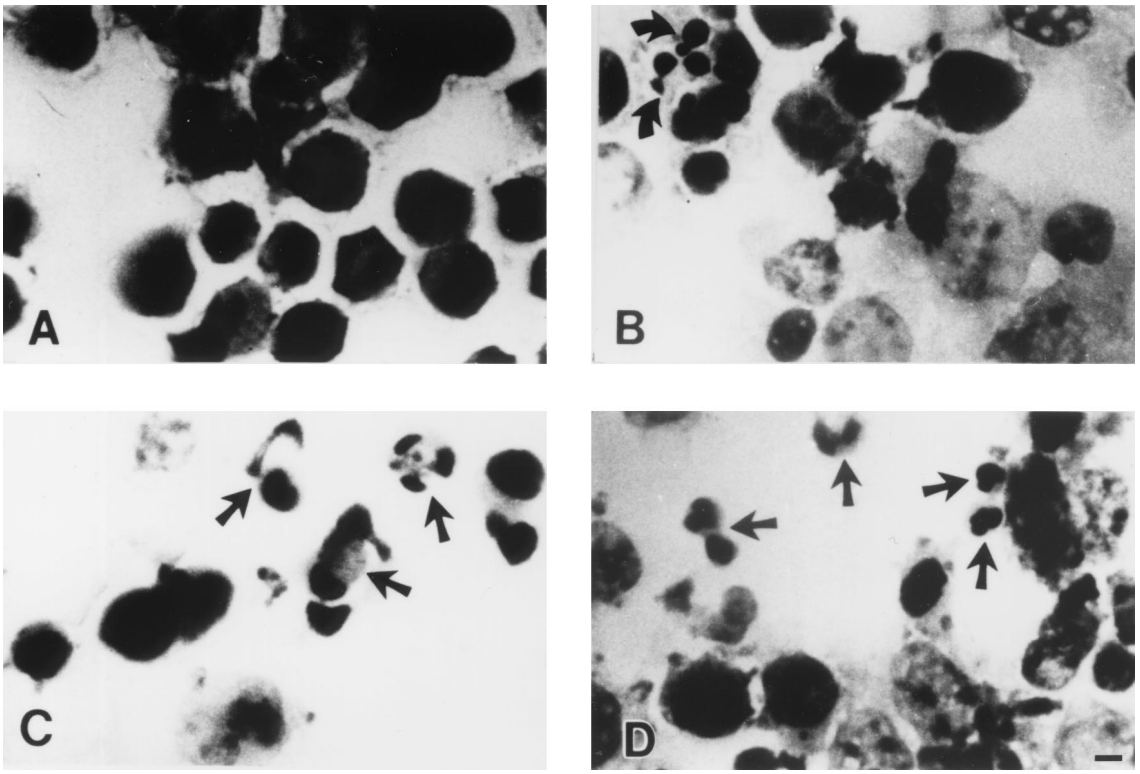


**FIG. 3. Morphological change of the nuclear chromatin of Hep3B cells after solamargine treatment. Hep3B cells were treated with solamargine (5 μg/mL) for (A) 0 hr; (B) 3 hr; (C) 7 hr; and (D) 12 hr. The cells were stained with haematoxylin and were inspected by light-microscopy (Olympus CX40, × $_{500}$). The apoptotic cells revealed cell shrinkage and chromatin condensation, as indicated by arrows. The scale bar indicates 5 μm.**

Fig. 8, the cytotoxicity to Hep3B cells was decreased in cyclosporin A-synchronized cells, while the cytotoxicity to colchicine- and genistein-synchronized cells was increased. The $IC_{50}$ values of solamargine for control, cyclosporin A ($G_0/G_1$)-, colchicine (M)-, and genistein ($G_2$/M)-synchronized cells were 5.0, > 10, 3.7, and 3.1 μg/mL, respectively. These results indicate that cells in the $G_2$/M phases are relatively susceptible to solamargine-mediated apoptosis.

### *Influence of Solamargine on the Expression of TNFR-I and -II in Hep3B Cells*

TNF receptors have been involved in the process of apoptosis. To clarify the apoptotic mechanism of Hep3B cells, specific gene expressions of TNFR-I and -II in the presence and absence of solamargine (5 μg/mL) were determined by single-tube RT-PCR. As shown in Fig. 9A, TNFR-I and -II genes were up-regulated within 30 min of solamargine treatment, and the genes were gradually degraded after 60 min. In addition, the surface receptors of TNFR-I and -II on Hep3B cells were assayed by fluorescent immunohistochemistry and radioimmunoassay. As shown in panels B and C of Fig. 9, the binding of unlabeled and $^{125}$I-labeled TNFR-I and -II antibodies to the receptors on Hep3B cells was increased after solamargine treatment. These results revealed that solamargine could elevate the gene and protein expressions of TNFR-I and -II of Hep3B cells.

### *Blockage of the Cytotoxicity of Solamargine by TNFR-I and -II Antibodies*

TNFR-I and -II neutralization antibodies especially recognize extracellular domains of type I and II receptors, and no cross-reaction occurs for either type of antibody (R&D Systems). To verify the role of TNFRs in solamargine-mediated cell death, the antibodies (5 and 10 μg/mL) were incubated with Hep3B cells for 3 hr prior to the addition of solamargine. Serial concentrations of solamargine were added to the cells, and the cells were incubated for 16 hr continuously. As shown in Fig. 10, the cytotoxicity of solamargine could be neutralized with either TNFR-I or -II specific antibody. However, combinations of TNFR-I and -II antibodies for the treatment of Hep3B cells had no synergistic effect on the neutralization. These results imply that the actions of TNFR-I and -II on Hep3B cells may be independent, and both are involved in the mechanism of solamargine-mediated apoptosis.

## DISCUSSION

Programmed cell death (apoptosis), the deletion of certain cells in tissues without concomitant inflammation, is advantageous in tissue homeostasis. Several antitumor agents have been described as inducing apoptosis [22–24]. The morphological features, e.g. apoptotic bodies, chromatin condensation, DNA fragmentation, and sub-$G_1$ peak in the

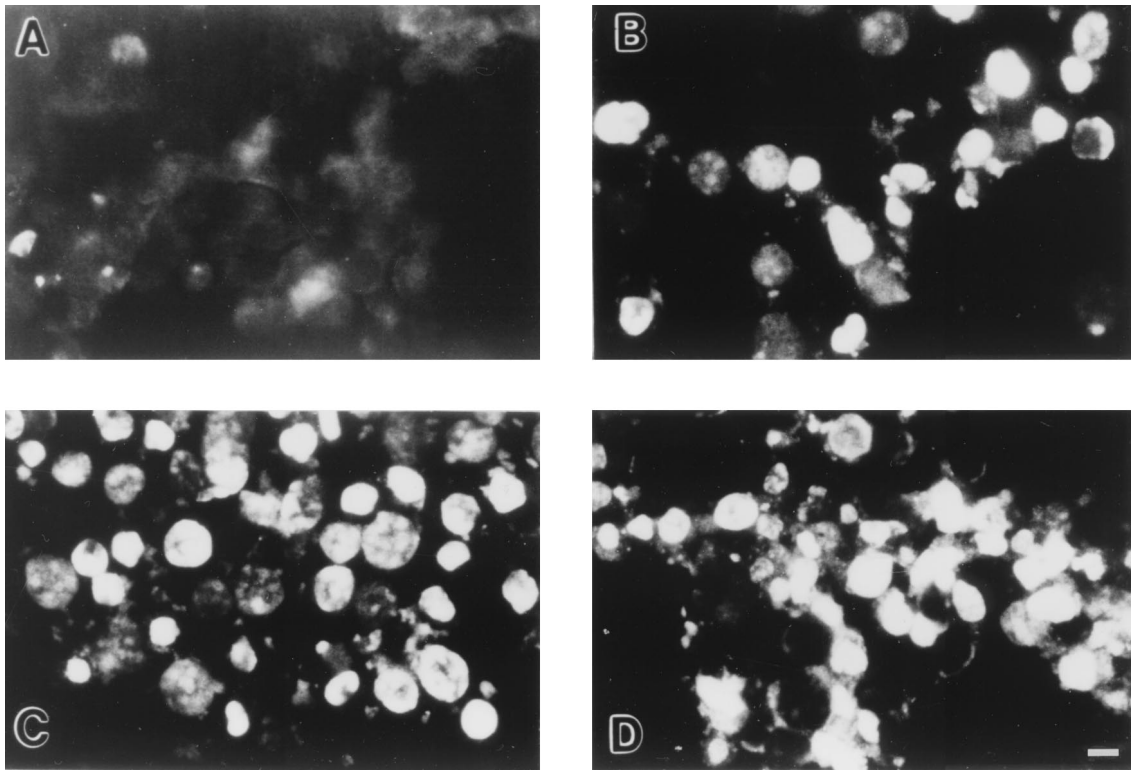


**FIG. 4. Determination of DNA fragmentation of Hep3B cells by the TUNEL assay. Hep3B cells were incubated with solamargine (5 μg/mL) for (A) 0 hr; (B) 3 hr; (C) 7 hr; and (D) 12 hr. The TUNEL assay was performed according to the manufacturer's protocol, and the intensity of fluorescence in the cells was determined by fluorescent microscopy. The scale bar indicates 5 μm.**

DNA histogram are generally employed as hallmarks of cell death by apoptosis [25–27]. The action of solamargine satisfied the criteria of apoptosis in Hep3B cells, indicating that solamargine induced cell death by apoptosis. Cells that undergo apoptosis in tissue usually degrade rapidly. As it is still not possible to measure all the features in most apoptotic cells *in vivo*, quantitating apoptosis remains an unsolved problem [28]. In the present paper, the time-courses of exhibition of apoptotic signals in Hep3B cells after solamargine treatment were monitored. Solamargine

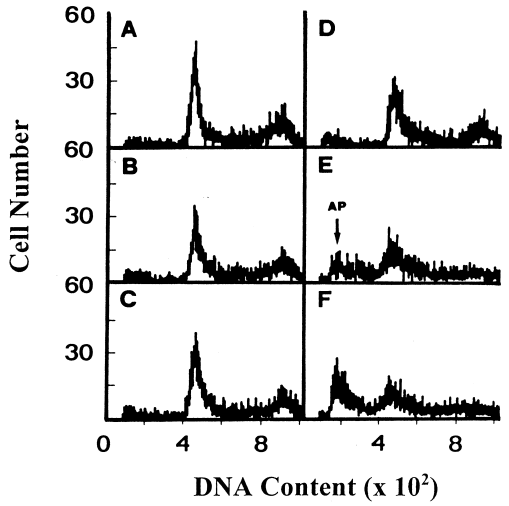


**FIG. 5. Flow cytometry of a DNA histogram of PI-stained Hep3B cells. The cells were incubated with solamargine (5 μg/mL) for (A) control; (B) 30 min; (C) 1 hr; (D) 3 hr; (E) 5 hr; and (F) 7 hr. After incubation, the cells were fixed and treated with PI and RNase before recording red fluorescence excited by a blue laser light. A sub-$G_1$ peak, labeled "Ap," appeared with the time of incubation.**

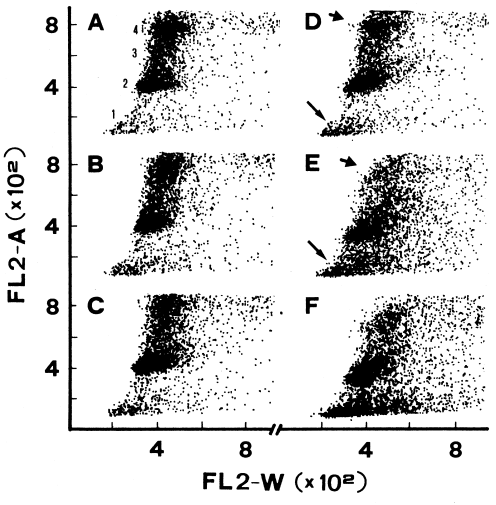


**FIG. 6. Flow cytometric cytograms of Hep3B cells after solamargine treatment. The cells were incubated with solamargine (5 μg/mL) for (A) control; (B) 30 min; (C) 1 hr; (D) 3 hr; (E) 5 hr; and (F) 7 hr. The cell populations 1, 2, 3, and 4 denote the cell cycle at sub-$G_1$, $G_0/G_1$, S, and $G_2$/M phase, respectively. Note that the cells in $G_2$/M and sub-$G_1$ phases at "D" and "E" were changed concurrently.**

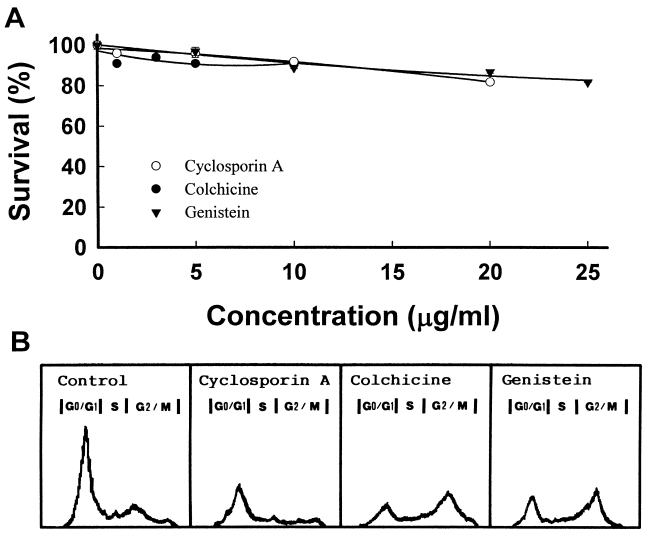


**FIG. 7. Effects of cell-phase-synchronization agents on Hep3B cells. Serially increased amounts of cyclosporin A, cochicine, and genistein were incubated individually with Hep3B cells to synchronize cell phase for 24 hr. (A) The cytotoxicity of the synchronization agents to Hep3B cells ($1 \times 10^4$) was determined by MTS assay. Data represent means ± SD of the proliferation (O.D. 490 nm) of solamargine-treated cells from quaternary determinations. (B) The DNA histogram of Hep3B cells after control, cyclosporin A (10 μg/mL), colchicine (8 μg/mL), and genistein (20 μg/mL) treatment was determined by flow cytometry. The cells were stained with PI followed by flow cytometric analysis.**

is a steroidal glycoalkaloid that diffuses rapidly into cells. The gene expressions of TNFR-I and -II could be up-regulated in 30 min of incubation with solamargine, and the genes were degraded gradually after 60 min. The result derived from the MTS assay indicated that Hep3B cells were dead within 2 hr after initiation of apoptosis by

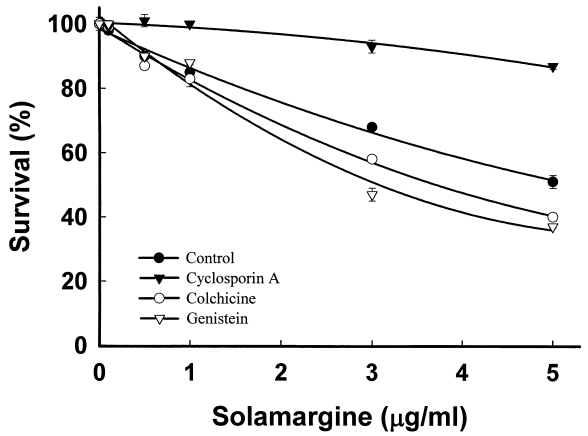


**FIG. 8. Susceptibility of phase-synchronized Hep3B cells to solamargine-mediated apoptosis. Hep3B cells were synchronized at defined cell phases by cyclosporin A (10 μg/mL), colchicine (8 μg/mL), and genistein (20 μg/mL) for 24 hr. Serial amounts of solamargine were then incubated with the cells ($1 \times 10^4$) for 5 hr. The solamargine-mediated cytotoxicity was determined by MTS assay. Each point represents the mean ± SD calculated from quaternary determinations.**

solamargine. In fact, the current apoptotic hallmarks are the final processes of cell death, because all these features appeared after 2 hr. Depending on the detection methods, the onsets of chromatin condensation, DNA fragmentation, and sub-$G_1$ peak in DNA content after cell death are difficult to distinguish precisely by conventional methods.

During the apoptotic process, the cell is activated by a cascade of events that ultimately lead to nuclear fragmentation [29, 30]. A sensitive method that directly transfers fluorescein-dUTP to the 3′-end of DNA segments was utilized to monitor DNA degradation. It demonstrated that DNA fragmentation of Hep3B cells could be initiated as early as 3 hr after irritation, and had become significant after 7 hr of incubation with solamargine. In principle, apoptosis cannot be determined merely by DNA fragmentation, because it also happens in cell necrosis, in which DNA is digested randomly by multiple endonucleases [31]. Although the DNA ladder in gel electrophoresis may be one example of the most common cell morphologies of apoptosis [32], in some cases morphological evidence of apoptosis occurs with the production of large DNA fragments only [33, 34] or in the absence of a detectable DNA ladder pattern [35, 36]. In the present paper, the DNA ladder was not detectable in solamargine-mediated apoptosis of Hep3B cells. Thus, to identify the manner of cell death, methods other than DNA ladder and TUNEL are essential to detect apoptosis. On the other hand, two common ways to determine cell proliferation are the MTS assay and [$^3$H]thymidine incorporation. MTS assay determines the capacity of mitochondrial dehydrogenase in viable cells to transform tetrazolium salt into colorimetric formazan [37]. The [$^3$H]thymidine method determines cell proliferation, which will incorporate [$^3$H]thymidine into newly synthesized DNA in cells. Theoretically, cells in either inhibition or death situations may not be distinguishable by means of [$^3$H]thymidine incorporation. Thus, to evaluate the anticancer effect, the cytotoxicity of solamargine was determined by MTS.

The changes in the cell cycle of Hep3B after solamargine (5 μg/mL) treatment were determined by flow cytometry. The cell population of the $G_2$/M phase was shifted drastically to the sub-$G_1$ population after 5 hr of incubation with solamargine, and no significant change of cell population in the $G_0/G_1$ phase was observed (Fig. 6). This indicates that solamargine might arrest Hep3B cells at the $G_2$/M phase, and primarily stimulate the cells from the $G_2$/M phase to the sub-$G_1$ phase of apoptosis. It has been reported that $G_2$ arrest occurs in response to many types of DNA damage. Manipulation of regulatory events at this checkpoint is one promising approach for improving the efficiency of cytotoxic drugs and overcoming drug resistance [38]. Several cell cycle synchronization agents were utilized to verify the susceptibility of Hep3B cells to solamargine-mediated apoptosis. Cyclosporin A is an immunosuppressant that has been reported to arrest *Saccharomyces cerevisiae* at the $G_0/G_1$ phase [39]. Colchicine interferes with the process of spindle separation during the M phase. Genistein, a tyrosine kinase

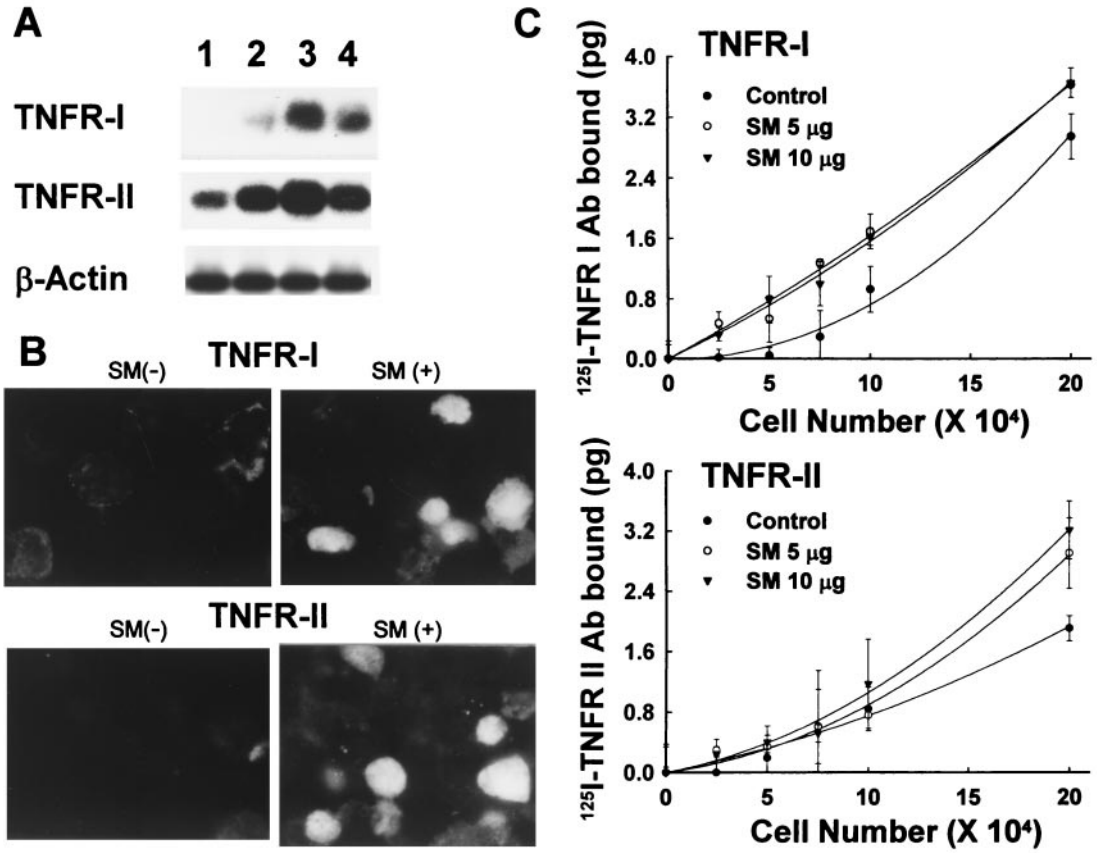


**FIG. 9. Expression of TNFR-I and -II on Hep3B cells after solamargine treatment. The expressions of TNFR-I and -II on cells were determined by RT-PCR (A), fluorescent immunohistochemistry (B), and radioimmunoassay (C). A constant amount of solamargine (5 μg/mL) was added to the cells. At various time intervals, the cellular RNAs of Hep3B cells were isolated and subjected to RT-PCR with TNFR-I and -II specific primers. The quantity of cDNA expression was determined by Southern hybridization using specific cDNA probes for human TNFR-I and -II. The RNA used for reverse transcription was controlled by the RT-PCR of human β-actin in the same conditions. Radioimmunoassay and fluorescent immunohistochemistry were carried out in accordance with the descriptions in Materials and Methods. Data represent means ± SD calculated from quaternary determinations. The cells were inspected by fluorescent microscopy (Olympus BX-50 with a 3CCD color camera). SM: solamargine.**

inhibitor, produces derangement in cell cycle progression, which culminates in $G_2$/M cell cycle arrest [40]. Figure 8 demonstrates that cells in $G_2$/M phases are relatively susceptible to solamargine-induced apoptosis. These experiments may provide important information for the study of correlation between cell phase and apoptosis in drug development.

Steroid molecules bind to intracellular receptor proteins by direct diffusion across the plasma membrane of target cells [41]. This binding activates the receptors, which then regulate the transcription of specific genes. It is rational that the irreversible action of solamargine in Hep3B cells may have resulted from an irreversible binding of solamargine receptor(s) to nuclear DNA. In addition, two distinct TNF receptors with molecular weights of 55 kDa (TNFR-I) and 75 kDa (TNFR-II) have been identified in all nucleated cells [16]. TNF-α exerts its cytotoxicity, antiviral activity, transcription factor activation, and immune response regulation by interacting with TNFR-I or its companion TNFR-II [18]. The extracellular domains of TNFR-I and -II are homologous, but the intracellular domains are different, and only TNFR-I contains a death domain (DD) [42]. TNFR-II does not contain a DD and is generally considered not to be involved in apoptosis, but overexpression of TNFR-II is able to mediate apoptosis [43, 44]. The mechanism of gene regulation of TNFR-I and -II has not been clarified. The expression of TNF receptors was upregulated by solamargine, and the solamargine-mediated cytotoxicity could be blocked by TNFR-I and -II antibodies. These results indicated that TNFR-I and -II were involved in the solamargine-mediated apoptosis. In general, biological action and signal cascade in human cells are usually complex. TNFR-I and -II antibodies could neutralize the cytotoxicity of solamargine; however, complete neutralization by the antibodies occurred only in the presence of a low concentration of solamargine. In fact, application of 5 or 10 μg/mL of TNFR-I and -II antibodies to each 96-well dish of cells ($1 \times 10^4$) should be sufficient, because an increase in antibodies to the cells did not elevate the effect of neutralization significantly (Fig. 10). These results imply that although TNFR-I and -II are involved in solamargine-mediated apoptosis, they may not

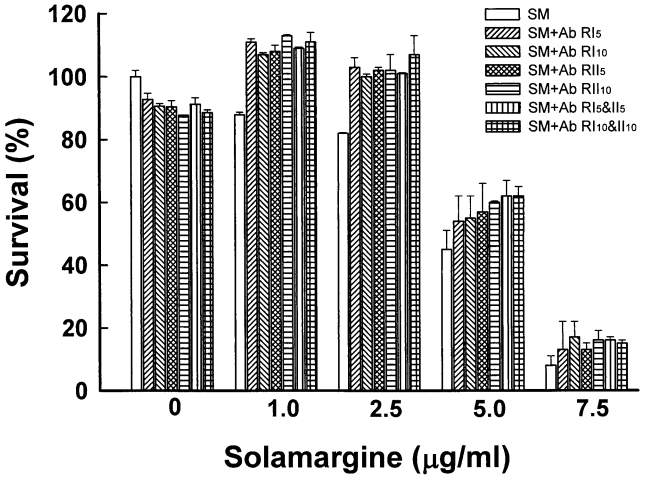


**FIG. 10. Neutralization of the effect of solamargine on Hep3B cells by TNFR-I and -II specific antibodies. The cells ($1 \times 10^4$) were incubated with constant amounts of TNFR-I and -II specific antibodies (5 and 10 μg/mL) for 3 hr, followed by incubation with serial concentrations of solamargine for 16 hr. The solamargine-mediated cytotoxicity was determined by MTS assay. Values represent means ± SD calculated from triplicate determinations. SM: solamargine.**

account for the total anticancer mechanism of solamargine. In conclusion, this paper characterizes the anticancer activity of solamargine in human hepatoma cells, and may forward the study of solamargine and other related steroidal glycoalkaloids as new agents for cancer therapy.

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