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The Rhamnose Moiety of Solamargine Plays a Crucial Role in Triggering Cell Death by Apoptosis

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Solamargine, solasodine and khasianine steroidal alkaloids are utilized to determine the role of carbohydrate moiety in the mechanism of apoptosis. The C_3 side chain of solamargine, khasianine and solasodine contains 4′Rha-Glc-Rha2′, 4′Rha-Glc and H, respectively. Solamargine possessed potent cytotoxicity to human hepatoma cells, while the cytotoxicity of khasianine was greatly diminished. Nevertheless, only solamargine could induce "sub-G1" of apoptotic feature in flowcytometry. Thus, the 2′Rha moiety of solamargine may play a crucial role in triggering cell death by apoptosis. In addition, the molecular modeling of solamargine indicated that the 2′Rha moiety was adjacent to the rigid steroid structure, and drastically changed the dihedral angle of the glycosidic bond. The regulations of TNFR I and II expression by different carbohydrate moieties were also distinct. It implied that the carbohydrate moieties of steroidal alkaloids might alter the binding specificity to steroid receptors and consequently regulate the gene expression in different manners. © 1998 Academic Press

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]. Recently, it has become a focus of interest in oncology because a disregulation of the apoptotic process can prompt malignancy of tumors [2, 3]. Solamargine, a new steroidal alkaloid glycoside, was isolated from the fruits of *Solanum incanum* L. The structure of solamargine has been determined [4]. Like other steroidal molecules, solamargine can exert its action by penetrating the cell membrane by simple diffusion. Once inside the cell, solamargine binds to steroid receptor which increases the molecule's affinity to particular DNA region and alters the gene expression [5]. Previous report showed that solamargine triggered gene expression of human tumor necrosis factor receptor (TNFR) I which might lead to cell apoptosis [6].

Solamargine (-L-Rhamnopyranosyl-(14)- -L-Rhamnopyranosyl-(12)--D-glucopyranosyl solasodine), khasianine (-L-Rhamnopyranosyl-(14)--D-glucopyranose solasodine) and solasodine steroidal alkaloids could be purified from *Sonanum* plants (7, 8). The difference among these alkaloids is carbohydrate moiety in the C_3 side chain of steroid structure. In order to investigate the role of carbohydrate moiety of solamargine in the mechanism of apoptosis, solamargine, solasodine and khasianine were utilized in the experiments. Since apoptosis is a deliberate and gene-controlled cellular response, the present paper demonstrates that the -L-Rhamnopyranosyl-(12) moiety of solamargine plays a crucial role in triggering cell death by apoptosis. The influence of the rhamnose moiety in the conformation of solamargine was illustrated by molecular modeling. The regulations of TNFR I and II by the carbohydrate moieties of alkaloids were also distinct. This is the first paper demonstrated that the carbohydrate moiety of steroidal alkaloid may mainly affect the binding specificity to steroid receptors, and thus regulates the gene expression in different characteristics.

MATERIALS AND METHODS

Cell lines and cell culture. Human hepatoma cell (Hep3B) was cultured in Dulbecco modified Eagle's medium (DMEM/F12) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 mg/ml streptomycin and 100 unit/ml penicillin at 37°C under humidified 5% CO_2 atmosphere.

Cytotoxicity assay. Hep3B cells (1x 10^4/ml) were seeded in each 1-ml well of 24-well multi-dishes with DMEM-10% fetal bovine serum for at least 24 h prior to use. Serial concentrations of solasodine, solamargine and khasianine were added into the cells at 37°C for 16h. [^{3}H]thymidine (2 μCi) was then added to each well and continuous incubation at 37°C for 4h. The incorporation was terminated by diluting with 1 ml of phosphate-buffered saline (PBS) containing cold thymidine (100 μg/ml). DNA was precipitated with cold 10% trichloroacetic acid (TCA) and washed once with 5% TCA, then 95%

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ethanol. [^{3}H]DNA was dissolved in 0.5 ml of 0.2 M sodium hydroxide solution and transferred to vials containing 3 ml of Ecolume scintillation fluid (ICN Biomedicals). [^{3}H]DNA was measured with a Packchard Model 2100TR scintillation counter.

Flow cytometry. Appropriate concentrations of solamargine, solasodine and khasianine were added to the cells (110^7 cells/ml). In various time intervals, the reactions were terminated by washing with PBS. The cells were fixed with 4% para-formaldehyde/PBS (pH 7.4) at room temperature for 30 min. After centrifugation at 1,000 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4°C for 2 min. Propidium iodide in PBS (10 mg/ml) was added to stain the cells at 37°C for 30 min. The intensity of red fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A minimum of 10,000 cells was collected for analysis by LYSIS II software.

RNA preparation. The RNA preparations were isolated from Hep3B cells treated with solamargine (5 g/ml), solasodine (5 g/ml) and khasianine (20 g/ml) for 30 minutes. The RNA was extracted by the RNA/DNA isolation kit (Maxim Biotech, USA) according to the manufacture's protocol.

RT-PCR. The cDNA was synthesized from 0.2-1g of total RNA with oligo $(dT)_{18}$ by Advantage RT-for-PCR Kit (Clontech, USA). Reverse transcription was carried out in a final volume of 20l reaction buffer containing 10 mM dNTP, recombinant RNase inhibitor and MMLV reverse transcriptase. The mixture was incubated at 42°C for 1 hr, then heated at 94°C for 5 min. The primer sequences used for detecting TNFR-I and II are 5′-AGTGCTGTTGCCCCTGGTCAT-TTTCTT-3′, 5′-ATTGTTTGT GGGAAATCGACACCTGAAA-3′ [9] and 5′TGGAAACTCAAGCCTGCACTCGG GAA-3′, 5′-GGGCTT-GCACACCACGTCTATGTTT-3′, respectively. The PCR was carried out with *Taq* DNA polymerase (Promega, USA) according to the standard procedure.

Southern hybridization. The cDNA products were electrophoresed on a 2% agarose gel and transferred onto nylon membrane (Bio-Rad, Richmond, USA). After prehybridization, the blot was hybridized with human TNFR I and II probes labeled with [^{32}P]-dCTP using randomly primed labeling procedure (Promega, Madison, USA).

Computer modeling. The molecular modeling was carried out using the Biosym/ MSI software (version 95.0) [10]. The energy of each structure was minimized with the CVFF (Consistent Valence Force Field). The conjugate gradient algorithm was used for the minimization until the energy of maximum derivative was less than 0.001 kcal/mol.

RESULTS AND DISCUSSION

Cytotoxicity and Apoptosis

The diagrams of solamargine, solasodine and khasianine steroidal alkaloids were shown in Fig. 1. The difference between solamargine and khasianine structures was the -L-Rhamnopyranosyl-(12) moiety in C_3 side chain of solamargine. Previous report showed that solamargine triggered gene expression of human TNFR I which might lead to cell apoptosis [6]. To explore the role of carbohydrate moiety in the mechanism of apoptosis, the cytotoxicities of these alkaloids were determined by [^{3}H]thymidine incorporation. As shown in Fig. 2, solamargine and solasodine exhibited potent cytotoxicity to human hepatoma cells, while the cytotoxicity of khasianine was greatly diminished (Fig. 2). The IC_{50} (dose that inhibits cell growth by 50 %) of solamargine, solasodine and khasianine were 3.0, 2.7 and larger than 20 g/ml, respectively. It suggests that the

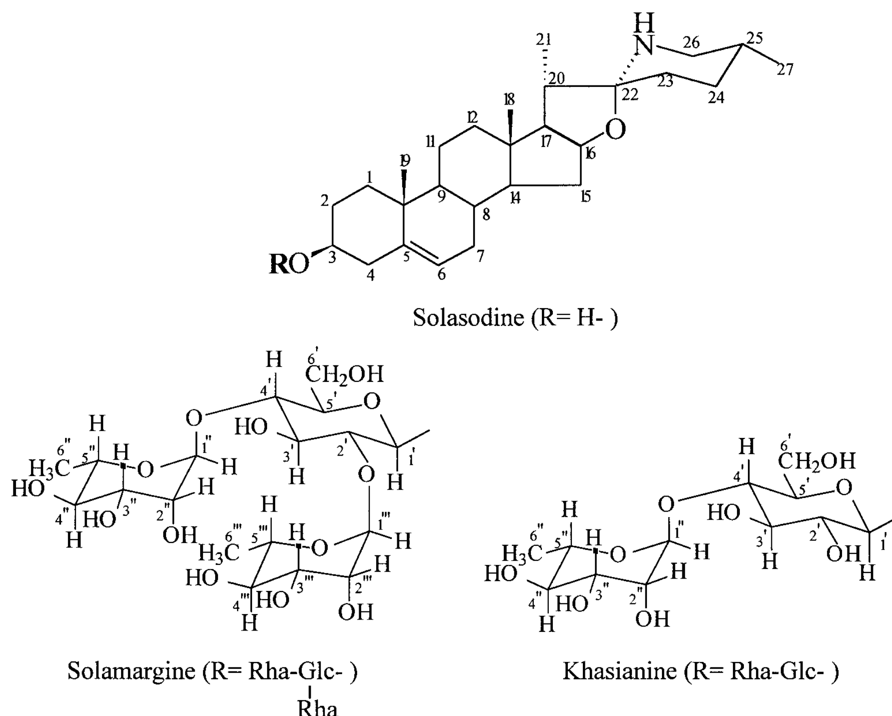

FIG. 1. Diagrams of the carbohydrate moieties of solamargine, solasodine and khasianine steroidal alkaloids.

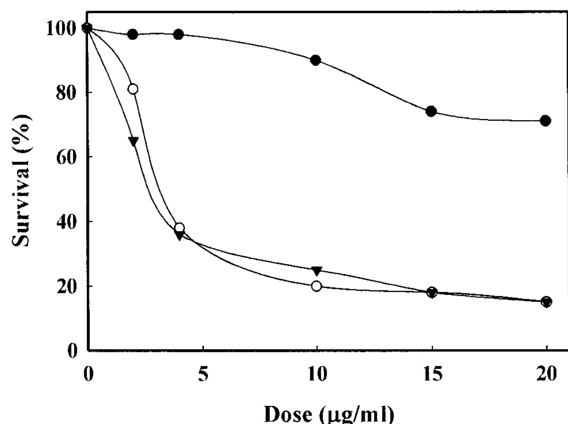

FIG. 2. Cytotoxicities of solamargine, solasodine and khasianine for Hep3B cells. Data represent means of percentage-survival rate of treated and untreated cells from triplicate determinations. ○, solamargine; ▼, solasodine and ●, khasianine.

carbohydrate moiety significantly affects the biological function of the steriodal glycoalkaloids. Comparing the carbohydrate moiety and cytotoxicity of solamargine and khasianine, the rhamnose (12) moiety was essential for the cytotoxicity of solamargine.

In addition, the morphological features of apoptotic bodies, chromatin condensation and DNA fragmentation are generally employed to characterize cell death by apoptosis [11–13]. Flow cytometry is the most sensitive method to distinguish apoptotic and necrotic cell death [14, 15]. A "sub-G1" peak in DNA histogram determined by flowcytometry is a hallmark of cell death undergoing apoptosis. In spite of the structural homology of solamargine, solasodine and khasianine, only solamargine could induce sub-G1 of apoptotic feature in the DNA histogram (Fig. 3). These results implied that the carbohydrate moiety might affect the action mechanism of steroidal alkaloid, and the -L-Rhamnopyranosyl-(12) moiety of solamargine plays a crucial role in triggering cell death by apoptosis.

Molecular Modeling of the Steroidal Alkaloids

To clarify the mechanism of rhamnose on solamargine, the absolute configurations of solamargine and khasianine were obtained using the molecular modeling program INSIGHT II system. Geometry optimization was performed by the DISCOVER program with the minimum energy of CVFF. As shown in Fig. 4, the rigid steroid structures of solamargine and khasianine were superimposed. It revealed that the -L-Rhamnopyranosyl-(12) moiety of solamargine was adjacent to the steroid structure, and consequently enforced a remarkable rotation of the glycosidic bond. The dihedral angles of 88.64($C_2C_3OC_{1'}$) and -148.29($C_4C_3OC_{1'}$) of khasianine were rotated to 138.19($C_2C_3OC_{1'}$) and -95.85($C_4C_3OC_{1'}$) of solamargine. Since solamargine and

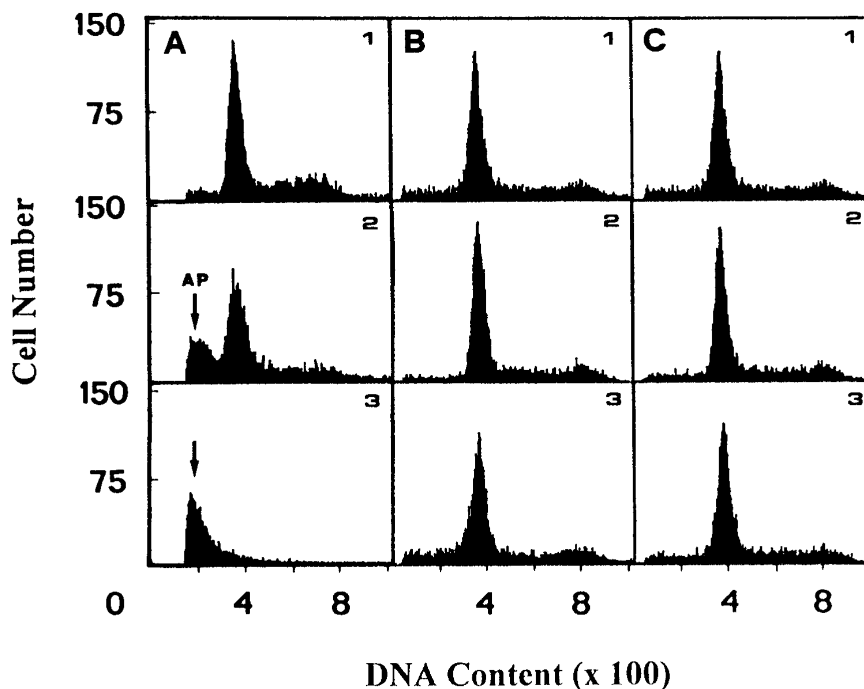

FIG. 3. The DNA histograms of Hep3B cells treated with various concentrations of solamargine (A), solasodine (B) and khasianine (C). The 1, 2 and 3 of panel A denote the concentrations of 0, 5 and 10 g/ml, respectively. The 1, 2 and 3 of panel B denote the concentrations of 0, 12 and 24 g/ml, respectively. The 1, 2 and 3 of panel C denote 0, 30 and 60 g/ml, respectively. A sub-G1 peak, labeled "Ap", appears with the increasing dose of solamargine.

khasianine displayed distinct cytotoxicity to Hep3B cells, the biological function of the glycoalkaloid might be influenced by the carbohydrate moiety. Moreover, different receptors for these steroidal alkaloids might exist in the cells, and they could be recognized by the specific carbohydrate moieties of glycoalkaloids.

Regulation of TNFR I and II Expression by the Steroidal Alkaloids

TNFR I is functional in almost every cell type and can independently transmit most biological activities of TNF. TNFR II seems so far largely restricted to cells of lymphoid origin, where it induces cell proliferation and cytokine production [16]. Both TNF receptors have been involved in the apoptosis [17]. The mechanisms that regulate the distinct gene expressions of TNF receptors and the relative contribution of TNF receptors to cell apoptosis are largely unknown [18]. In our experiments, the gene expression of TNFR I and II could be up-regulated by solamargine, solasodine and khasianine alkaloids. However, the extents of regulation by the alkaloids were varied (Fig. 5). The intensities of the hybridization bands in figure 5 were then integrated by a densitometer. The ratios of TNFR II/I expression for the control, solamargine, solasodine and khasianine were 1.37, 1.49, 1.53 and 0.95, respectively. In contrast to the highest up-regulation of TNFR I by khasianine, the cytotoxicity of khasianine to Hep3B cell was almost diminished, suggesting that the signaling through TNFR I pathway might not be significant in the cytotoxicity of khasianine. Thus, the carbohydrate moiety of steroidal alkaloid may be crucial in affecting the binding specificity and affinity to steroid receptors.

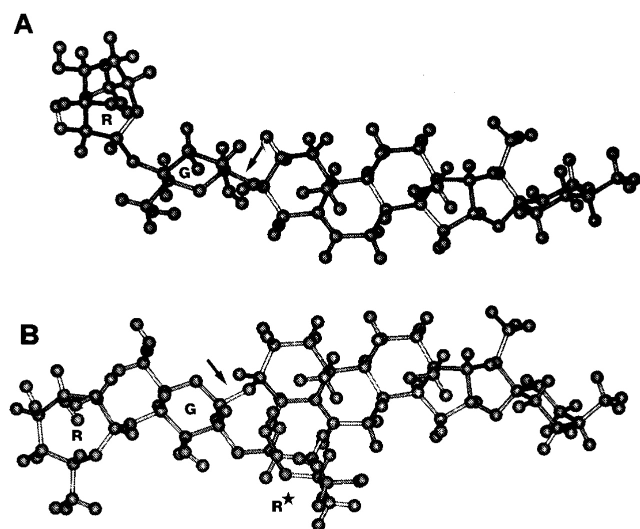

FIG. 4. Molecular modeling of the configurations of solamargine and khasianine. A, khasianine and B, solamargine. The C_3 glycosidic bond is indicated by arrow. G, glucose; R, rhamnose. The -L-Rhamnopyranosyl-(12) moiety of solamargine is indicated by a star.

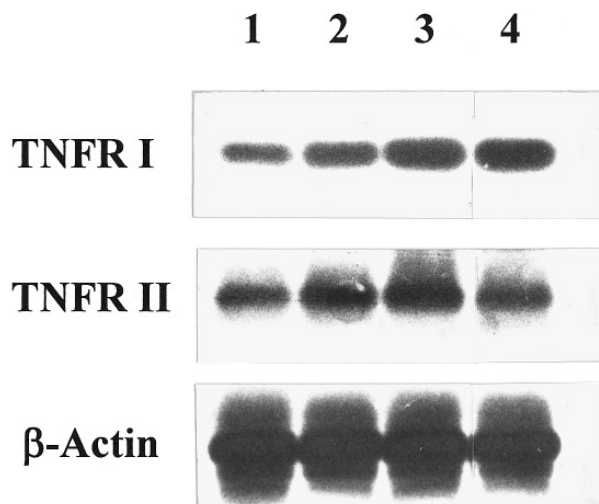

FIG. 5. Gene expression of TNFR I and II of Hep3B cells after treatment with the steroidal alkaloids. The amount of solamargine (5 μg/ml), solasodine (5 μg/ml) and khasianine (20 μg/ml) was individually added to the cells for 30 minutes. The cellular RNA of Hep3B was isolated and employed for RT-PCR with the specific primers. The quantity of cDNA was determined by southern hybridization using [^{32}P] labeled human TNFR I and II cDNA probes. The quantity of RNA used for the reactions was verified with the RT-PCR of human β-actin in the same condition. 1, control; 2, solamargine; 3, solasodine and 4, khasianine.

This structure-function analysis of the carbohydrate moiety of solamargine might provide insight into the apoptotic mechanism of steroidal alkaloid, and might shed light on the role of carbohydrate moiety in steroidal glycoalkaloids.

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