BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 1–5 (1996)
ARTICLE NO. 1748

Solamargine Purified from *Solanum incanum* Chinese Herb Triggers Gene Expression of Human TNFR I Which May Lead to Cell Apoptosis

Shu-Hui Hsu,*,† Tong-Rong Tsai,† Chun-Nan Lin,†
Ming-Hong Yen,† and Kou-Wha Kuo*,[1]

**Department of Biochemistry and †School of Pharmacy, Kaohsiung Medical College, Kaohsiung 807, Taiwan*

Received September 16, 1996

Solamargine possessed a potent cytotoxicity to human hepatocyte (Hep3B) and normal skin fibroblast. The inhibition curves of solamargine to the both cells were essentially overlapped, suggesting a parallel effect for the cell death. To define mechanism of cytotoxicity of solamargine, the changes of morphology and DNA content in cells were studied. A sub-G_1 cell stage was drastically increased after 3-h incubation with solamargine. The results evidence that solamargine arises cell death by apoptosis. In addition, the gene expression of TNFR I were up-regulated within 30 min of solamargine treatment. Since TNF Receptor I has been involved in apoptosis, the overexpression of TNF receptor I may be related with the mechanism of cytotoxicity of solamargine. This communication is the first report that a component of Chinese herbs triggers gene expression of human TNFR I which may lead to cell apoptosis.

Chinese herbs compose of active components that exhibit the capability to regulate cell activities [1, 2]. Previous treatments of diseases with herbs were empirical more than theoretical. In order to identify active ingredients from Chinese herbs, purification and structural characterization of components were extensively studied [3, 4]. Solamargine, a new steroidal alkaloid glycoside, was isolated from the fruits of *Solanum incanum* L. [5]. It showed a novel liver protective effect against CCl_4-induced liver damage, and inhibited the growths of JTC-26 and human PLC/PRF/5 hepatoma cells [6]. The structure of solamargine has been determined [5]. However, the mechanism of cytotoxicity of solamargine is largely unknown.

Apoptosis (programmed cell death) and necrosis are two fundamental types of cell death [7]. The mechanism of necrosis is relatively well understood, whereas that of apoptosis is presently subject to be intense investigation [8, 9]. An effective compound inducing apoptosis appears to be a relevant strategy to suppress tumors [10].

TNF receptors have been involved in the process of cell apoptosis [11, 12]. In this communication, we characterized the mechanism of cytotoxicity of solamargine by the changes of cell morphology and DNA content. The gene expression of TNF receptor I regulated by solamargine was also investigated. The present study is the first report of a component of Chinese herb that mediates its cytotoxicity through apoptosis.

MATERIALS AND METHODS

Cell lines and cell culture. Normal human skin fibroblast and hepatocellular carcinoma (Hep3B) cells were kindly provided by Dr. L.C. Chiang in the department of microbiology, Kaohsiung Medical College, Taiwan. The cells were cultured at 37°C in a humidified atmosphere of carbon dioxide-air (5:95). The culture medium consisted of Dulbecco modified Eagle's medium (DMEM) (GIBCO, Grand Island NY, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazelton Product, Denver, PA, U.S.A.), 100 μg/ml streptomycin and 100 unit/ml penicillin. The

[1] To whom correspondence should be addressed.
Abbreviation: TNFR, tumor necrosis factor receptor.

0006-291X/96 $18.00

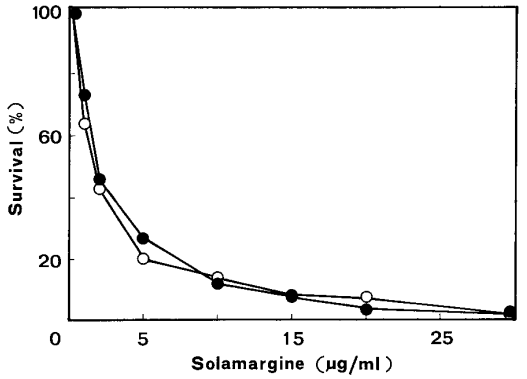

FIG. 1. Cytotoxicities of solamargine for Hep3B and normal skin fibroblast cells. [^{3}H]thymidine incorporation assay was performed according to the procedure in ''Materials and Methods''. Data represent means of percentage-survival rate of treated and untreated cells from triplicate determinations. ●, Hep3B; ○, Normal skin fibroblast.

cells (1×10^4 cells/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.

Cytotoxicity assay. Serial concentrations of solamargine were added into the cells at 37°C for 16 h. [^{3}H]thymidine (2 mCi) was then added to each well and continuous incubation at 37°C for 4 h. 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% 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 using a Packchard Model LS 6800 scintillation counter.

Flow cytometry. Solamargine (5 μg/ml) was added to the cells (1×10^7 cell/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 μg/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 5,000 cells were collected for the analysis by LYSIS II software.

RNA preparation. The RNA was isolated from U937 cell lines by the guanidine thiocyanate/cesium chloride gradient ultracentrifugation method (9). All the preparations were treated with DNase, and were stored in liquid nitrogen prior to use.

RT-PCR. RT-PCR was carried out essentially according to the manual protocol of Perkin Elmer. A 50 μl reaction buffer containing 100 mM Tris-HCl pH 8.3, 1 mM dNTP, 1 μM antisense primer and 100 ng RNA template. The RNA was denatured at 94°C for 2 min, and annealed with antisense primer to the template at 55°C for 2 min. Reverse transcription was performed with reverse transcriptase (2.5 U) and 2 μl of 10 mM $MnCl_2$ at 75°C for 5 min. 4 μl of chelating buffer (50% glycerol (v/v), 100 mM Tris-HCl pH 8.3, 1 M KCl and 7.5 mM EGTA/0.5% Tween 20) was added to stop the reaction. After the addition of 3 μl of 25 mM $MgCl_2$ and 1 μM sense primer, amplification was produced on a thermocycler (model 9600, Perkin Elmer), 94°C/55°C/75°C 1 min each, for a total 35 cycles.

RESULTS AND DISCUSSION

Cytotoxicity Assay

Solamargine was purified from *Solanum incanum* herb according to the previous report [5]. The cytotoxicity of solamargine to Hep3B and normal skin fibroblast cells were determined by [^{3}H]thymidine incorporation method [13]. As shown in Fig. 1, the ED_{50} (dose that inhibits cell growth by 50%) for Hep3B and normal fibroblast cells were 1.9 and 1.7 μg/ml, respectively. Both the inhibition curves of solamargine to cells were essentially overlapped, suggesting a parallel effect for the cell death.

Morphology and Flow Cytometry Analysis of DNA Content of Cells

To define the mechanism of cytotoxicity, the changes of cell morphology and nucleus DNA contents of Hep3B after solamargine (5 μg/ml) treatment were analyzed. It is in contrast to

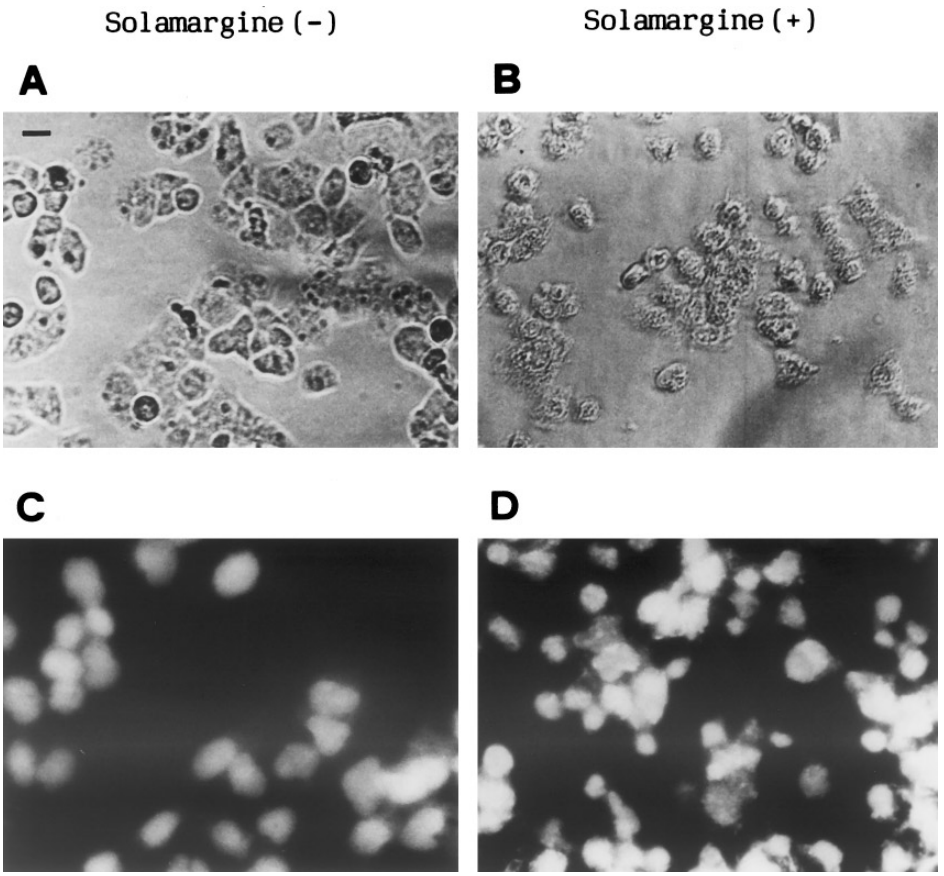

FIG. 2. Morphology of solamargine-treated and untreated Hep3B cells. A and B indicate the light microscopic morphologies of Hep3B before and after solamargine treatment, respectively. C and D denote fluoro-spectroscopic morphologies of nucleus chromatin before and after solamargine treatment, respectively. Chromatin DNA of cell was stained with propidium iodide (10 μg/ml) for 30 min prior to the examinations. Note that the apoptotic cells (D) are reduced in size compared with the non-apoptotic cells (C). Scale bar=10 μm.

cell necrosis, where one of the earliest changes is loss of membrane function and its structural integrity [14]. The solamargine-treated Hep3B cell remained integral, and formed membrane blebbing (zeiosis) in the cells (Fig. 2B). The size of treated and untreated Hep3B cells measured by Culter counter were not significantly different, showing 9 μm and 10 μm for the treated and untreated cells, respectively. Moreover, condensed chromatin which provides the morphological hallmark of the apoptotic nucleus consists of fragmented chromatin molecular [15]. The chromatin condensation in the apoptotic cells after solamargine treatment was viewed under fluorescence on a confocal microscope (Fig. 2D).

It has been recognized that apoptotic cells reduce DNA stainability with a variety of fluorochromes [16, 17]. The appearance of cells with low DNA stainability forms ''sub-G_1 peak'' which has been considered to be the marker of cell death by apoptosis [18]. In the experiment, constant amount of solamargine (5 μg/ml) was added to Hep3B cells for different intervals. As shown in Fig. 3B, a sub-G_1 peak, indicated by ''Ap'', was detected in the DNA histograms of solamargine-treated Hep3B cells by flow cytometry. The apoptotic cells calculated from the area of sub-G_1 peak was drastic increased after 3-h incubation with solamargine. However, a maximum of 50% apoptotic cells was detected at 10 h. No further increase apoptosis could

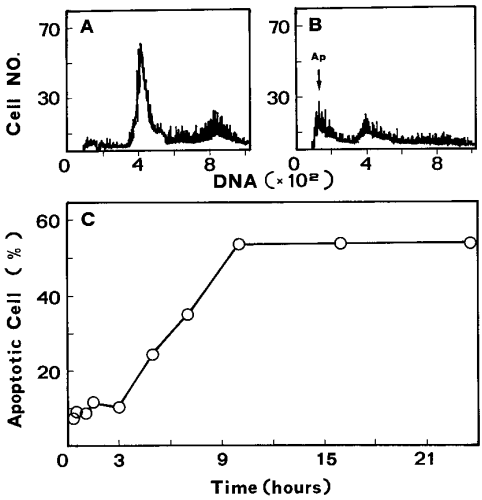

FIG. 3. DNA contents of propidium iodide (PI)-stained Hep3B cells. The cells were fixed and incubated with PI and RNase before recording red fluorescence excited by blue light. A, untreated cells; B, DNA histogram of solamargine-treated (6h) cell. A sub-G_1 peak, labelled ''Ap'', appears with the time of incubation. C, The kinetics of solamargine-induced apoptosis in Hep3B cells. The percentage of apoptotic cells were measured by a flow cytometry following the calculation by LYSIS II software.

be observed after prolonged incubation up to 24 hours (Fig. 3C). Thus, these results evidence that solamargine mediates its cytotoxicity through apoptosis, and the action of solamargine to the cells might be irreversible.

Gene Expression of TNF Receptor I in Solamargine-Treated Cells

Until recently the function of TNF receptors for induction of cell apoptosis was unclear. TNFR I can mediate apoptosis via TRADD signal pathways [11]. Elevated cytosolic phospholi-

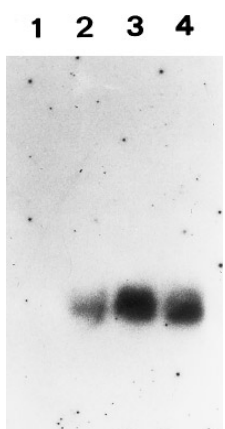

FIG. 4. Southern hybridization of TNFR I cDNA in Hep3B cell after solamargine treatment. Constant amount of solamargine (5 μg/ml) was added to the cells. At various time intervals, the cellular RNA of Hep3B was isolated and employed for RT-PCR. The numbers: 1, 2, 3 and 4 denote the solamargine treatments for 0, 15, 30 and 60 min, respectively. The primer used for the detection of human TNFR I was described as previous report [19]. The quantity of cDNA was determined by southern hybridization using human TNFR I cDNA as probe. The RNA used in RT-PCR was calculated by measuring A_{260nm} and was further verified with the amount of β-actin in the same conditions.

pase A2 level enhances apoptosis mediated by TNF receptors [12]. In our experiment, the gene expressions of TNFR I were up-regulated within 30 min of solamargine treatment (Fig. 4), indicating that the overexpression of TNFR I might be related with the mechanism of cytotoxicity of solamargine. However, the apoptotic cells were drastically increased after 3 hours incubation (Fig. 3B). It implies that a signal transduction cascade from TNFR I or another apoptotic pathways after solamargine addition might be proceeded.

ACKNOWLEDGMENTS

This work was supported by the Department of Health, Executive Yuan of Taiwan (DOH Grant 85-CM-036 to K.W. Kuo).

REFERENCES

1. Kaku, H., Tanaka, Y., Tazaki, K., Minami, E., Mizuno, H., and Shibuya, N. (1996) *J. Biol. Chem.* **271,** 1480–1485.
2. Zhang, L., Chang, C. J., Bacus, S. S., and Hung, M. C. (1995) *Cancer Res.* **55,** 3890–3896.
3. Fang, S. C., Shieh, B. J., Wu, R. R., and Lin, C. N. (1995) *Phytochemistry* **38,** 535–537.
4. Gan, K. H., and Lin, C. N. (1993) *J. Nat. Prod.* **56,** 15–21.
5. Lin, C. N., Lu, C. M., Cheng, M. K., and Gan, K. H. (1990) *J. Natural Prod.* **53,** 513–516.
6. Lin, C. N., Chung, M. I., and Gan, K. H. (1988) *Planta Med.* **54,** 222.
7. Maximilian, L., Marsha, L., and Edwin, H. (1993) *Arch. Pathol. Lab. Med.* **117,** 1208–1214.
8. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26,** 239–257.
9. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) *Int. Rev. Cytol.* **68,** 251–306.
10. Hickman, J. A. (1992) *Cancer Metast. Rev.* **11,** 121–139.
11. Grell, M., Zimmermann, G., Hulser, D., Pfizenmaier, K., and Scheurich, P. (1994) *J. Immunol.* **153,** 1963–1972.
12. Vandenabeele, P., Declercq, W., Vanhaesebroeck, B., Grooten, J., and Fiers, W. (1995) *J. Immunol.* **154,** 2904–2913.
13. Kuo, K. W., Yeh, H. W., Chu, D. Z. J., and Yeh, Y. C. (1991) *J. Chromatogr.* **543,** 463–470.
14. Lennon, S. V., Martin, S. J., and Cotter, T. G. (1991) *Cell Proliferat.* **24,** 203–214.
15. Arends, M. J., and Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32,** 223–254.
16. Ojeda, F., Guarda, M. I., Maldonado, C., and Folch, H. (1990) *Cell Immunol.* **125,** 535–539.
17. Afanasev, V. N., Korol, B. A., Mantsygin, Y. A., Nelipovich, P. A., Pechtnikov, V. A., and Umansky, S. R. (1986) *FEBS Lett.* **194,** 347–350.
18. Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992) *Cytometry* **13,** 795–808.
19. Kuo, K. W., Leung, M., and Leung, W. C. (1996) *Mol. Cell. Biochem.* (in press).