Tohoku J. Exp. Med., 1999, **189**, 51-57

Gossypol Induced Apoptosis in the Human Promyelocytic Leukemia Cell Line HL 60

AYŞE BALCI, FERIDE İFFET ŞAHİN and ABDULLAH EKMEKCİ

Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, 06510 Beşevler, Ankara, Turkey

BALCI, A., ŞAHİN, F.İ. and EKMEKCİ, A. *Gossypol Induced Apoptosis in the Human Promyelocytic Leukemia Cell Line HL 60.* Tohoku J. Exp. Med., 1999, **189** (1), 51-57 —— In human promyelocytic leukemia cell line HL60, apoptosis was induced by treatment with gossypol that is an inhibitor of protein kinase C. Gossypol acetic acid was added to HL 60 cells at 50, 100, 150 and 200 μM concentrations for six hours. Morphological features of apoptosis as well as internucleosomal DNA fragmentation were evaluated by light microscope, agarose gel electrophoresis and spectrofluorometric quantitation. Our results indicated that with the effective concentrations of gossypol (50 and 100 μM), apoptosis was induced in HL 60 cells. ———— apoptosis; gossypol; protein kinase C © Tohoku University Medical Press

Apoptosis is a signal-regulated process whereby a cell activates a set of genes which mediates its own death. It is characterized by certain morphological and biochemical features. Characteristic morphological changes are chromatin condensation, cell shrinkage, membrane blebbing and finally, disintegration of the cell into membrane-bound fragments called apoptotic bodies. In addition, several biochemical changes also occur, such as internucleosomal DNA fragmentation and protein degradation mediated by Ca^{2+} and Mg^{2+} dependent endonucleases, and proteases (Kerr et al. 1972). Morphological changes in cells undergoing apoptosis are easily recognizable using electron or light microscopy.

Many diseases such as malignant diseases, AIDS, autoimmune diseases may trigger apoptotic process (Dipasquale and Youle 1991; Terai et al. 1991; Carson and Ribeiro 1993). In order that the process of apoptosis may be manipulated in the prevention and treatment of these diseases, much effort has been expended in the search for many compounds which mediate the induction of this pathway (Oren 1992; Chorváth and Sedlák 1996).

In hematopoietic cells, apoptosis has been reported to be regulated by signal transduction pathways, especially by Ca^{2+} and lipid-dependent serine-threonine

Received April 1, 1999; revision accepted for publication September 14, 1999.

Address for reprints: Feride İffet Şahin, M.D., Ph.D., Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, 06510 Beşevler, Ankara, Turkey.
e-mail: feridesahin@hotmail.com

kinase, and protein kinase C (PKC) (Grant et al. 1996; Hunáková et al. 1996). Protein kinase inhibitors are a broad family of enzyme inhibitors able to induce or modulate the in vitro cellular proliferation and differentiation, and apoptosis in a variety of neoplastic cell lines (Chorváth and Sedlák 1996).

In the present study, we used gossypol that is a potent inhibitor of phospholipid-sensitive Ca^{2+} dependent protein kinase (Kimura et al. 1985). In addition, since gossypol irreversibly blocks cells in the S phase, it has also been used as an antitumor drug (Nakadate et al. 1988). The aim of our study was to investigate induction of apoptosis in HL60 cell line and examination of apoptotic cells morphologically and biochemically.

Materials and Methods

Cell cultures and gossypol treatment

The human promyelocytic leukemia cell line HL-60 cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (Seromed), 200 mM L-glutamine (Seromed) and 100 U/ml penicillin, 100 μg/ml streptomycin (Seromed). The cultures were maintained in a 37°C, 5% CO_2, humidified incubator, and passed twice weekly.

Gossypol acetic acid (Sigma, St. Louis, MO, USA) was added to the cultures during logarithmic growth phase at different concentrations ranging from 50 to 200 μM, for 6 hours. Aceton (Carlo Erba, Rodano, Italy) was added as the solvent control.

Evaluation of the HL-60 cell model of apoptosis

Cell morphology, agarose gel electrophoresis and spectrofluorometric quantitation of DNA fragments were used in assessing apoptosis. Cell viability was determined by trypan blue exclusion.

Cell morphology

Morphological evaluation of different stages of apoptosis in cells were made by staining in 10% Giemsa solution (Merck, Darmstadt, Germany). Membrane blebs, apoptotic bodies in cells were regarded as apoptosis 500 cells/different concentrations of the drug were evaluated and the percentage of apoptotic cells were determined.

Agarose gel electrophoresis

DNA fragmentation was analysed by agarose (Sigma) gel electrophoresis as described previously (Jarvis et al. 1994). Small molecular weight DNA from cell extracts was loaded onto 2% agarose gels and visualised by ethidium bromide. One hundred twenty three base pair ladder (Sigma) was run in parallel with DNA samples.

Spectrofluorometric quantitation of DNA fragments

After exposure to gossypol small molecular weight DNA fragments in cell lysate and medium samples were determined by quantitative bisbenzimide spectrofluorometry (FP-550, Jasco, Tokyo) (Kimura et al. 1985; Grant et al. 1996). A total number of 5×10^6 cells for each drug concentration were centrifuged and lysed in cell lysis buffer. Lysate and medium preparation were diluted in Tris Sodium EDTA (TNE) buffer containing 1 mg/ml bisbenzimide trihydrochloride (Sigma). DNA quantitation was made by spectrofluorometry with excitation at 365 nm and emission at 460 nm. Purified calf thymus DNA was used as the calibration standard.

Results

Cell morphology

Apoptotic features such as cell shrinkage, nuclear condensation, membrane blebbing, cytoplasmic granularity were observed in cells after 6 hours exposure to

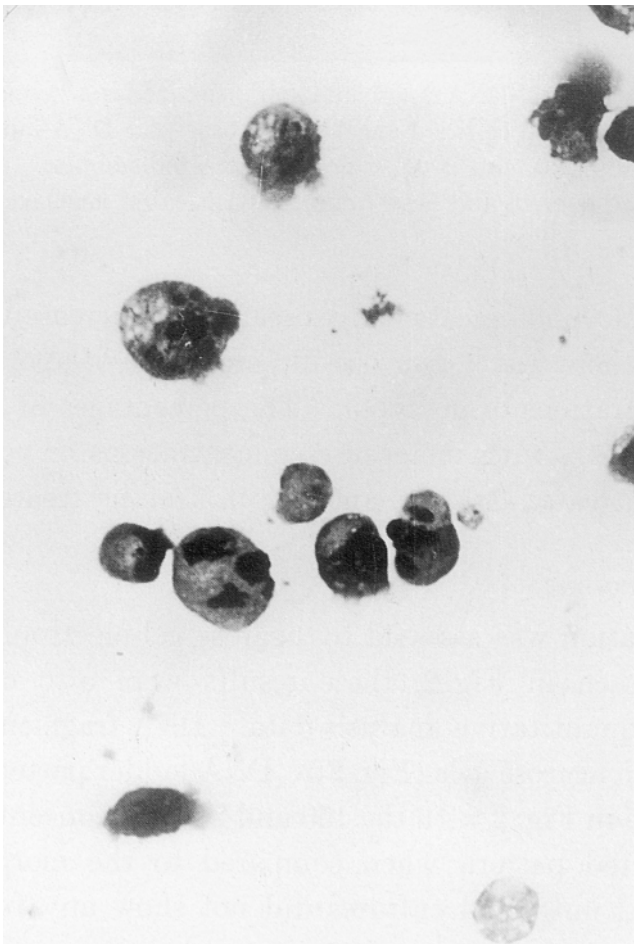

Fig. 1. Morphological features of 100 μM gossypol treated HL 60 cells (630×). M, Membrane blebbing; N, DNA condensation and disintegration of the nucleus; C, Cytoplasmic granularity.

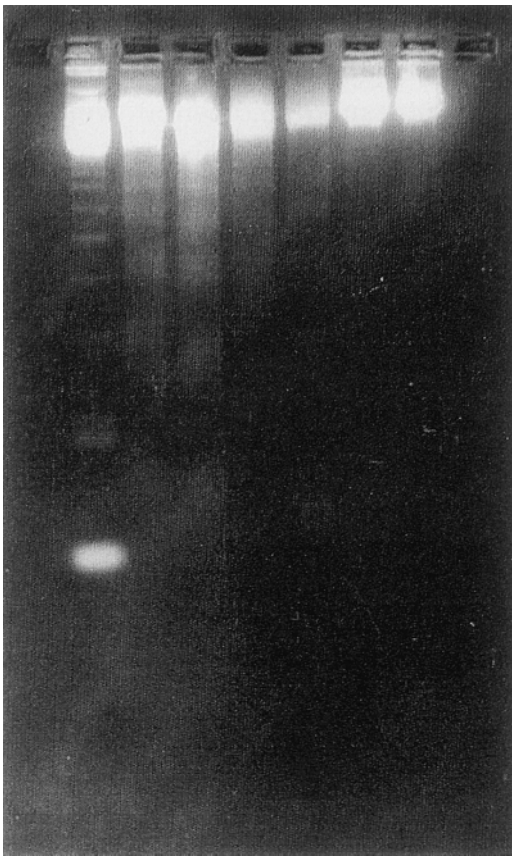

Fig. 2. Internucleosomal DNA fragmentation visualized in 2% agarose gel electrophoresis from HL 60 cells. Lane 1 is 123 base pair DNA ladder. Lanes 2-5 contain 50, 100, 150, and 200 μM gossypol treated samples. Lane 6 contains aceton treated control and lane 7 contains untreated negative control sample.

gossypol (Fig. 1). Morphological changes seemed to increase with 50 and 100 μM concentrations and there was a decrease in percentage of apoptotic cells with 150 and 200 μM concentrations of gossypol. The percentages of apoptotic cells ranged from 92.7% to 38% with increasing concentrations of gossypol. Apoptotic cells were 2% in untreated cultures and 3% in acetone treated cultures.

Agarose gel electrophoresis

DNA fragmentation was assessed by agarose gel electrophoresis as a qualitative method. As seen in Fig. 2, these results were also consistent with the morphological and quantitative analysis data. DNA fragments were observed in a laddered pattern in agarose gels (Fig. 2). DNA ladder pattern became fainter as seen in lanes 4 and 5 in Fig. 2 with the 150 and 200 μM concentrations of gossypol. This was an expected pattern when compared to the morphological findings. Acetone control and untreated cultures did not show any fragmented DNA, as expected.

Spectrofluorometry

Quantitative analysis of fragmented apoptotic DNA was made by bisben-

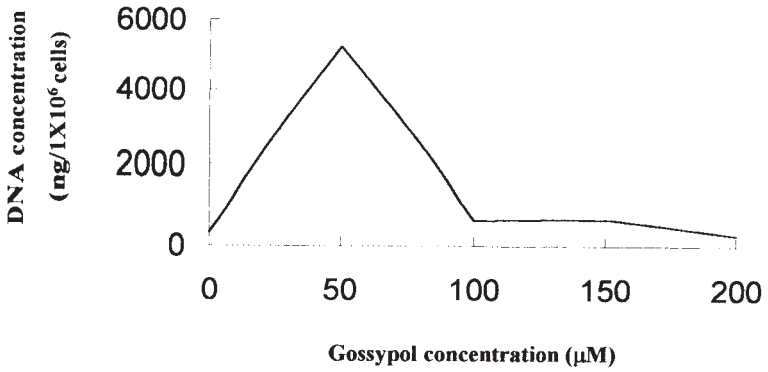

Fig. 3. Graph showing quantitative analysis of DNA fragmentation by spectrofluorometry. DNA fragmentation is maximum with the 50 μM concentration of gossypol, which is decreased to the basal concentration of the untreated cultures with the increasing concentrations of the drug.

zimide spectrofluorometry. Double stranded DNA concentration was 360 ng/10^6 cells in untreated cultures (Fig. 3). After 6 hours treatment with gossypol DNA fragmentation was found to be 5250 ng/10^6 cells when 50 μM drug concentration was used. This was the maximum concentration of the fragmented DNA. With the increasing concentrations of gossypol, DNA amount declined and with 200 μM concentration of the drug, DNA amount was detected to be 310 ng/10^6 cells (Fig. 3). This concentration was near to the fragmented DNA concentration in untreated cultures.

Discussion

Apoptosis is induced and regulated by many different pathways. It is very important to know how apoptosis is triggered and this may be necessary for successful treatment of cancer. The studies about drug-induced apoptosis of cancer cells have provided an experimental background for improvement of therapeutic strategies (Fisher 1994; Schuler et al. 1994; Hunáková et al. 1996).

A nonspecific inhibitor of PKC, gossypol is used in this study as an apoptotic inducer of HL 60 cells. Gossypol inhibits PKC by acting at both the catalytic and regulatory domains of the enzyme (Nakadate et al. 1988; Jarvis et al. 1994). Our results show that with the effective concentrations of gossypol (50–100 μM), apoptosis is induced in HL60 cells. With the increasing concentrations of the drug (150–200 μM), apoptotic DNA fragmentation seemed to decrease and with the 200 μM concentration it became nearly to the basal values. DNA fragmentation was maximum with the 50 μM concentration of the drug. The decrease in apoptotic cells with the increasing concentrations of the drug may be because of several different mechanisms leading to cell death, but not apoptosis. It has been reported that reductions in DNA damage are not associated with restoration of cell viability, instead with other lethal events (Jarvis et al. 1994).

In our study, morphological findings correlated with low molecular weight DNA amounts by spectrofluorometry and agarose gel electrophoresis visualisation

of DNA fragmentation. These findings are similar to the other reports (Jarvis et al. 1994; Grant et al. 1996). Indeed, in the present study 38% apoptotic cells was observed by examination on light microscope at 200 μM concentration of gossypol. But DNA fragmentation was not seen at the same rate at 200 μM concentration of the drug in spectrofluorometic and agarose gel electrophoretic analysis. It might be explained by the same mechanism as studies suggesting that some of the characteristic morphological changes of apoptotic cells can occur without endonuclease activity (Cohen et al. 1992; Jacopson et al. 1994).

Treatment failures and cancer cell resistance to chemotherapeutic drugs are important problems in cancer therapy. Various agents, such as 2-deoxyglucose and antioxidants like vitamin E and retinoids have been investigated for their activitiy in drug induced apoptosis (Halicka et al. 1995; Turley et al. 1995). In addition, many different type of PKC inhibitors such as staurosporine, chelerythrine, hypericin and H7 have been reported that induce apoptosis in HL 60 cells (Jarvis et al. 1994; Hunáková et al. 1996). Based on the previous reports and our study, it might be suggested that PKC-dependent signaling processes may play important role in apoptotic process. Cellular differentiation does not seem to be important in the apoptotic effects of gossypol, chelerythrine, calphostin C and H7 (Jarvis et al. 1994).

Finally, according to our and other investigators' results, gossypol seems to induce apoptosis in effective concentrations and could be investigated as a stimulator of drug induced apoptosis. The mechanisms playing role in this effect need detailed investigations.

References

1) Carson, D.A. & Ribeiro, J.M. (1993) Apoptosis and disease. *Lancet*, **341**, 1251–1254.
2) Chorváth, B. & Sedlák, J. (1996) Protein kinase inhibitors: Induction of apoptosis and drug resistance modulation in neoplastic cell lines. *Neoplasma*, **43**, 3–9.
3) Cohen, G.M., Sun, X., Snowden, R.T., Dinsdale, D. & Skilleter, D.N. (1992) Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.*, **286**, 331–334.
4) Dipasquale, B. & Youle, R.J. (1991) Programmed cell death and the immune system: Physiopathological implications of apoptosis. *Journal of Immunological Research*, **3**, 149–154.
5) Fisher, D.E. (1994) Apoptosis in cancer theraphy: Crossing the threshold. *Cell*, **78**, 539–542.
6) Grant, S., Turner, A.J., Freemerman, A.J., Wang, Z., Kramer, L. & Jarvis, W.D. (1996) Modulation of protein kinase C activity and calcium-sensitive isoform expression in human myeloid leukemia cells by bryostatin 1: Relationship to differentiation and Ara-C-induced apoptosis. *Exp. Cell Res.*, **228**, 65–75.
7) Halicka, H.D., Ardelt, B., Li, X., Melamed, M.M. & Darzyn-Kiewicz, Z. (1995) 2-Deoxy-D-glucose enhances sensivitiy of human histiocytic lymphoma U937 cells to apoptosis induced by tumor necrosis factor. *Cancer Res.*, **55**, 444–449.
8) Hunáková, L., Şuliková, M., Duraj, J., Sedlák, J. & Chorváth, B. (1996) Stimulation of 1-(beta-D-arabinofuranosyl)cytosine (AraC)-induced apoptosis in the multidrug resistant human promyelocytic leukemia cell lines with protein kinase inhibitors.

Neoplasm, **43**, 291-295.

9) Jacopson, M.D., Burne, J.F. & Raff, M.C. (1994) Programmed cell death and Bcl-2 protection in the absence of nucleus. *EMBO J.*, **13**, 1899-1910.
10) Jarvis, W.D., Turner, A.J., Povirk, L.F., Traylor, R.S. & Grant, S. (1994) Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Res.*, **54**, 1707-1714.
11) Kerr, J.F.R., Wyllie, A.H. & Currie, A.R. (1972) Apoptosis: biological phenomenon with wide-ranging implication in tissue kinetics. *Br. J. Cancer.*, **26**, 239-257.
12) Kimura, K., Sakurada, K. & Katoh, N. (1985) Inhibition by gossypol of phospholipid-sensitive Ca^{++}-dependent protein kinase from pig testis. *Biochim. Biophys. Acta.*, **839**, 276-280.
13) Nakadate, T., Jeng, A.Y. & Blumberg, P.M. (1988) Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochem. Pharmacol.*, **37**, 1541-1545.
14) Oren, M. (1992) The involvement of oncogenes and tumor suppressor genes in the control of apoptosis. *Cancer Metastasis Rev.*, **11**, 141-148.
15) Schuler, D., Szende, B., Borsi, J.D., Marton, T., Bocsi, J., Magyarossy, E., Koós, R. & Csóka, M. (1994) Apoptosis as a possible way of destruction of lymphoblasts after glucocorticoid treatment of children with acute lymphoblastic leukemia. *Pediatr. Hematol. Oncol.*, **11**, 641-649.
16) Terai, C., Kornbluth, R.S., Pauza, C.D., Richman, D.D. & Carson, D.A. (1991) Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. *J. Clin. Invest.*, **87**, 1710-1715.
17) Turley, J.M., Funakoshi, S., Ruscett, F.W., Kasper, J., Murphy W.J., Longo D.L. & Birchenall-Roberts, M. (1995) Growth inhibition and apoptosis of RL human B-lymphoma cells by vitamin E succinate and retinoic acid: Role for transforming factor beta. *Cell Growth Differ.*, **6**, 655-663.