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Ar-Turmerone and β-atlantone induce Internucleosomal DNA Fragmentation associated with Programmed Cell Death in Human Myeloid Leukemia HL-60 cells

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In the course of a search for antitumor agents, we found that the extract of *Curcuma longa* was effective in inducing apoptosis or programmed cell death (PCD) in human myeloid leukemia cells (HL-60). Active compounds for PCD were isolated from the hexanic extraction of the rhizome of *Curcuma longa*. With the several chromatographies, and spectral data, they were identified as ar-turmerone and β-atlantone. The present results demonstrate that the exposure of human myeloid leukemia HL-60 cells to clinically achievable concentrations of ar-turmerone (TU) or β-atlantone (AT) produced internucleosomal DNA fragmentation of approximately 200 base-pair multiples, and the morphological changes characteristic of cells undergoing apoptosis or PCD. This findings suggest that these agents may exert their antitumoral activity, in part, through induction of apoptosis(PCD).

Key words : ar-Turmerone, β-atlantone, Anticancer activity, Apoptosis, Programmed cell death

INTRODUCTION

In 1972, Kerr *et al.* reported "apoptosis", which was different from necrosis, is a process in which cells die in a controlled manner, in response to specific stimuli, following an intrinsic program (Kerr *et. al.*, 1972). Apoptosis constitutes a systematic means of cell suicide within an organism during normal morphogenesis, tissue remolding and in response to pathogenic infections or other irreparable cell damage (Kerr *et. al.*, 1987). Inappropriate apoptosis may lead to human diseases such as Alzheimer's, Parkinson's and Huntington's diseases, immune deficiency and autoimmune disorders, leukemias, lymphomas and other cancers (Barr and Tomei, 1994). And, there were a number of reports that described the possibility of apoptosis inducers for a promising cancer chemotherapy (Dive *et. al.*, 1992; Hickman, 1992). Since then, the concept of an apoptosis in tumors has attracted the interest of tumor cell biologists and experimental cancer chemotherapists. Spontaneous and induced apoptosis have been identified in many malignant cell types including lymphomas, leukemias, embryonal carcinomas, hepatic neoplasmas, small cell lung cancer, ascites tumors, sarcomas and tumors of the colon, prostate and breast (Dive *et. al.*, 1992; Wyllie, 1985). It is becoming increasingly evident that spontaneous and induced tumor cell loss occurs via apoptosis and that many currently used anticancer agents can induce apoptosis in various tumor cells (Barry *et. al.*, 1990; Bhalla *et. al.*, 1993; Hickman, 1992; Jarvis *et. al.*, 1994).

Based on the earlier studies, this work was designed to investigate the apoptotic effect of ar-turmerone and β-atlantone on human myeloid leukemia cells, HL-60 cells.

METHODS

Isolation procedure

The rhizome of *Curcuma longa* were purchased at Oriental herbal medicine market. The rhizome of *C. longa* (200 g) were extracted with MeOH, the solvent was concentrated in vacuo, and the residue was partitioned between EtOAc and H_2O. The EtOAc extract showed apoptosis induction activity in HL-60 cells. The EtOAc extract was concentrated in vacuo and the residue partitioned between Hexane and MeOH. The hexanic extract gave apoptosis induction activity. It was chromatographed on 500 g of Si gel using gradients of 1-20% EtOAc in Hexane. Fractions(20 ml) were collected, and fraction combinations were made based on simility of normal phase TLC patterns and

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Rf values. Active fractions were eluted with 5% EtOAc in Hexane. They contained two major components and gave significant apoptosis induction activity. After further chromatography on Si gel, eluting with Hexane-EtOAc (20 : 1), pale yellow oily liquid was obtained. The IR-, UV-spectral data and ^{1}H-, ^{13}C-NMR spectral data were all in accord with those of the known ar-turmerone and β-atlantone, previously isolated from *Curcuma longa* (Itotawa *et. al.*, 1985).

Drugs

Ar-turmerone and β-atlantone were isolated from *Curcuma longa*. They were made fresh for each experiment. It was dissolved in MeOH to make a stock solution of 100 mM which was diluted with medium to obtain the desired concentrations for the experiments. In no conditions did the MeOH concentration exceed 2%. Vehicle controls of 2.5% MeOH were consistently found to be ineffective in induction of DNA fragmentation in HL-60 cells.

Cell culture and test exposure

The human myeloid leukemia HL-60 cells were derived from the original lines (Gallegher *et. al.*, 1979), and maintained in suspension culture as previously described (Bhalla *et. al.*, 1984). Briefly, HL-60 cells were grown in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Cansera International Inc. Canada), 100 IU/ml penicillin G, 100 μg/ml streptomycin and 2 mg/ml sodium bicarbonate; all cultures were maintained under a fully humidified atmosphere of 95% room air-5% CO_2 at 37°C. All cultures were passaged twice weekly and routinely examined for *Mycoplasma* contamination. For experimental incubations, HL-60 cells in log-phase growth were suspended at a density of 5×10^5 cells/ml, and maintained as described above. Cells were exposed to TU or AT in complete medium for 6 hours. Test incubations were terminated by gentle pelleting of the cells at 400×g for 10 minutes and withdrawal of the medium. Following determination of cell density and viability, the cells were prepared for assessment of DNA fragmentation by agarose gel electrophoresis and examination of cell morphology as described below.

Trypan blue exclusion

Cells were assessed for viability by trypan blue exclusion test. Exclusion was assayed with aliquots of the cell preparation used for electrophoresis. Cells resuspended in culture medium were stained by addition of an equal volume of 0.4% trypan blue in 0.7% saline and counted on a hemocytometer.

Analysis of DNA fragmentation

The internucleosomal DNA fragmentation was assayed by a modification of previously described method (Kyprianou and Isaacs, 1989). Briefly, at the end of the culture, cells (10×10^6 cells/ml) were washed twice in PBS at 4°C. The pellets were lysed with 0.2 ml of lysis buffer (5 mM Tris-HCl, pH 8/ 20 mM EDTA, pH 8/ 2% SDS), incubated at 37°C for 10 minutes and chilled on ice for 10 minutes. To precipitate proteins, 0.2 ml of 5 N NaCl solution was added and tubes were left on ice for 5 minutes. Precipitates were centrifuged at 10000 rpm for 15 minutes to separate intact from fragmented chromatin. RNase was then added supernatants at a final concentration of 20 μg/ml and incubation at 37°C was continued for 15 minutes, and 50 μg/ml of proteinase K was added and incubated at 50°C for 1 hour. Samples were extracted with equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1). High molecular weight DNA was then pelleted at 15000 rpm for 15 minutes, and the low molecular weight DNA in the supernatant was removed and precipitated overnight in two volumes of ice-cold ethanol at -70°C. DNA was spun-down at 15000 rpm for 15 minutes, air-dried at room temperature for 5 minutes, resuspended in 50 μl of TE buffer (0.01 M Tris-HCl, pH 8/ 1 mM EDTA) and stored at 4°C. Prior to electrophoresis, loading buffer(containing 0.25% bromophenol blue/ 0.25% xylene cyanol/ 15% ficoll) was added to each sample at a 1 : 5 (v/v) ratio. DNA was electrophoresed in a 2.0% agarose gel with 1 μg/ml ethidium bromide at 6 V per cm of gel in TBE buffer (2 mM EDTA, pH 8.0/ 89 mM Tris/ 89 mM boric acid). *A Hind* III-digest of λ-DNA was applied to each gel to provide molecular size standards of 23.5, 9.6, 6.6, 4.3, 2.2, 2.1 and 0.5 Kbp. Following electrophoresis, the stained gel was viewed by transillumination with UV light (302 nm) and photographed for permanent records.

RESULTS AND DISCUSSION

Several currently used anticancer agents have been shown to produce the oligonucleosome-sized DNA fragments and the morphologic changes of apoptosis in leukemic cells. The internucleosomal DNA fragmentation is a biochemical hallmark of cells undergoing apoptosis (Arends *et. al.*, 1990). Fig. 1 shows that the exposure to various concentrations of ar-turmerone(TU) or β-atlantone (AT) for 4 hours results in the characteristic ladder of DNA fragments of approximately 200 base-pair multiple on agarose gel electrophoresis of DNA stained by ethidium bromide. Such a ladder of DNA fragments was not observed when the cells were exposed to TU concentration less than 0.1 μM and exposed to AT concentration less than 0.05 μM. And more than 1 mM

of AT was so cytotoxic to HL-60 cells that no DNA bands could be observed (data not shown). The untreated, control HL-60 cells demonstrated no significant fragmented DNAs.

Fig. 2 and figure 3 demonstrate the percentage of HL-60 cells with morphologic appearance of apoptosis treated with TU or AT. Exponentially growing HL-60 cells were treated with TU or AT at concentration from 0.01 μM to 1 mM for 4 hours, washed and then incubated at 37°C in drug-free media for an additional 5 days. During exposure of these agents, the percentage of apoptotic cells monitored by observing morphological characteristics of apoptosis, i.e., cell shrinkage, cell membrane blebbing and apoptotic body formation (Fig. 4). Membrane integrity of the cells was also monitored by their ability to exclude trypan blue dye. Trypan blue staining of these cells revealed that less than 10% of cells were unable to exclude the dye. All of these three morphologic changes were observed in more than 30% of cells at 0.5-100 μM TU and 0.1-10 μM AT(Fig. 2). This result was consistent with the result obtained in the DNA fragmentation assay. These results demonstrated that minimum concentration of TU and AT required to induce apoptosis in HL-60 cells were 0.5 μM and 0.1 μM, respectively. After cultured in suspension with 0.5 μM TU or 0.1 μM AT, HL-60 cells presented detectable appearance of apoptosis from day 2 and maximum percentage of apoptotic morphology at day 3 (Fig. 3).

In the present study, we were able to demonstrate that TU and AT, main active components of *C. longa* extract directly induce internucleosomal DNA fragmentation and morphologic changes characteristic of apoptosis at concentrations as low as 0.1 μM. The LC_{50}

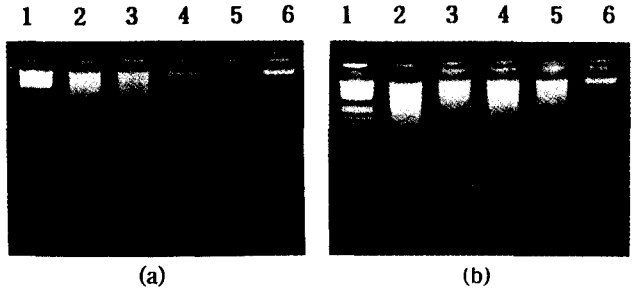

Fig. 1. Induction of internucleosomal DNA fragmentation by ar-turmerone(a) and β-atlantone(b). HL-60 cells were exposed to various concentrations of these agents for 4 hours, and the formation of oligonucleosomal fragments was determined by agarose gel electrophoresis as described in the text. a) Lane 1 : *Hind* III marker, 2 : 100 μM, 3 : 10 μM, 4 : 1 μM, 5 : 0.1 μM, 6:control, b) Lane 1 : *Hind* III marker, 2 : 100 μM, 3 : 10 μM, 4 : 1 μM, 5 : 0.1 μM, 6 : control

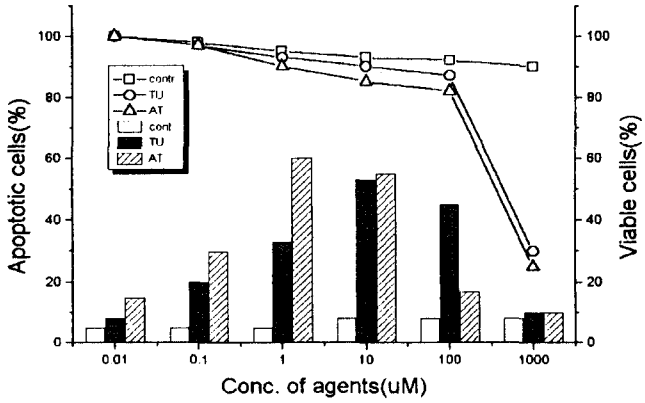

Fig. 2. Percentage of HL-60 cells with morphologic appearance of apoptosis after culture for 5 days in the presence of TU or AT at concentrations from 0.01 μM to 1 mM. The results are shown as mean of three experiments.

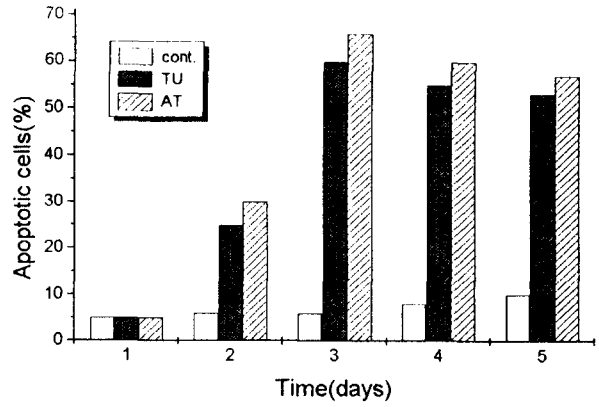

Fig. 3. Time course of appearance of apoptotic cells in the presence or absence of 1 μM TU or 0.1 μM AT. The results are shown as mean of three experiments.

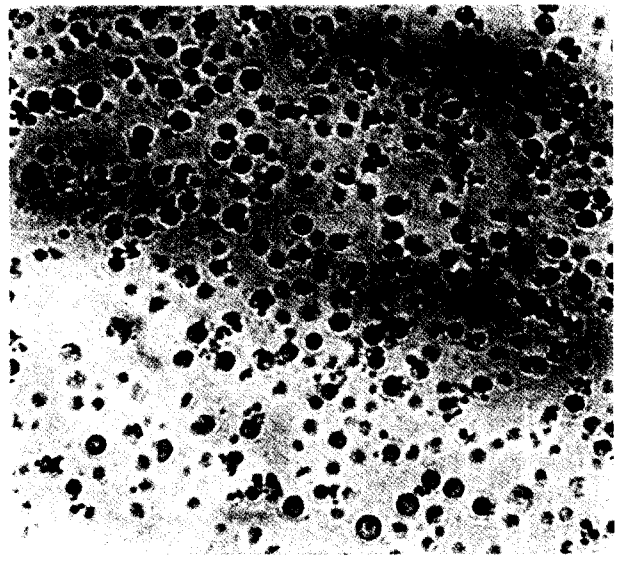

Fig. 4. Morphological appearance of HL-60 cells treated for 3 days with TU or AT. Note the morphology of apoptotic cells with their apoptotic bodies, membrane blebbing and cell shrinkage. This picture was taken by Leitz FLUOVERT inverted microscope (125 X).

value of TU and AT in this study is considered to be useful in clinical situations. And their toxicity against normal cells was not investigated because of their apparent low toxicity; i.e., it is already a common ingredient of oriental medicines.

Since apoptosis, different from necrosis, was identified by Kerr *et al.*, various inducers of apoptosis and the mechanisms of apoptosis have been reported. However, to our knowledge, it has never been reported that TU and AT induces apoptotic effects on HL-60 cells. Although, several previous studies reported that TU and AT represented antitumor activity against some tumor cell lines (Ahn *et. al.*, 1989; Itokawa *et. al.*, 1985; Matthes *et. al.*, 1980), none of them indicated the possible mechanism of thier antitumor acitivity.

Here, we are trying to report that ar-turmerone and -atlantone, main components of the rhizome of *C. longa* are effective in inducing apoptosis in human leukemia cells, HL-60 cells and that their antitumoric mechanism might be related to induction of apoptosis activity.

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