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THE ANTITUMOR ACTIVITY OF ELEMENE IS ASSOCIATED WITH APOPTOSIS

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Elemene, isolated from the Chinese medicinal herb Rhizoma Zedoariae was shown to exhibit antitumor activity *in vitro* and *in vivo* to human and murine tumor cells. This novel antineoplastic agent has been demonstrated to have substantial clinical activity against various tumors. In this paper, the mechanisms of antitumor activity of elemene are reported. The *in vitro* effect of elemene on the growth of leukemia cells was evaluated by MTT assay. The IC_{50} values of elemene for promyelocytic leukemia HL-60 cells and erythroleukemia K562 cells were found to be 27.5 μg/ml and 81μg/ml, respectively, while IC_{50} for peripheral blood leukocytes (PBL) was 254.3 μg/ml. The inhibitory effect of elemene on proliferation of HL-60 cells was associated with cell cycle arrest from S to G_2M phase transition and with induction of apoptosis. The apoptosis of tumor cells was confirmed by DNA ladder formation on gel electrophoresis and characteristic ultrastructural alternations. These results indicate that induction of apoptosis contributes to the mechanisms of antitumor activity of elemene.

Key words: Elemene, Apoptosis, Leukemia

β-elemene (1-methyl-1-vinyl-2, 4-diisopropenyl-cyclohexane) is a naturally occurring compound that can be isolated from the traditional Chinese medicinal herb, Rhizoma Zedoarie, native to South China. Elemene has been show to have variety of pharmacological effects in animal experiments and clinical trials.[1-3] This recent antineoplastic agent has been demonstrated to have substantial clinical activity in treatment of various tumors, but less side-effects and no signs of bone marrow suppression,[4-6] the mechanism of which is unclear. In this paper, we determine if the growth inhibitory effects of elemene on human leukemia cells involved in the process of proliferation (cell cycle progression) and/or cell death (apoptosis).

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MATERIALS AND METHODS

Chemicals

ELEMENE EMULSION INJECTION (elemene) was originally provided by Dalian Jin Gang Pharmaceutical Co., Ltd. (Dalian, P. R. China). DAPI and other laboratory reagents were purchased from Sigma (St. Louis, MO. USA.). Cisplatin (CDDP) was purchased from Jinzhou Pharmaceutical factory (Jinzhou, P.R. China).

Cell Culture and Treatment

The human promyelocytic HL-60 cells were obtained from Shanghai Institute for Cell Biology, Academia Sinica. The human leukemia K562 cells were kindly provided by Dr. Steinmann (Kiel University, Germany). HL-60 and K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in our lab. Human peripheral blood mononuclear cells (PBMCs) were isolated from

the healthy donors using Ficoll-Hypaque gradient centrifugation. Cultures of 5 × 10^4 /ml were generally incubated with elemene, which was diluted in culture medium to obtain the desired concentration. Cells were incubated with various concentration of elemene for 2 to 48 hours. The same aliquot of drug-free emulsion along was added to the control cells.

Cytotoxicity and MTT Assay

IC_{50} value calculation for each cell line and PBMNs were determined by MTT assay as described.[7] For MTT assay, cells were seeded at 5 × 10^4 cells/well and incubated overnight, then treated with equal volumes of medium containing elemene final concentrations from 0.01 to 320 μg/ml. All experiments were repeated at least twice, each in duplicate.

Flow Cytometry Analysis

Cell sample preparation and DAPI staining for flow cytometry analysis were performed according to the method as reported previously.[8,9] Cell cycle distribution was determined using a CA-II instrument (Partec, Germany). Resulting DNA distribution were then analyzed for proportion of cells in apoptosis. Data was analyzed by Multicycle software (Phoenix Flow System, San Diego, CA).

Electronic Microscope Examination

HL-60 cells treated for 24 hours with different concentrations of elemene. Cell samples were conventionally fixed.[10] Cells were examined and photographed using Philips EM410 transmission electron microscope (TEM).

Agarose Gel Electrophoresis of Apoptotic DNA

Cells were treated with various concentrations of elemene then harvested and washed with phosphate-buffered saline (PBS). 1 × 10^6 Cell pellets were suspended in 1 ml of lysis solution containing 5 mmol/L Tris-HCl pH 8, 0.25% NP-40, 1 mmol/L EDTA, 10mg/ml RNase at 37 ℃ for 1 hour. 25 μl of proteinase K (20 mg/ml) was added to the above suspension for 1 hour at 37 ℃. The supernatant of the cell lysat was analyzed by electrophoresis in a 1.5% agarose gel containing 0.2% μg/ml ethidium bromide (EB), and visualized under UV illumination, recorded by a IS-1000 Digital Image System.[11] Dexamethasone-induced apoptotic DNA of rat thymus cells was used as positive control in agarose gel electrophoresis.

RESULTS

Inhibitory Effect of Elemene on the Proliferation of Human Leukemia Cells

Cell proliferation of leukemia cells treated with elemene were evaluated by MTT assay. When leukemia cells in culture were treated with elemene for 72 hours, the growth of HL-60 leukemia cells and K562 leukemia cells was significantly inhibited. The IC_{50} values of elemene for HL-60 and K562 cells were found to be 27.5 μg/ml and 81 μg/ml, respectively, while treated with elemene for 72 hours, IC_{50} for peripheral blood leukocytes (PBL) from normal blood donor was 254.3 μg/ml.

Effect of Elemene on Cell Cycle Progression of Tumor Cells

When HL-60 cells treated with 10 μg/ml of elemene, the proportion of cells at G_2 M phase was relatively decreased for 4 hours treatment. However, during 24 hours to 48 hours treatment, no significant effect of 10 μg/ml elemene on cell cycle could be observed. When treated with 20 μg/ml elemene, the proportion of cells at G_2 M phase was markedly decreased and dependent on the time of drug exposure. In the histogram, the distinct apoptotic feature of sub-G1 peak (Ap peak) were observed (Figure 1). The percentage of apoptotic cells for the treatment of 4, 24, 48 hours were 41.5%, 35.3% and 47.7%, respectively. When the dose of elemene increased to 40 μg/ml, total cells were undergoing death completely beginning from 4 hours treatment. Cell cycle analysis by flow cytometry revealed that elemene has great influence on cell cycle progression of HL-60 cells and its effect is dependent on the dose and time of drug exposure. The inhibitory effect of elemene on proliferation of HL-60 cells was associated with cell cycle arrest from S to G_2 M phase transition and reduced the proportion of cells at the G_2 M phase, consequently inhibiting mitosis of tumor cells. On the other hand, the elemene-treated tumor cells were rapidly induced undergoing apoptosis.

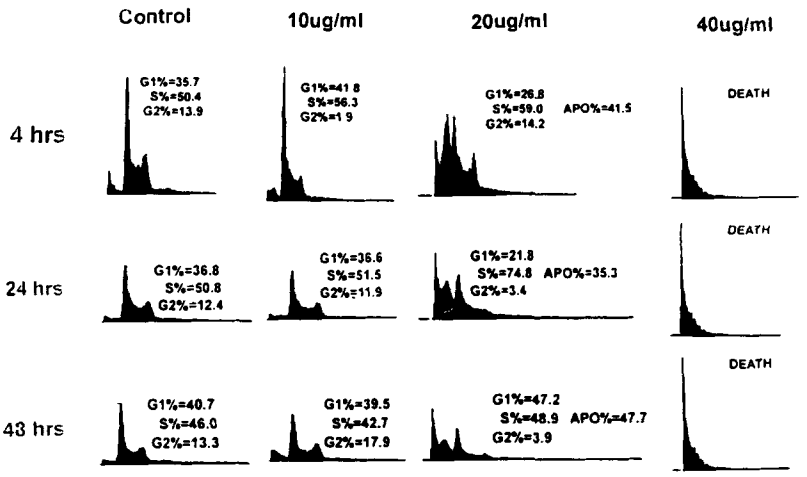
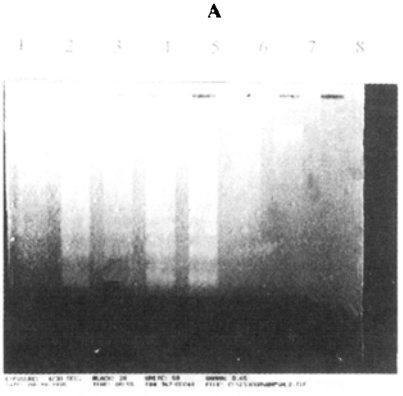


Fig. 1. Effect of elemene on cell cycle progression of tumor cells.

Cell cycle phase arrest and subsequent apoptosis induced by varying concentrations of elemene in HL-60 cells. The cultures were treated for 4 hr, 24 hr, 48 hr. At various time, cells were analyzed for apoptotic cell populations and for alterations in cell cycle stages by flow cytometry. Apoptotic cells have fractional DNA content and are represented by a "sub-G1" peak. Cells treated with 20 μg/ml of elemene beginning from 4 hr arrested in S phase and also demonstrated significant apoptotic responses.

Apoptosis of HL-60 Cells Induced by Elemene

An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180 bp units producing a characteristic ladder on agarose gel electrophoresis.[12] To characterize elemene-induced apoptotic cell death in HL-60 cells, internucleosomal DNA fragmentation was analyzed after the cells were exposed to different concentrations of elemene from 2 hours to 24 hours. The distinct internucleosomal DNA fragmentation ladder could be observed in the elemene-treated HL-60 cells for 2 hours (Figure 2A). Moreover, in comparison with 2 hour treatment, the dose of elemene for triggering DNA fragmentation was quite lower than that for 24 hours treatment (Figure 2 B). When morphological changes in the 24 hours elemene-treated HL-60 cells were examined by TEM, the distinct apoptotic features were observed, which were condensed chromosomes and apoptotic bodies (Figure 3A-3E). The characteristic ultrastructural alternations in the elemene-treated HL-60 cells were associated with the dose of drug exposure and very similar to those of the Cisplatin-treated HL-60 cells (Figure 3F).[15,16]



B

1 2 3 4 5 6 7 8

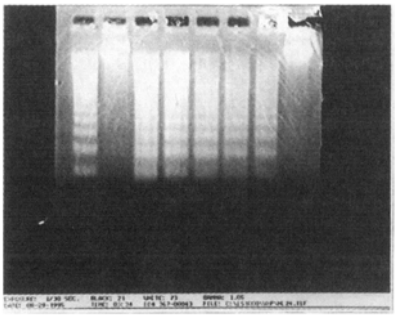
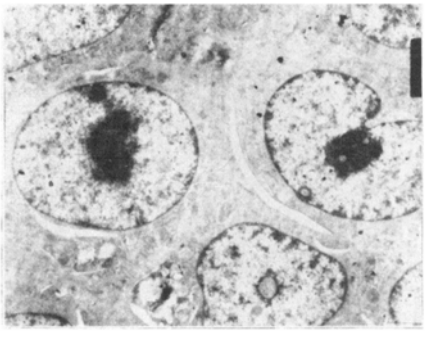


Fig 2. Dose-response of elemene-induced internucleosomal DNA fragmentation.

(A) HL-60 cells were treated with various concentrations of elemene for 2 hr. control (lane 1), elemene 10 μg/ml (lane 2), 50 μg/ml (lane 3), 100 μg/ml (lane 4), 200μg/ml (lane 5), 500 μg/ml (lane 6), 1000 μg/ml (lane 7). (B) HL-60 cells treated with various concentrations of elemene for 24 hr. Dexanmethasone-induced apoptotic DNA of rat thymus cells (lane 1), control (lane 2), elemene 10 μg/ml (lane 3), 50 μg/ml (lane 4), 100 μg/ml (lane 5), 200 μg/ml (lane 6), 500 μg/ml (lane 7).

A



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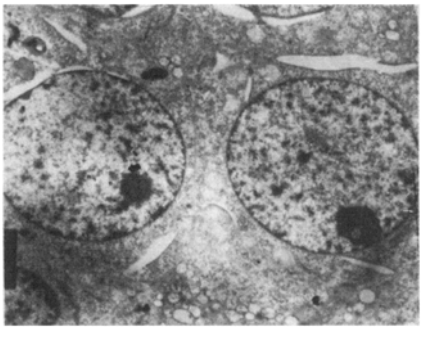

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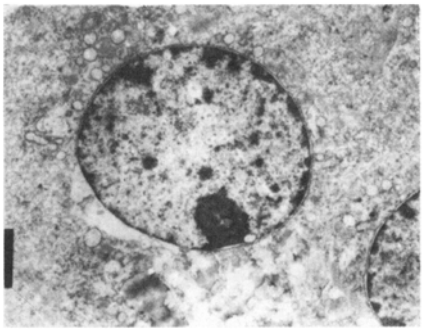

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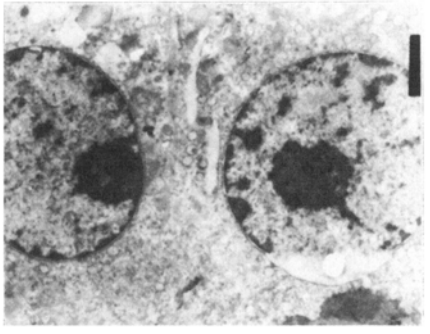

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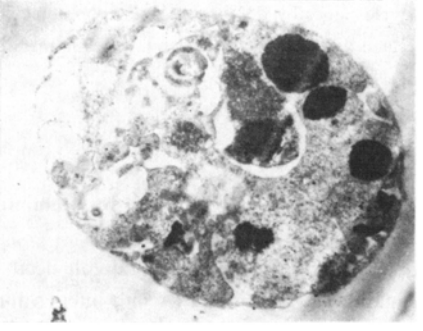

F

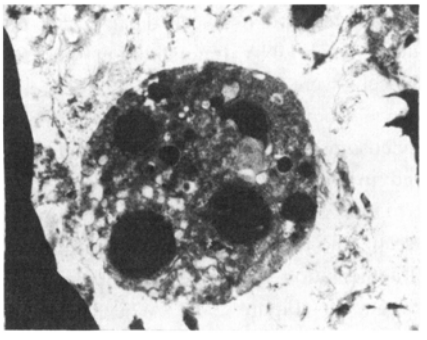

Fig 3. Analysis of morphological changes of HL-60 cells induced by elemene

Control of HL-60 leukemia cell (A), cells treated with 10 μg/ml of elemene, structure of nuclear still in good condition (B), cells treated with 20 μg/ml of elemene, chromatin anchored to inner side of nucleus membrane, numbers of endoplasmic reticulum (ER) increased, mitochondrion in good condition (C), cells treated with 20 μg/ml of elemene, local lesion of nucleus membrane demonstrated (D), cells treated with 30 μg/ml of elemene, chromosome condensation, rudimental nucleus membrane and vacuolization of degenerative cells (E), cells treated with 10 μg/ml of CDDP, karyopyknosis and apoptotic body surrounded by nucleus membrane (F).

DISCUSSION

The main active component of ELEMENE INJECTION, a newly approved anticancer drug, is β-elemene, which has been demonstrated effective not only for treatment of several tumors on the body surface but also for the tumors of lung, liver, brain and others. Less side-effects of ELEMENE INJECTION were reported in clinical trial. No overt signs of elemene-induced toxicity of the function of liver and kidney and no suppression of bone marrow were observed.

Pharmacological study indicated that β-elemene was active against a variety of tumor cell lines *in vitro* tests. It was found that elemene possesses direct cell killing activity via inhibition of the synthesis of DNA, RNA and protein in tumor cells but the mechanism by which elemene leads to cell death is not entirely clear. We are currently investigating the possibility that human tumor cells may undergo apoptosis after the treatment of β-elemene. Our results[13] indicate that β-elemene causes inhibition of the growth of tumor cells and an apparent block in cell cycle progression from S to G_2 M phase, subsequently resulting in apoptosis in tumor cells. Our data suggest that the induction of apoptosis contributes to the mechanisms for antitumor activity of β-elemene. These data also indicate that the growth inhibitory effect of β-elemene on tumor cells were much stronger than that on normal cells based on the comparison of IC_{50} between the leukemia cells and normal peripheral blood leukocytes. In our study, HL-60 cells were more susceptible than K562 cells to β-elemene. It means that the sensitivity to β-elemene of various tumor cells may be different, although antitumor spectrum of this agent is broad. It is noteworthy that several factors may influence the effect of β-elemene therapy, which besides the difference in sensitivity of various tumors, also include methods of giving medicine and pharmacokinetics. Therefore more attention should be paid to these factors in the β-elemene therapy. On the other hand, further studies are needed to determine the effect of β-elemene on multidrug-resistant (MDR) tumors or reversal of drug resistance in combination with β-elemene and other chemotherapeutic agents.

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