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Basic Investigations

INDUCTION OF APOPTOSIS OF HUMAN LEUKEMIA CELLS BY α-ANORDRIN

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The apoptosis-inducing effect of α-anordrin (ANO) was investigated in this study. ANO 10–50 μM inhibited the growth of both human leukemia HL-60 and K562 cells by 19–52%. Electron microscopy showed that ANO-treated cells exhibited the drastic changes including cell shrinkage, chromatin condensation, nuclear fragmentation, typical of apoptosis. Gel electrophoresis of DNA extracted from both HL-60 and K562 cells treated with ANO revealed characteristic "ladder" pattern. ANO 50 μM for 48 h caused approximately 50–70% apoptosis. Cycloheximide (CHX) and actinomycin D (Act D) did not prevent ANO-induced apoptosis in K562 cells, however, apoptosis of HL-60 cells in the presence of ANO was partially blocked by these two agents. Moreover, it was found that tamoxifen synergically potentiated and estradiol partially antagonized ANO-induced apoptosis in HL-60 cells. Results demonstrated that ANO could induce tumor cell apoptosis, which might contribute to its anticancer action.

Key words: Apoptosis, α-anordrin, Leukemia cells.

Anordrin is a contraceptive agent which was first developed in China. Previous study showed that α isomer of anordrin (ANO) exhibited antitumor effect both *in vitro* and *in vivo*.[1,2] It was also found that at small doses, ANO could induce differentiation of human promyelocytic leukemia HL-60 cells.[3] However, the mechanism of its antitumor activity was still unclear. In this work, we further investigated the tumor-inhibitory effect of ANO to explore the possible mechanisms of its action.

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MATERIALS AND METHODS

Drug

ANO was produced by Shanghai No. 19 Pharmaceutical Factory and purified in our institute.[4] Its stock solution was made in ethanol at the concentration of 50 mM. The final concentration of ethanol was less than 0.1% which had no significant effect on the parameters measured in the present experiments.

Cell Culture

Human leukemia HL-60 and K562 cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin at 37 ℃ in a humidified atmosphere containing 95% air/5% CO_2. Exponentially growing cells were used in all experiments.

Morphological Examination

Cell morphological changes were observed

under electron microscope.

DNA Gel Electrophoresis

Analysis of DNA fragmentation was performed by agarose gel electrophoresis according to the method described by Kamesaki, et al.[5]

Cell Cycle Analysis

The cells untreated and treated were harvested by centrifugation, washed in PBS, and fixed in ice-cold 70% ethanol overnight. Following fixation, the cells were centrifugated and the cellular DNA was stained with 50 μg/ml propidium iodide (Sigma) dissolved in PBS containing RNase A 20 μg/ml (Boehringer Mannheim), 0.1% Triton X-100 (Sigma). The fluorescence of individual cells was measured by FACStar plus flow cytometer (Becton Dickinson).

RESULTS

Cell Growth

The dose-response effect of ANO on the growth of human leukemia HL-60 and K562 cells was examined. Exposure of these two cell lines to ANO 2.5–50 μM for 48 h resulted in dose- and time-dependent growth inhibition (Figure 1). HL-60 cells were more sensitive to ANO than K562 cells as reflected by the IC50 value (26 μM for HL-60 cells and 38 μM for K562 cells).

Morphological Assessment

Electron microscopic observation showed that ANO-treated HL-60 cells exhibited cell shrinkage, chromatin condensation, and nuclear fragmentation, characteristic of apoptosis (Figure 2). K562 cells had the same morphological changes as that in HL-60 cells.

DNA Gel Electrophoresis

Agarose gel electrophoresis of DNA extracted from ANO-treated cells revealed typical "ladder" pattern in agarose gel, suggesting that DNA fragmentation preferentially occur at internucleosomal or linker DNA regions which was a major hallmark for apoptosis (Figure 3, 4). DNA electrophoresis also showed that ANO-induced apoptosis of K562 cells was not prevented by protein synthesis inhibitor cycloheximide (CHX) (Figure 3) and RNA synthesis inhibitor actinomycin D (Act D) (data not shown), in contrast, these two agents enhanced the extent of apoptosis. Interestingly, HL-60 cell apoptosis in the presence of ANO was partially blocked by CHX and Act D. The blocking effect of CHX was stronger than that of Act D (Figure 4).

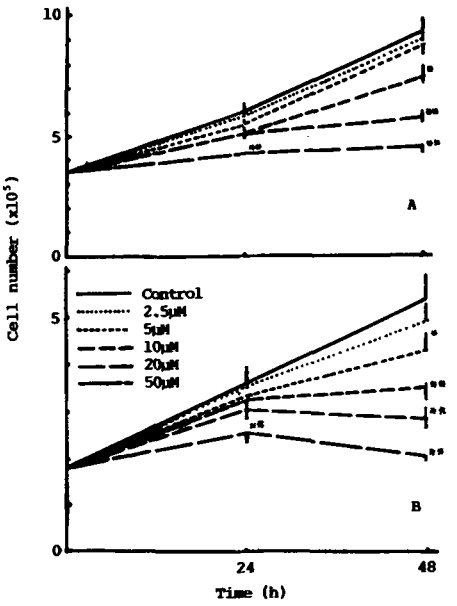

Fig. 1. Effect of α-anordrin (ANO) on the growth of K562 (A) and HL-60 cells (B). Cells were incubated with ANO 2.5–50 μM for 24–48 h. n=3, $\bar{x}\pm s$. $^{*}P<0.05$, $^{**}P<0.01$ vs control.

Cell Cycle Analysis

It has been widely accepted that apoptotic cells have reduced DNA stainability following staining with a variety of fluorochromes. So, the appearance of cells with low stainability, lower than that of G_1 cells ("sub-G_1 peak") in cultures has been considered to be the marker of apoptosis.[6] ANO treatment led to the formation of "sub-G_1 peak" in DNA content frequency distribution histogram (Figure 5) and caused approximately 50–60% cell apoptosis after exposure of HL-60 or K562 cells to 50 μM ANO for 48 h. Apoptosis-inducing effect was dose- and time-dependent.

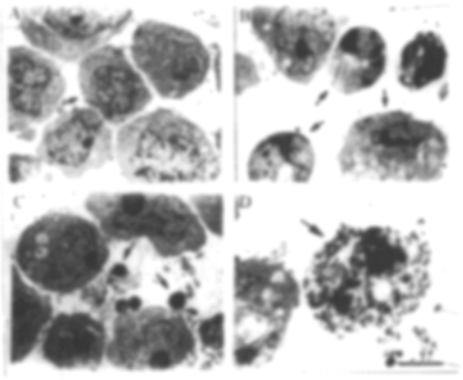

Fig. 2. Morphology of HL-60 cells. Cells were untreated (A) and treated with α-anordrin 50 μmol L-1 for 48 h (B, C, D). Arrow: A, normal HL-60 cells; B, apoptotic cells; C, apoptotic body; D, necrotic cells. (Electron micrograph, original magnification: A, B, C, × 3100; D, × 4700; Bar=5 μm.

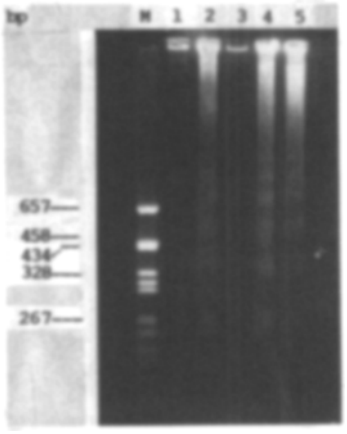

Fig. 3. Agarose gel electrophoresis of DNA extracted from K562 cells, untreated (lane 1) or treated with cycloheximide (CHX) 1 μM (lane 2), α-anordrin (ANO) 50 μM (lane 3), ANO 50 μM+CHX 1 μM (lane 4), CHX 400 μM (lane 5) for 24 h, respectively. M: molecular weight marker.

ANO possessed antiestrogenic activity which was related to its contraceptive action.[7] In this work, we investigated whether or not the apoptosis-inducing effect of ANO could be antagonized by estradiol (E_2) and potentiated by estrogen receptor (ER) antagonist tamoxifen (TAM). As expected, TAM synergestically augmented and E_2 partially suppressed apoptosis caused by ANO, which was analyzed by median-effect principle software provided by Prof. Chou.[8] In addition, both TAM and E_2 could induce HL-60 cell apoptosis, altered cell cycle distribution (Figure 5).

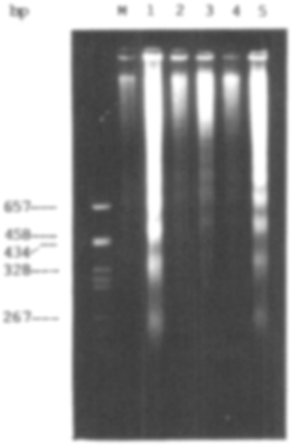

Fig. 4. Agarose gel electrophoresis of DNA extracted from HL-60 cells. HL-60 cells were untreated (lane 1) or treated with α-anordrin (ANO) 50 μM (lane 2), cycloheximide (CHX) 1 μM (lane 3), ANO 50 μM+CHX 1 μM (lane 4), actinocycin D (Act D) 0.005 μM (lane 5), and ANO 50 μM+Act D 0.005 μM (lane 6) for 24 h. M:molecular weight marker.

DISCUSSION

Antineoplastic agents exert their antitumor activity by different mechanisms including growth inhibition, differentiation-induction, immune response modification, and recently defined mechanism: induction of apoptosis of tumor cells. Among them, apoptosis is one of the most attractive area of cancer research in recent years. Evidence accumulated demonstrated that the antitumor potency of virous antitumor drugs was closely associated with their ability to induce target cell apoptosis.[9] So, investigation of the characteristics of a drug in triggering apoptosis will provide important information for cancer chemotherapy. The results obtained in the present study clearly showed that ANO could induce apoptosis in both HL-60 and K562 cells which was confirmed by the appearance of the internucleosomal

DNA fragmentation and typical apopotic morphological changes after treatment with ANO.

Apoptosis is a tightly controlled gene-directed process, during which a number of related genes express their product to initiate the cell death program.[10] Thus, apoptosis occurring in a number of circumstances could be abrogated or delayed by inhibitors of macromolecular synthesis.[11,12] Indeed, CHX and Act D did diminish the extent of apoptosis induced by ANO in HL-60 cells, but in K562 cells, it was not observed. Even CHX and Act D enhanced ANO-induced apoptosis. These data suggested that diverse regulatory mechanisms of apoptosis exist in different cell lines.

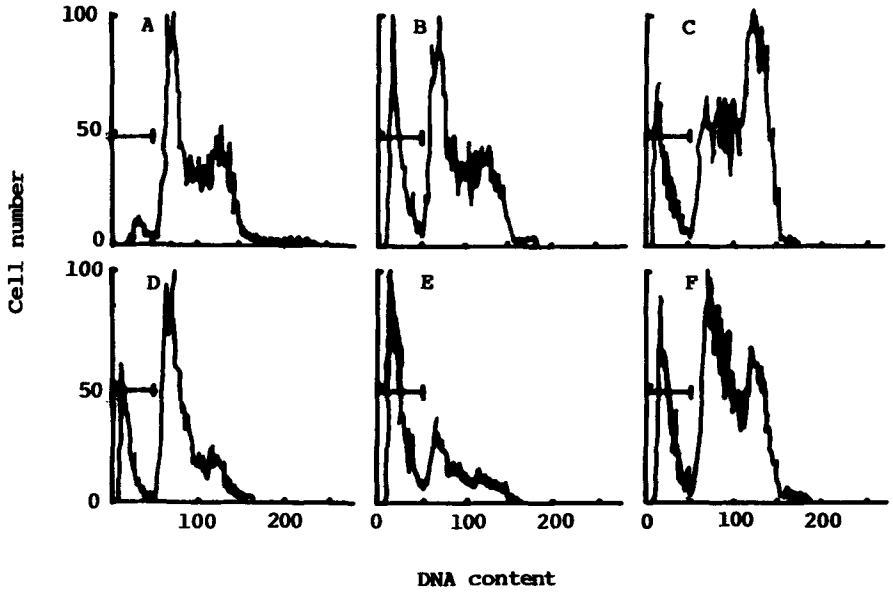

Fig. 5. Effect of α-anordrin (ANO), tamoxifen (TAM), estradiol (E2) each alone or in combinaton on apoptosis and the cell cycle distribution of HL-60 cells. Cells were treated with respective drug for 8 h. A: control, G_1=35%, S=51%, G_2/M=14%, Ap=4%; B: TAM 10 μM, G_1=42%, S=46%, G_2/M=12%, Ap=26%; C: E2 10 μM, G_1=9%, S=38%, G_2/M=53%, Ap=17%; D: ANO 25 μm, G_1=52%, S=44%, G_2/M=4%, Ap=20%; E: ano 25 μm+tam 10 μm, G_1=38%, S=58%, G_2/M=4%, Ap=58%; F: ano 25 μm+e2 10 μm G_1=38%, S=44%, G_2/M=18%, Ap=21%, respectively.

ANO-induced apoptosis was substantially enhanced by ER antagonist TAM and partially abolished by ER agonist E_2, it is indicated that ER played an important role in mediating this effect. But the fact that E_2 was unable to abrogated the apoptosis completely implied that other pathways might be involved in this process. Sutherland et al.[13-15] have reported that E_2 could not reverse the rapid cytotoxic effect of high concentration TAM and identified a high affinity antiestrogen binding site different from the classic ER. Considering the antiestrogenic activity of ANO, it appears to be likely that ANO exerts its cytotoxicity also in part through its interaction with the antiestrogen binding site.

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