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Ara-C INDUCED APOPTOSIS IN HUMAN MYELOID LEUKEMIA CELL LINE HL-60: INDUCING APOPTOSIS IS THE PRIMARY MECHANISM OF CHEMOTHERAPY

Zhou Jianfeng 周剑锋 Chen Yan 陈燕 Li Chongyu 李崇渔 Xiang Jianping 向建平
Yu Dongjiao 喻冬娇 Zou Ping 邹萍 Wang Bianming 王辩明

Institute of Hematology, Union Hospital, Tongji Medical University, Wuhan 430022

Objective: To elucidate the pattern of chemotherapy drugs induced apoptosis and its role in chemotherapy of acute leukemia. Methods: Apoptosis induced by Ara-C in human myeloid leukemia cell line HL-60 was investigated by applying light microscope, electron microscopy combined with DNA electrophoresis and flow cytometry analysis techniques. Results: Apoptosis persisted throughout 36 h following addition of Ara-C with a gradual augmentation. Efficiency of apoptosis was enhanced in a dosedependent pattern, HL-60 treated with six other chemotherapy drugs and peripheral white blood cells from a AML case undergoing DA regimen chemotherapy exhibited typical DNA ladder pattern. Further investigation indicated that chemotherapy drugs apoptosis came into being possibly by downregulating the expression of c-*myc* and *bcl*-2 oncogenes. Conclusion: Chemotherapy induced apoptosis is the primary mechanism of chemotherapy.

Key words: Apoptosis, HL-60, Chemotherapy, Ara-C.

Chemotherapy is now generally the treatment of primary choice for acute leukemia (AL). To design ideal chemotherapy regimen of acceptable toxicity but vastly improved potency, the mechanism of killing AL cells must be understood further. Although we know a great deal about genotoxicity of antitumor drugs, we still know little the origin of the antitumor activity. In most clinical context, the genotoxicity of antitumor drugs can not account for the factual killing efficiency observed.[1] Recently, emerging evidences demonstrated chemotherapeutic drugs differing in mechanism of action can induced apoptosis in AL cells, which implicated induced apoptosis may play certain role in AL chemotherapy.[2] We here reported the patterns of Ara-C induced apoptosis in human myeloid leukemia cell line HL-60 and evaluated the role of apoptosis in chemotherapy.

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MATERIALS AND METHODS

Cell Line and Reagents

HL-60 cell line was purchased from American Type Culture Collection (ATCC). rhGM-CSF Monoclonal antibodies (McAb) *bcl*-2, c-*myc*, APAAP second and third antibodies were kindly provided by Dr. Herrmann in Boehringer Mannheim company. RPMI-1640, fetal calf serum (FCS) were supplied by Gibco (USA). Ara-C was from Farmitilia Company (Italy).

Methods

Culture of HL-60 Cells

HL-60 cells were cultured in 15% FCS RPMI-1640. After growing exponentially, passage was performed. The cellular activity was 98% according to

0.4% trypan blue staining. All experiments at least repeated once to confirm the results.

The Patterns of Ara-C Induced Apoptosis

HL-60 cell concentration was 1×10^6/ml. The concentration of Ara-C was calculated by the formula.[3] 60×dosage used in clinical practice (mg/kg day)×2×10^3/5000. *In vitro* 10 μg/ml, 1000 μg/ml, 2000 μg/ml Ara-C was equal to low, routine, intermediate and high dose *in vivo* respectively. To observe the time pattern of Ara-C induced apoptosis, 100 μg/ml final concentration of Ara-C was added. From zero to 36 h, at an interval of 4 h, cellular viability was judged by typran blue exclusion test; The apoptosis rates were determined by morphologic observation under light microscope according to criteria: (1) cell shrank obviously with intact cytoplasm membrane; (2) cellular nuclear and cytoplasm condensed highly; (3) cellular cytoplasm blebbed extensively; DNA from treated HL-60 cells was analyzed by gel electrophoresis. The cell cycle distri-bution was examined by flow cytometry (FACS). Positive cell rate and immunofluorescence of c-*myc*, *bcl*-2 antigens were determined by FACS and APAAP technique 24 h before and after Ara-C treatment. To observe the apoptosis efficiency produced by varied doses of Ara-C, Ara-C at final concentration of 10, 50, 100, 500, 1000, 2000, μg/ml was added respectively. 6 h after addition of Ara-C, DNA was extracted and analyzed by gel electrophoresis. The cell cycle distribution and apoptotic rate was examined by FACS.

Apoptosis Induced by Other Chemotherapeutic Drugs

HL-60 cells at density of 1×10^6/ml were treated by Harringtonine (H, 10 μg/ml), Methotrexate (MTX, 10 μg/ml), Aclacinomycin (ACR 0.4 μg/ml), Mitoxantrane (Mito, 10 μg/ml), Adriamycin (ADR, 20 μg/ml) and Etoposide (VP_{16} 10 μg/ml) for a duration of 10 h. Peripheral white blood cells from one case AML undergoing DA (DNR, Ara-C) regimen chemotherapy were isolated. DNA were extracted and analyzed by gel electrophoresis.

DNA Gel Electrophoresis

Pretreated 2×10^6 cells were fixed in cooled 10 ml 70% ethanol for at least 24 h. The cells were collected by centrifugation. After 3 times wash with PBS, 80 μl phosphate-citrate buffer (PC) (0.2 mol/L Na_2HPO_4:0.1 mol/L citrate= 192:8, pH 7.8) was added to cell pellets and incubated for 1 h at 37 ℃. After centrifugation at 1000 g for 5 min, the supernatant was collected for DNA isolation by routine acetate-ethanol precipitation method and the cell pellets were applied to FACS analysis for cell cycle distribution and apoptosis rates. 3 μl 0.25% NP40, 3 μl RNase (2 mg/ml) were added to extracted DNA and incubated for 30 min at 37 ℃. 3 μl Protein K (1 mg/ml) was added and incubated for another 30 min at 37 ℃. 10 μl loading buffer was added and 1.5% gel electrophoresis was performed at voltage of 1 V/cm. 3 h after, the gel was stained in 0.5 μg/ml ethidium bromide for 25 min and photographed under UV light analyzer PBR 322/HinfI was used as markers.

HL-60 Leukemia Colony Forming Unit (CFU-L)

Total culture volume was 1 ml, which containing 2, 105 HL-60 cells, 20% FCS, 0.8% methycellulose, 0.5 ng/ml rhGM-CSF and RPMI-1640. 200 μl media was used for CFU-L culture with triplicate. After incubation at 37 ℃, 5% CO_2 atmosphere for 7 days, colonies were counted under inverse microscope.

Alkaline Phosphatase Anti-alkaline-phosphatase Technique (APAAP)

The cells were fixed for 5 min with 100% acetone. The c-*myc*, *bcl*-2 McAb, second and third antibodies were added in turn. The samples were stained with fast red, washed by PBS, and stained with hematoxylin again. Total 200 cells were counted under microscope.

Detection of FACS

After the routine pretreated procedures, the cell cycle distribution and apoptosis rates was analyzed. The positive cell rate and immunofluores-cence were determined.

RESULTS

Morphologic Features of Apoptotic Cells

Morphologically apoptotic cell under light

microscope appeared as single small shrank body with intact cytoplasm membrane and highly condensed chromatin and cytoplasm. The chromatin of apoptotic cell collapsed to form a stretch of highly condensed mass along periphery. The cytoplasm membrane showed extensively blebbing, some of which were budding off to produce apoptotic bodies (Figure 1). Apoptosis cell under electron microscope showed specific nuclear and cytoplasm condensation resulting in forming of apoptotic bodies.

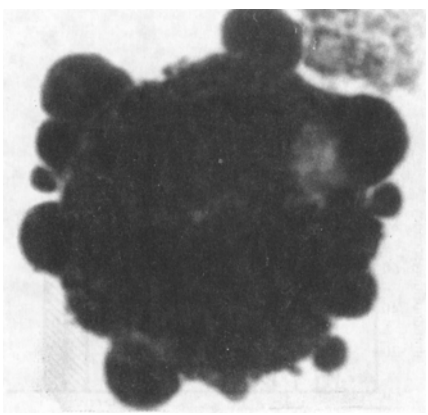

Fig. 1. Ara-C induced apoptotic HL-60 cell as stained by May-Grunwald-Gimesa stain

Apoptosis Induced by Varying Lengths of Time of Ara-C Incubation

Under light microscope only 4 h after addition of Ara-C, typical apoptotic cells appeared. 12 h after Ara-C treatment, the apoptotic rate reached a peak (40% apoptotic rate) and then decreased with time going on accompanying by the increase of morphologically necrotic cells. Necrosis cells defined as typran blue staining appeared slower than apoptosis cells did. 12 h after addition of Ara-C the necrosis rate was only 10% and thereafter increased with time proceeding (Figure 2). The results analyzed by DNA gel electrophoresis and FACS showed that apoptosis persisted throughout 36 h with a gradual augmentation following addition of Ara-C (Figure 3).

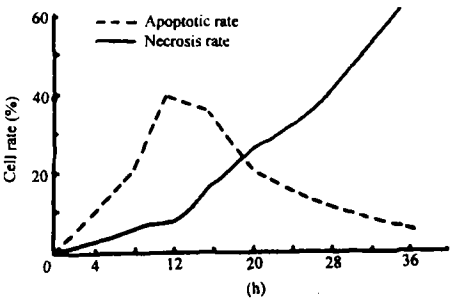

Fig. 2. Ara-C induced apoptosis for different lengths of time incubation observed by May-Grunwald-Gimesa stain and typran blue exclusion test.

Apoptosis Induced by Different Doses of Ara-C

The results of DNA gel electrophoresis showed efficiency of apoptosis was enhanced in a dose-dependent pattern within a dose range from 10 to 2000 μg/ml (Figure 4). The simultaneous FACS analysis showed the same pattern (Table 1) of killing rate induced by 8 h length of Ara-C treatment: 8 h after Ara-C treatment, the necrotic rate was only 4%, while the apoptotic rate was 20%. The killing rate of CFU-L was 40% at this time point of Ara-C treatment. And so, apoptosis, but not necrosis, at this time was the main contributory mode to killing of HL-60 leukemic colonies.

Table 1. Apoptosis induced by different doses of Ara-Canalyzed by FACS

Ara-C dose (μg/ml)	Apoptotic rate	G_1	S	G_2/M
0	1.5	60.3	21.3	14.2
10	5	61.8	20.8	10.0
50	7	61.8	20.8	6.0
100	12	62.8	18.6	6.0
500	16	64.0	10.6	4.2
1000	24	63.0	7.4	5.3
2000	30	59.0	6.0	7

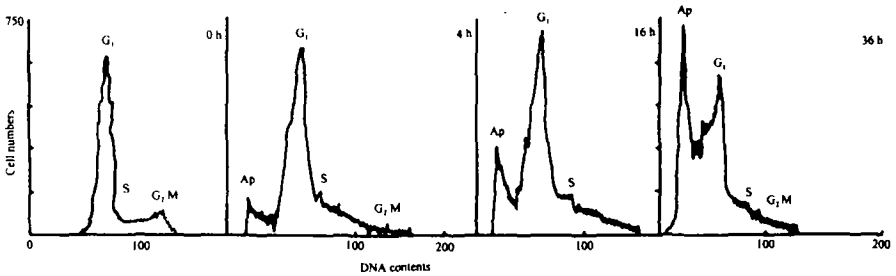

Fig. 3. Ara-C induced apoptosis for different lengths of time incubation observed by FACS analysis

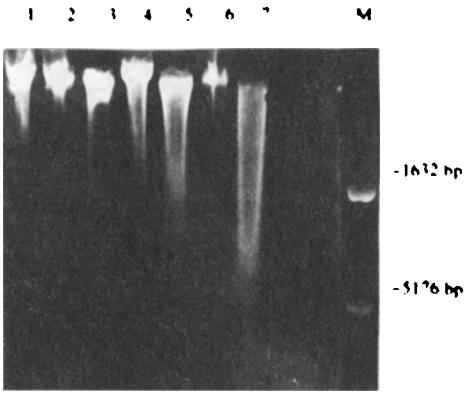

Fig. 4. Apoptosis induced by different doses of Ara-C, dose from 1 to 7 was 10, 50, 100, 500, 1000, 2000 μg/ml. Lane M was marker PBR 322/HinfI.

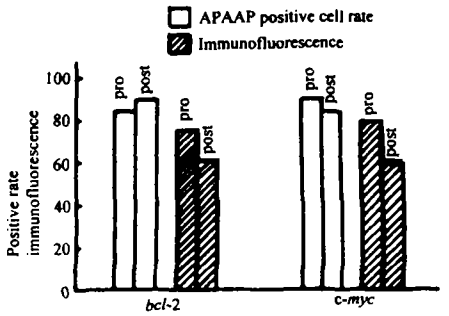

Fig. 5. Ara-C induced regulated expression of *bcl*-2 and c-*myc* (positive cell rate, immunofluorescence)

Apoptosis Induced by Other Chemotherapeutic Drugs Both *in Vitro and in Vivo*

The results of DNA gel electrophoresis showed that all six cytotoxic agents used induced apoptosis in HL-60 cells. The DNA extraction from one case of AML undergoing DA regimen chemotherapy also manifest typical DNA ladder pattern. These findings demonstrated chemotherapeutic drugs of different action shared the common mechanism of killing AL cells by inducing apoptosis both *in vitro* and *in vivo*.

Ara-C Induced Regulated Expression of C-*myc* and *Bcl*-2 Antigens

The Figure 5 showed 24 h after treated by Ara-C the immunofluorescence of c-*myc*, *bcl*-2 protein and the c-*myc* positive cell rate decreased, while the *bcl*-2 positive cell rate increased.

DISCUSSION

Apoptosis is a mode of cell death involved an active participation of the cell in the self destruction that distinguish it from necrosis. In contrast to apoptosis, the alternative mode of cell death, necrosis, is a passive, catabolic and degenerative process. Necrosis is characterized by swelling of cell and early rupture of cytoplasm membrane. The necrotic cells failed to exclude typran blue dye during the early phase of onset of cell death, which distinguished from apoptotic cells. One of the most characteristic features of apoptosis is activation of an endonuclease which has preference to the linker DNA sections. The products of apoptosis induced DNA degradation are discontinuous DNA stretches of the size equivalent of mono- and oligo-nuclesomes that form a typical "DNA ladder" during gel electrophoresis. These biochemical events are paralleled by changes in cell morphology. The loss of intracellular water is

reflected very early by cell shrinkage and condensation of the cytoplasm. This is accompanied by condensation of chromatin, which starts from the nuclear periphery. The cytoplasm blebs to detach from the cell as apoptotic bodies. In our present experiment, typical apoptosis in HL-60 cells induced by Ara-C was manifested both by morphologic observation under light electron microscope and gel electrophoresis techniques.

Apoptosis, as determined by DNA gel electrophoresis and FACS, persisted throughout 36 h following addition of Ara-C with a gradual augmentation, which contradicted with the results revealed by morphologic observation. The possible reason for these paradoxical results might well be due to the appearance of secondary necrosis as a results apoptotic cells failing to be phagocytized *in vitro*. And so, the conclusion drew by Lotem et al.[4] According to the results of morphologic observaton that apoptosis might only play a role in the early phase of chemotherapy should be taken cautious. In our study, HL-60 cells treated by Ara-C for a duration of 8 h resulted in a 40% HL-60 CFU-L killing in spite of only a 4% necrosis rate, which implicated apoptosis might well account for the effective killing of HL-60. Administration of many chemotherapeutic drugs of different action both *in vivo* and *in vitro* all triggered apoptosis as a common mechanism of killing AL cells. These observation implicated apoptosis as the primary mechanism of chemotherapy.

Ara-C has long been considered to be an S-phase -specific drug. Yet, the administration of intermediate or high dose Ara-C regimen produces a rapid and substantial reduction in the leukemia blast counts and have been successfully used in the treatment of AML to overcome resistance to conventional doses of Ara-C. This implicates Ara-C administrated at these doses may involve other killing mechanism of operative in non S-phase of the cell cycle. We here observed that the efficiency of Ara-C induced apoptotsis were enhanced in a Ara-C dose-dependent way, which suggested intermediate and high doses Ara-C pon-tentiated apoptosis was an important contributors mechanism to good therapy response. These findings have potential clinical implications that modulating the propensity of cells to respond by apoptosis could be a promising strategy of chemotherapy.

Chemotherapy induced apoptosis were accompanied by downregulated expression of *bcl*-2 and c-*myc* oncoprotein, which was supported by our results of FACS immunofluorescency analysis. However, 24 h after Ara-C treatment, the *bcl*-2 positive cell rate showed upregulation. This might be caused by the preferring killing of Ara-C to cell subpopulations with low or negative *bcl*-2 expression and resulted in a uprising of *bcl*-2 positive cell rate. The minor residual leukemic cells with high expression of *bcl*-2 may well be contribute a lot to the development of drugs resistance.

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