Apoptosis of Primary Acute Leukemic Cells Induced by Aclacinomycin in Vitro

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Abstract **Objective** To elucidate the inhibition to proliferation of acute leukemic(AL) cells induced by aclacinomycin(ACM) in vitro and its mechanism. **Methods** The proliferation inhibition of leukemic cells of 37 patients with AL was examined by MTT assay. The apoptosis of leukemic cells of 20 patients with AL was analyzed with the method of TUNEL. **Results** Cytostatic effect induced by ACM was significant. The apoptosis of leukemic cells induced by ACM was confirmed from the change of cell morphology and DNA agar glucose electroresis. When AL cells were cultured with ACM for 15 hours, the rate of TUNEL-positive AL cells was significantly higher than that in the control group (30.89±15.90% vs 14.85±15.90%, $p<0.01$). The TUNEL-positive rate was correlated closely with MTT proliferation inhibition of AL cells induced by ACM ($\gamma=0.326, p=0.04$). **Conclusions** These findings suggested that ACM can induced apoptosis of primary AL cells, that might be one of the mechanism of ACM-induced proliferation inhibition of leukemic cells.

Key Words aclacinomycin; leukemic cells, primary; apoptosis

Aclacinomycin(ACM) was spreadly used to treat various human leukemia. Recently, low-dose ACM-based therapeutic regimens was focused to treated relapsed and refractory or aged leukemia[1,2]. We explored the effect of low-dose ACM on the proliferation of primary acute leukemic cells and its possible mechanism.

Patients and cells

37 patients with acute leukemia(AL) diagnosed by standard morphologic, cytochemical, immunologic and cytogenetic criteria, including 23 male,14 female, ageing from 14 to 69 years old(medium age was 31.9 years). Of the 37 patient, 11 cases (L2 subtype 10 cases, 1 case with lymphocyte-dominated acute hybrid leukemia) with acute lymphocytic leukemia (ALL) and 26 cases(various subtypes included M0 1 case, M1 2 cases, M2 7 cases, M3 8 cases, M5 7 cases, M6 1 case) with acute non-lymphocytic leukemia (ANLL). Before treatment, 2-3ml bone marrow fluid anti-coagulated with ACD (1:1) was aspirated, and mononuclear cells (MNCs) were separated by centrifugation on Ficoll-Hypaque. Greater than 99 % MNCs were exclusive to 0.2% trypan blue, furthermore beyond 80 % MNCs were leukemic cells.

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Materials

ACM was purchased from hangzhou pharmaceutical factory, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) from sigma, TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling) reagent kit from Boehringer Mannheim, Agarose and RNase from shanghai bioengineering company, and Proteinase K from shanghai huamei company.

Reference formula of similar blood concentration of chemotherapeutic agents[3]

$$\text{maximal blood concentration} = \frac{\text{drug dose/weight} \times \text{weight}}{\text{weight} \times 0.08 \times 1000}$$

For a 60-kg-weight patient, the maximal blood concentration of ACM was 4ug/ml, once injected with ACM 20mg by intravenous drip. Drug concentration in vitro was similar to maximal blood concentration.

Drug cytotoxicity of leukemic cells assessed by MTT assay

Drug cytotoxicity was determined with MTT assay. Cells were cultured in 96-well microculture

plates (Danish product). In 0.2ml medium, final concentration of 4ug/ml ACM was used to treat cells with equal volume RPMI 1640 medium free FBS as control group. Cells(1×10^6/ml) were cultivated in RPMI 1640 medium supplemented with 20% heat-inactivated FCS for up to 15 hours at 37℃, saturated humidity, 5% carbon dioxide atmosphere. Each sample was triple. MTT (5mg/ml) 20ul was added into the medium during a final 4-hour incubation, centrifugated and removed the supernatant, added dimethyl sulfoxide (DMSO) 0.2ml to the wells and mixed thoroughly. The optical density (OD) can be determined spectrophotometrically at 562 nm. Final OD was the average of the triple.

$$\text{MTT inhibition rate}=\frac{\text{OD of the control}-\text{OD of the ACM}}{\text{OD of the control}}\times100\%$$

Apoptosis

Cell morphology ACM-induced cells collected, prepared smear and Wright-Giemsa staining.

DNA extract and DNA agar glucose electroresis Cells (2×10^6/ml)were harvested and washed with PBS, lysed in lysis buffer (final concentration of 10 mmol Tris-Hcl, 10 mmol EDTA, 10 mmol NaCl, 0.5% SDS, 0.5 mg/ml proteinase K). Incubation for an hour at 50℃, and RNA digested by incubation with 50μg/mL RNAase for 2 hours at 37°C. Added 2 mol NaCl and removed by centrifugation and the supernatant subjected to extraction with 95% ethanol. Low molecular weight chromosomal DNA was precipitated and dissolved in TE (10mmol/L Tris, pH 8.1 mmol/L EDTA) 10ul. An equal volume of each sample was then loaded onto 1.8% agarose gels (1V/cm)for 5 hours and DNA laddering was visualized by ethidium bromide staining. The control of apoptosis-positive were HL60 cells treated with 0.5umol/L ACM and 0.5umol/L daunorubicin(DNR)[4,5].

Apoptosis quantified by TUNEL Boehringer Mannheim kit following the manufacturer´s instructions. Yellow-brown staining in the nucleus or cytoplasm was apoptosis-positive cells by light microscopy. Counted 500 cells and calculated the percent of apoptosis-positive cells.

Statistical analysis

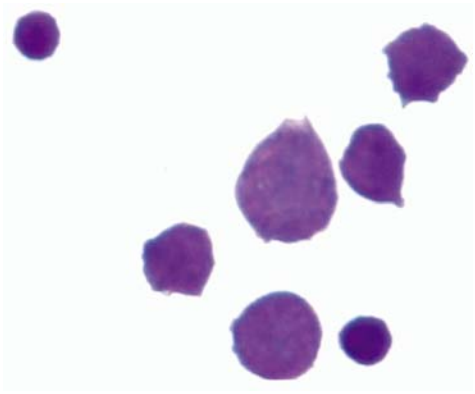
Statistical analysis was performed with the 2-tailed t test. An analysis of correlation was performed with the Kendall method. SPSS 10.0 software was used for the statistical analysis of data. $P<0.05$ was considered as statistically significant.

ACM-induced proliferation inhibition of primary leukemic cells

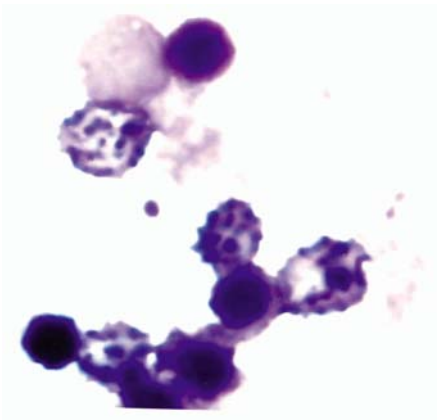
Incubated with ACM for 15 hours, the average proliferation inhibition rate of leukemic cells in 37 AL patients was 32.94±23.49%. of them, in 11 ALL patients was 36.64±22.34%, and 26 ANLL patients was 31.37±24.21%.

ACM-induced morphologic change of apoptosis

Primary AL cells incubated with ACM for 15 hours appeared apoptotic features, chromatin condensation and shattered nuclear and apoptotic bodies by Wright-Giemsa staining(Fig. 1).



(A)



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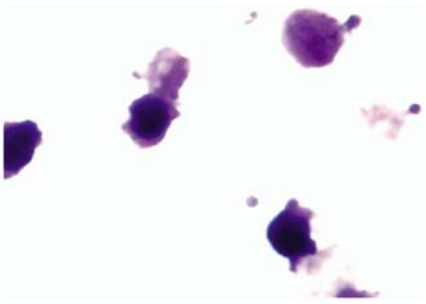


(C)

Fig. 1 Morphological change of primary AL cell by Wright–Giemsa staining

(A)Control. (B) and (C) ACM–induced. Morphologic changes showing features of apoptosis in primary AL cell after treatment with ACM for 15 hours (original magnification×1000).

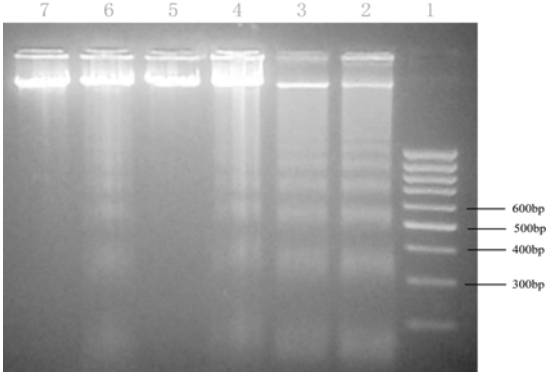


Fig. 2 DNA agar glucose electroresis
Estimation of DNA fragmentation after induction of ACM.

1. Marker;
2. Positive control(0.5umol/L DNR–treated HL60 cells);
3. Positive control (0.5umol/L ACM–treated HL60 cells);
4. ACM–treated AL cells of case A;
5. Control of case A;
6. ACM–treated AL cells of case B;
7. Control of case B.

ACM–induced DNA fragmentation

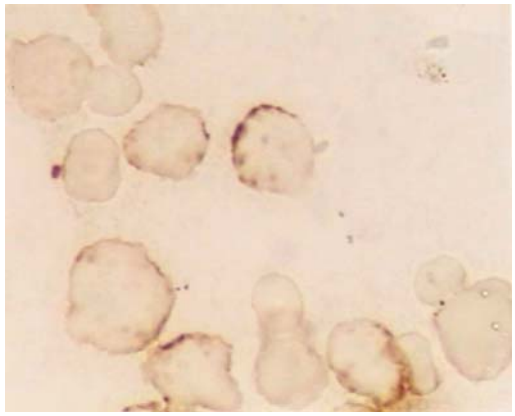
AL cells treated with ACM for 15 hours, DNA fragmentation(typical apoptotic ladder) was seen by agar glucose electroresis. No apoptotic band in the control group(Fig.2).

ACM–induced apoptosis

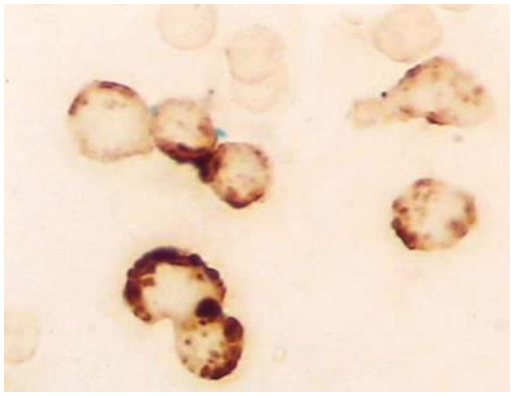
By TUNEL method, yellow–brown staining in the nucleus or cytoplasm was apoptosis –positive cells. ACM–treated AL cells for 15 hours appeared increased apoptosis (Fig.3). TUNEL–positive AL cells were significantly higher than that of the control (t=3.589; p=0.001). TUNEL–positive cells in the group of ALL and ANLL were also significantly higher than that of its control (t=2.499 p=0.028; t=2.657 p=0.014, respectively)(Table 1).

The correlation of ACM –induced cell apoptosis and cytotoxicity

AL cells of 20 patients treated with ACM for 15 hours, its TUNEL–positive rate was correlated closely with MTT proliferation inhibition rate (γ= 0.326, p=0.04). That indicated the effect of ACM–induced apoptosis might play a vital role in inhibiting the proliferation of leukemic cells.



(A)



(B)

Fig. 3 Apoptotic change of ACM–treated AL cells (TUNEL method)

(A) The control. (B) ACM–treated. Yellow brown staining in the nucleus or cytoplasm were apoptotic cells (original magnification×1000).

ACM was a kind of effective chemotherapeutic agent. Standard dose ACM was of 3 days of 40~60mg/m2/d. Standard dose ACM was restricted in the treatment of the aged leukemia because of higher tonic and side effects. On the other hand, relapsed and refractory leukemia, having accepted

Table 1 ACM-induced apoptosis of primary leukemic cells (TUNEL)

Group	n	TUNEL-positive cells (%)	Group	n	TUNEL-positive cells (%)
AL	20	30.89±15.90**	Al control	20	14.85±15.90
ALL	7	35.53±13.55*	ALL control	7	18.26±12.28
ANLL	13	28.38±17.00*	ANLL control	13	13.01±12.10

* The difference was significant(p<0.05)
** The difference was significant(p=0.001)

for a long term chemotherapy, appeared poor chemotherapy tolerance. That might be associated with more severe treatment-related morbidity [6]. Therefore, lower-dose ACM, 10~14mg/m2/d (7~14 days) was suggested to the treatment of aged or relapsed and refractory leukemia by monitoring the blood cell count and bone marrow proliferation . Better treatment effect and higher tolerance showed clinical value of lower-dose ACM.

Dartsch[7] thought ACM-inducing apoptosis was a kind of mechanism of the proliferation inhibition of HL60 and Jurket leukemic cell. Gieseler[8] and Larsen [9] thought apoptosis of ACM-treated HL60 was related to inhabit the activity of nuclear enzyme DNA topoisomerase II. Our data suggested ACM can induced apoptosis of primary AL cell. Apoptotic morphologic feature of primary AL cells including: reduce in volume or vacuolation in plasma, marginal distributed or shattered nuclear, condensation or breakage or budding of chromatin and apoptotic bodies, etc. ACM-induced AL cells also appeared biochemical characteristics of apoptosis, apoptotic ladder by agar glucose electroresis that was because of 180~200bp and its multiple DNA fragmentation formation from nucleosome joint-DNA degradation induced by endogenous endonuclease. With TUNEL method, it was discovered that higher apoptotic rate of AL cells treated with ACM for 15 hours than that in the control(30.89±15.90% vs 14.85±15.90%, p=0.001). TUNEL-positive cells in the group of ALL and ANLL were also significantly higher than that of their control (p=0.028, 0.014, respectively) (Table 1). And TUNEL-positive rate was correlated closely with the proliferation inhibition rate by MTT method (γ=0.326, p=0.04). The result indicated ACM-induced apoptosis may play a vital role in inhibiting the proliferation of primary AL cells. It was worth exploring the mechanism of ACM-induced primary AL apoptosis.

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