

Caspases promoted DADAG-induced apoptosis in human leukemia HL-60 cells

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KEY WORDS diacetyl(dianhydrogalactitol; apoptosis; DNA fragmentation; flow cytometry; caspases; HL-60 cells

ABSTRACT

AIM: To investigate the roles of caspases in diacetyl-dianhydrogalactitol (DADAG)-induced apoptosis in human leukemia HL-60 cells. **METHODS:** Inhibition of proliferation was measured by MTT assay. DADAG-induced apoptosis in HL-60 cells was observed by electron microscopy, flow cytometry, and DNA fragmentation assay. Caspase-3 activity was determined by ApoAlert CPP32 colorimetric assay kit. The cleavage of substrates of caspases was detected by Western blot. **RESULTS:** DADAG exhibited potent antiproliferative activity and induced apoptosis in HL-60 cells. After treatment with DADAG 8 mg/L for 24 h, caspase-3 activity increased markedly and the cleavage of poly-(ADP-ribose) polymerase (PARP), lamin B, and DFF45 appeared. All of the apoptotic signals were suppressed by z-VAD-fmk (a general inhibitor of caspase activities), whereas z-DEVD-fmk, a selective inhibitor of caspase-3 activity, only induced partial reversion of the apoptotic effects. **CONCLUSION:** DADAG induced apoptosis in HL-60 cells by activating caspases. Caspases promoted apoptosis through the cleavage of substrates of PARP, lamin B, and DFF45.

INTRODUCTION

Apoptosis is the ordered dismantling of the cell, in response to suicide signals, resulting in its "silent" demise and subsequent removal of packaged cell fragments by phagocytic cells. The vital role of

apoptosis in normal animal development and its aberrations in pathology led to an intensive search for the components of the program. A key to the mechanism of programmed cell death was the discovery that, irrespective of the lethal stimuli, death results in identical apoptotic morphology that includes cell and organelle dismantling and packaging, DNA cleavage to nucleosome sized fragments, and engulfment of the fragmented cell. While the biochemical events underlying these changes have not been fully characterized, a knowledge of these processes would help to identify potential targets for the therapeutic regulation of apoptosis.

A family of cysteine proteases (designated caspases) related to the *C elegans* CED-3 protein has been identified in mammalian cells⁽¹⁾. Caspase activation is induced by a wide array of death signals and leads to cleavage of target proteins and execution of the apoptotic program⁽²⁾. Repression of caspase enzymatic activity by viral proteins, p35 from baculovirus and CrmA from cowpox virus, as well as by synthetic peptides inhibits apoptosis induced by stimulation of members of the tumor necrosis factor (TNF) family of cell surface death receptors⁽³⁾. These observations suggest that the execution phase of the apoptotic process induced by cell death receptors requires caspase activity. Understanding caspases regulation is intimately linked to the ability to rationally manipulate apoptosis for therapeutic gain.

In 1957, Vargha *et al*⁽⁴⁾ initially used sugar as the carrier of alkylating agent to synthesize mannomustine (BCM) which was effective to leukemic lymphadenosis and lymphadenoma. It led to the study of cytostatic sugar derivatives known as the hexitols. In this series, diacetyl(dianhydrogalactitol (DADAG) exhibited the higher antitumor activity and less toxicity and was more amphiphilic than other hexitols and easily penetrated the blood brain barrier into brain⁽⁵⁻⁷⁾. So, DADAG was the most promising member in the hexitols. Despite the fact that the antitumor effects of EADAG have been studied by many authors, the mechanism of its action

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remains unclear. This study was to investigate the apoptosis induced by DADAG in HL-60 cells and the relationship between apoptosis and caspases.

MATERIALS AND METHODS

Cell culture and reagents Human leukemia HL-60 cells were maintained in RPMI-1640 medium supplemented with 10 % fetal calf serum, glutamine 2 mmol/L, benzylpenicillin 100 KU/L, and streptomycin 100 mg/L at 37 °C in a 5 % CO₂ atmosphere. DADAG was supplied by Guangxi Institute of Traditional Chinese Medicine. DADAG was dissolved in 0.9 % NaCl solution and was prepared immediately prior to use. *z*-VAD·fmk (0.1 mmol/L) and *z*-DEVD·fmk (0.1 mmol/L) were purchased from Santa Cruz Biotechnology.

Antiproliferative activity of DADAG *in vitro*

The logarithmically growing HL-60 cells were plated to 96-well plates. DADAG was added to the wells for the desired final concentration. After 72 h treatment with DADAG, cell survival was analyzed by MTT assay and the absorbance (*A*) was measured on DG-3022 ELISA microplate Reader at 570 nm. IC₅₀ value was determined using a Bliss Software.

Electron microscopy HL-60 cells treated with DADAG 8 mg/L for 24 h were collected. Cells were fixed in half-strength Karnofsky's fixative, postfixed in 1 % collidine-buffered osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide, and embedded in LR White Resin. Sections were cut and stained with both uranyl acetate and Reynold's lead stain. Electron micrographs were obtained with a JEM-1200 electron microscope.

Internucleosomal DNA damage HL-60 cells treated with DADAG 8 mg/L for various time were centrifuged and washed once with PBS, and cell pellet was solubilized in 400 μL lysis buffer. The total DNA in cells was extracted with phenol-chloroform-isopropanol (25:24:1). DNA was separated on 1.8 % agarose gel electrophoresis, stained with ethidium bromide, and photographed with UV illumination.

Flow cytometry Treated with various drug dilution for various time, cells were collected, washed with PBS, and resuspended in a final volume of 100 μL of ice-cold PBS. One milliliter of 70 % (v/v) ethanol in PBS was added to the resuspended cells with vigorous mixing. Fixed cells were stained for 5 min with propidium iodide, incubated in the dark at 4 °C for 30

min before flow cytometric analysis. Cells were assessed with respect to their red fluorescence profile (575 nm ± 26 nm) using 488 nm excitation at 150 mW from a Coherent Enterprise laser of a FACS vantage instrument (Becton Dickinson). Resulting DNA histograms were acquired using LYSIS II software.

***In vitro* assay of caspase-3 activity** HL-60 cells were lysed for 1 h as reported in the manufacture's instructions and centrifuged at 15 000 × *g* for 15 min at 4 °C. Assays were performed in 96-well plates using the ApoAlert CPP32 colorimetric assay, which is based on spectrophotometric detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate of the enzyme, DEVD-*p*-nitroanilide. The manufacture's instructions were followed.

Western blot analysis Cells were lysed for 30 min at 4 °C. Cell lysates were centrifuged at 15 000 × *g* for 20 min at 4 °C. Equivalent amounts of protein (50 μg) were resolved by 12 % SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) for detection with antibodies. The primary antibodies used were: (a) rabbit polyclonal antihuman PARP (1:1000); (b) goat polyclonal antihuman lamin B and DFF45 (1:200). All antibodies were from Santa Cruz Biotechnology.

Statistical analysis The data were subjected to statistical analysis using analysis of variance (ANOVA) followed by Dunnett's *t* test. A level of *P* < 0.05 was considered to be statistically significant.

RESULTS

Antiproliferative activity of DADAG *in vitro*

DADAG was found cytotoxic to HL-60 cells in a concentration-dependent manner. At concentrations of 2.56, 3.20, 4.00, 5.00, and 6.25 mg/L, inhibitory rates of DADAG on HL-60 cells were 16.7 %, 38.5 %, 69.2 %, 74.4 %, and 83.3 % (Tab 1). IC₅₀ value was 3.7 (3.4–4.0) mg/L.

Apoptosis of HL-60 cells After HL-60 cells were incubated with DADAG 8 mg/L for 24 h, typical morphological changes of apoptosis were observed using electron microscope. The cytoplasm shrank and the chromatin of cells became condensed and margined (Fig 1). The integrity of DNA was assessed by agarose gel electrophoresis. Incubation of HL-60 cells with DADAG 8 mg/L for 24 h elicited a characteristic "ladder" of DNA fragments representing integer multiples of the internucleosomal DNA length (about 180–200 base pair). DNA ladder was observed in a time-

dependent manner (Fig 2).

Tab 1. Antiproliferative action of DADAG on HL-60 cells after treatment with DADAG (2.56 – 6.25 mg/L). $n = 4$. $\bar{x} \pm s$.

Drug $\text{mg} \cdot \text{L}^{-1}$	A_{570}	Inhibitory rate/%
Control	0.78 ± 0.03	0.0
2.56	0.65 ± 0.03	16.7
3.20	0.48 ± 0.04	38.5
4.00	0.24 ± 0.03	69.2
5.00	0.20 ± 0.04	74.4
6.25	0.132 ± 0.014	83.3

$IC_{50} = 3.7(3.4 - 4.0) \text{ mg/L}$

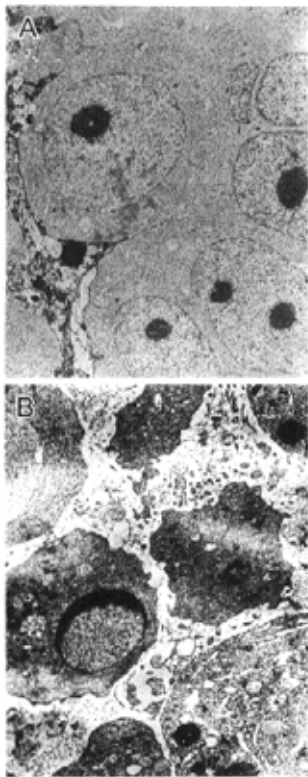


Fig 1. Morphological profiles of DADAG-induced apoptosis in HL-60 cells for 24 h under electron microscope. A: Control; B: Treatment with DADAG 8 mg/L. $\times 3000$.

Effect of DADAG on the activity of caspase-3 in HL-60 cells HL-60 cells treated with DADAG 8 mg/L for 3 h, 6 h, 9 h, 12 h, 24 h, 36 h, and 48 h were collected. The samples were divided into four groups. They were control, DADAG plus inhibitor,

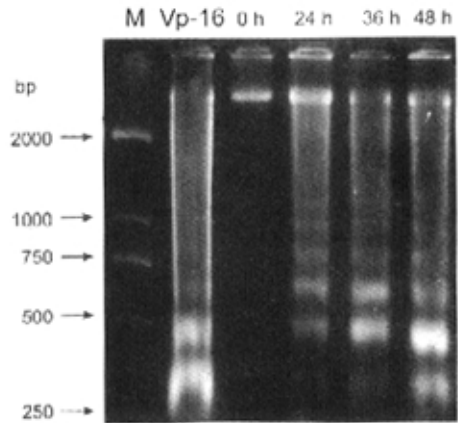


Fig 2. Internucleosomal DNA fragmentation in HL-60 cells treated with DADAG 8 mg/L for indicated time. Land M: DNA marker.

DADAG minus substrate, and DADAG groups. The direct estimation showed that the activity of the caspase-3 slowly increased from 3 h to 12 h and reached a peak at 24 h after exposure to DADAG 8 mg/L in HL-60 cells. Thereafter, the activity of caspase-3 began to decrease (Fig 3).

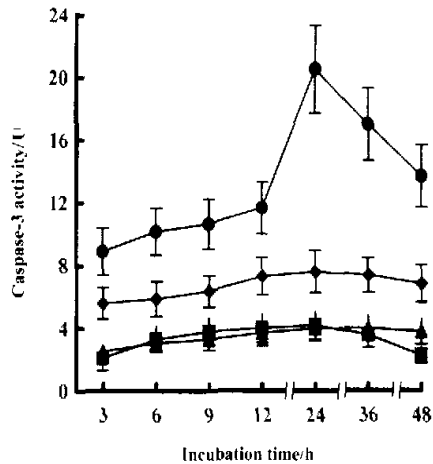


Fig 3. Effect of DADAG 8 mg/L on the activity of caspase3 in HL-60 cells. (◆) Control. (■) DADAG + inhibitor. (▲) DADAG - substrate. (●) DADAG. $n = 3$ experiments (3 samples in each experiment, cell density $1 \times 10^4/\text{L}$). $\bar{x} \pm s$.

Effect of DADAG on the activities of caspases in HL-60 cells Western blot analysis revealed that treatment of HL-60 cells with DADAG 8 mg/L for 24 h induced the proteolytic cleavage of $M_r 116\ 000$

PARP protein, M_r 67 000 lamin B protein, and M_r 45 000 DFF45 protein to yield the characteristic M_r 85 000, M_r 46 000, and M_r 11 000 fragment respectively (Fig 4).

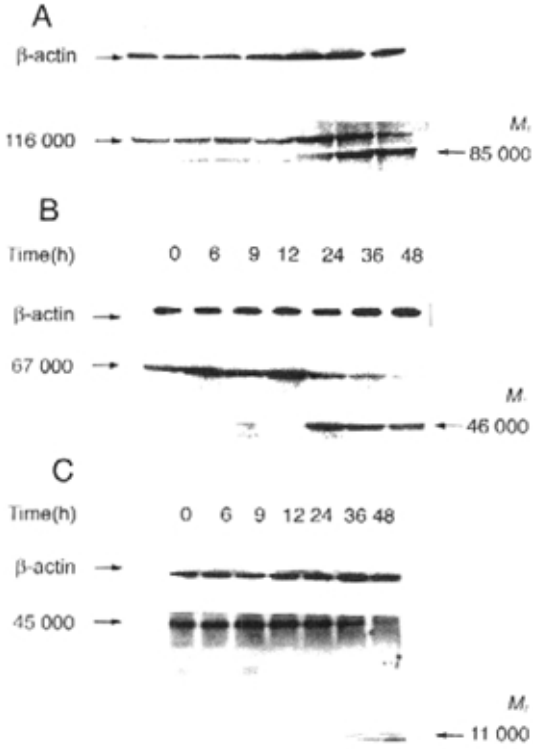


Fig 4. Western blot analysis of caspases substrate of PARP(A), lamin B(B), and DFF45(C) in HL-60 cells treated with DADAG 8 mg/L for indicated time. The expression of β -actin was used as an internal reference.

Effects of the inhibitors of caspases activity on DADAG-induced apoptosis in HL-60 cells To clarify the effect of caspases and the caspases cascade in DADAG-induced apoptosis in HL-60 cells, the cells were treated for 3 h with caspase-3 inhibitor of z-DEVD·fmk 0.1 mmol/L or the general caspases inhibitor of z-VAD·fmk 0.1 mmol/L before DADAG treatment. Flow cytometric analysis showed that in HL-60 cells, DADAG induced an arrest of the cell cycle at the G_2/M phase. The effect, which was already evident at 6 h of treatment, increased with time, so that at 24 h in the presence of DADAG 8 mg/L, about 74 % of cells were arrested at the G_2/M phase, while the apoptotic cells corresponded to about 22.0 %. z-DEVD·fmk, a selective inhibitor of caspase-3, did not decrease the time-

dependent accumulation of cells in the G_2/M phase following DADAG exposure for 6 h, 9 h, 12 h, and 24 h (Tab 2). But z-DEVD·fmk partially counteracted the

Tab 2. Percentage of cells in the G_2/M phase after treatment with DADAG 8 mg/L following pretreatment with caspase-3 inhibitor z-DEVD·fmk 0.1 mmol/L or the general caspases inhibitor z-VAD·fmk 0.1 mmol/L for 3 h. $n=3$. $\bar{x} \pm s$. * $P < 0.01$ vs control.

Group	Incubation time/h			
	6	9	12	24
Control	8.3 ± 1.2	10.0 ± 1.7	8.2 ± 1.0	32 ± 0.11
DADAG	15.5 ± 2.3*	32 ± 3*	37 ± 4*	74 ± 5*
DADAG + z-DEVD·fmk	14.9 ± 1.9*	30.8 ± 2.9*	51 ± 3*	68 ± 4*
DADAG + z-VAD·fmk	15.2 ± 1.8*	29.9 ± 2.5*	49 ± 3*	62 ± 4*

apoptotic effects induced by DADAG. After treatment with DADAG 8 mg/L for 24 h, 36 h, and 48 h, the apoptotic percentage in HL-60 cells were 15.5 %, 16.6 %, and 20.6 % respectively (Fig 5). z-VAD·fmk, a general inhibitor of caspases, similarly did not

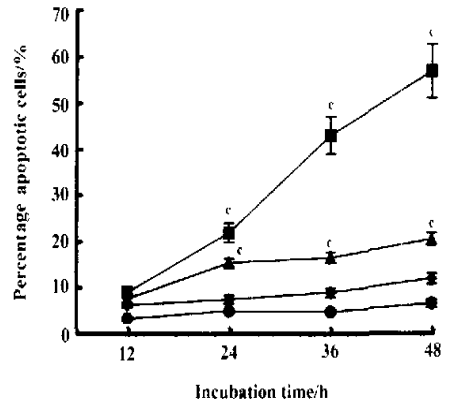


Fig 5. Percentage of apoptotic cells in HL-60 cells after treatment with DADAG 8 mg/L following pretreatment with caspase-3 inhibitor z-DEVD·fmk 0.1 mmol/L or the general caspases inhibitor z-VAD·fmk 0.1 mmol/L for 3 h. (◆) Control; (■) DADAG; (▲) DADAG + z-DEVD·fmk; (●) DADAG + z-VAD·fmk. $n=3$. $\bar{x} \pm s$. * $P < 0.01$ vs control.

block the accumulation of cells in the G_2/M phase (Tab 2), but it was capable of entirely suppressing apoptosis induced by DADAG. After treatment with DADAG 8 mg/L for 24 h, 36 h, and 48 h, the apoptotic percentage in HL-60 cells were 5.0 %, 4.8 %, and 6.6 % respectively (Fig 5). Western blot analysis showed that

the effect induced by DADAG in HL-60 cells on the cleavage of PARP was suppressed by the addition of z-DEVD·fmk. The appearance of lamin B and DFF45 fragment was not entirely inhibited by z-DEVD·fmk and was also seen after DADAG treatment for 48 h (Fig 6).

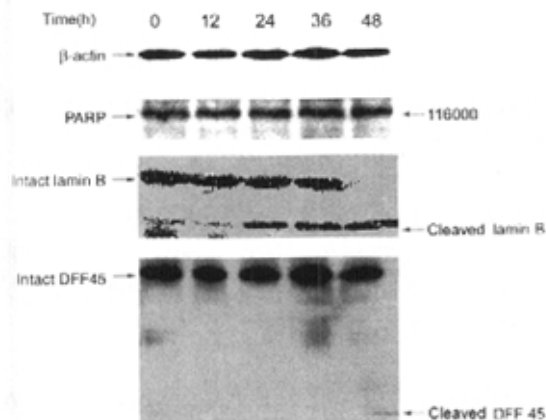


Fig 6. Western blot analysis of caspase substrate of PARP, lamin B, and DFF45 in HL-60 cells treated with DADAG 8 mg/L for indicated time in the presence of z-DEVD·fmk 0.1 mmol/L.

DISCUSSION

Previous studies demonstrated that many chemotherapeutic agents could induce apoptosis in HL-60 cells, such as securinine^[8]. This study demonstrates that DADAG is a good inducer of apoptosis in HL-60 cells. DADAG-induced apoptosis was preceded by the accumulation of HL-60 cells in the G₂/M phase, which was clearly observed by flow cytometric assay, showing that HL-60 cells exhibited a time-dependent accumulation of cells in the G₂/M phase following just 6 h of DADAG exposure and the maximal accumulation of cells in the G₂/M phase was observed at 24 h. With caspase-3 inhibitor of z-DEVD·fmk 0.1 mmol/L or the general caspases inhibitor of z-VAD·fmk 0.1 mmol/L before DADAG treatment, they did not decrease the time-dependent accumulation of HL-60 cells in the G₂/M phase following DADAG exposure. The results indicated that DADAG could induce a G₂/M phase arrest in HL-60 cells first of all. It might be related to the antiproliferative action of DADAG on HL-60 cells.

Based on their structure and order of action in the death pathway, caspases can be divided into initiator caspases and effector caspases. Initiator caspases reside

at upstream and activate effector caspases by proteolytic cleavage. Effector caspases promote apoptosis through the cleavage of death substrates^[9]. The identification and evaluation of caspase substrates is now a healthy growth industry. Poly (ADP-ribose) polymerase is a protein of M_r 116 000 that is associated with DNA damage repair and is cleaved in a number of cell death systems by caspase-3 to yield two fragments of M_r 85 000 and M_r 24 000^[10]. In the mammalian cell, the nuclear lamina consists mainly of three proteins, namely lamin A, B, and C. Disassembly of the lamina precludes any further attempts to replicate or repair DNA and is required for packaging of the nuclear material into apoptotic bodies^[11]. Lamin B is a substrate of caspase-6^[12]. DFF45 is a subunit of the DNA fragmentation factor that is cleaved by active caspase-3 and caspase-7 into two proteolytic fragments. DFF45 can mediate DNA fragmentation^[13,14]. In the present study, we provided evidence that the DADAG-induced apoptosis in HL-60 cells was regulated by caspases. During the process of DADAG-induced apoptosis, the activation of caspase-3 was clearly observed at 24 h by direct estimation of its activity. Activation of caspase-3 was accompanied by degradation of PARP with the production of the M_r 85 000 fragment. All these events were in accordance with the morphological signs of apoptosis. The observation that in HL-60 cells, DADAG also provoked the degradation of lamin B, and DFF45, a substrate of caspase-6 and a substrate of caspase-3 and caspase-7^[12-14], suggested that other caspases besides caspase-3 could be involved in DADAG-induced apoptosis. This was confirmed by the findings that when HL-60 cells were treated with DADAG plus z-DEVD·fmk, a selective inhibitor of caspase-3, apoptosis was reduced but not entirely suppressed and the appearance of lamin B fragment and DFF45 fragment still appeared, whereas when z-VAD·fmk, a general inhibitor of caspases, was capable of entirely suppressing apoptosis induced by DADAG. So, in the execution of apoptosis, caspase-3 played a central role and caspase-6 and -7 might play secondary roles. These caspases promoted apoptosis through the cleavage of death substrates of PARP, lamin B, and DFF45 and these finally led to apoptotic events.

The induction of apoptosis has been recognized as an effective tool in the therapeutic treatment of many forms of tumors, and apoptosis can be triggered by a number of chemotherapeutic agents. A thorough understanding of the molecular events that result in activation of apoptosis,

and whether these can be modulated by specific caspase inhibition, is vital to both understanding the diseases and designing appropriate therapies to counter them. These studies will provide theoretical basis for DADAG clinical application and for improving therapeutic index of tumors.

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Caspases 家族成员促进 DADAG 诱导的人白血病 HL-60 细胞凋亡

R96 A
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关键词 二乙酰二脱水 D-核糖醇; 细胞凋亡; DNA
断片; 流式细胞术; caspases; HL-60 细胞

目的: 研究 caspases 家族成员在二乙酰二脱水 D-核糖醇 (DADAG) 诱导人白血病 HL-60 细胞凋亡中的作用。
方法: MTT 法观察 DADAG 的体外抗增殖作用; 透射电镜、DNA 梯形条带和流式细胞仪检测 HL-60 细胞凋亡; caspase-3 检测试剂盒和 Western blot 法分析 caspases 家族成员。
结果: DADAG 明显抑制 HL-60 细胞增殖和诱导细胞凋亡。DADAG 处理 HL-60 细胞 24 h 后, caspase-3 酶活性达峰值, 同时聚腺苷二磷酸核糖聚合酶 (PARP)、lamin B 和 DFF45 蛋白开始出现断裂片段。Caspase-3 抑制剂 z-DEVD·fmk 可部分逆转 DADAG 诱导的 HL-60 细胞凋亡, 而 caspases 广谱抑制剂 z-VAD·fmk 可完全逆转此作用。
结论: Caspases 在 DADAG 诱导 HL-60 细胞凋亡中起重要作用, 它们通过酶解底物 PARP、DFF45 和 lamin B 促进细胞凋亡。

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