APOPTOSIS INDUCED BY DIACETYLDIANHYDROGALACTITOL AND ITS MECHANISM IN HL-60 LEUKEMIA CELLS

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ABSTRACT: **AIM** To investigate the apoptosis induced by diacetyldianhydrogalactitol (DADAG) and its mechanism in human HL-60 leukemia 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 . The levels of Bcl-2 family proteins were detected by Western blotting . Caspase-3 activity was determined by ApoAlert CPP32 colorimetric assay kit . **RESULTS** DADAG exhibited potent antiproliferative activity and induced apoptosis in HL-60 cellls . After treatment with DADAG 8 μg· mL^{-1} for various times , the Bcl-X_L protein level decreased in a time-dependent manner , while the Bad protein level was up-regulated . The caspase-3 activity increased markedly after treatment with DADAG for 24 h . The apoptotic signals were suppressed by z-VAD .fmk (a general inhibitor of caspases) , whereas z-DEVD .fmk , a selective inhibitor of caspase-3 , only induced partial reversion of the apoptotic effects . **CONCLUSION** DADAG-induced apoptosis in HL-60 cells required caspase-3 activation and caspase-3 activation was related with Bcl-2 family members .

KEY WORDS: diacetyldianhydrogalactitol ; apoptosis ; HL-60 cells
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In 1957 , Vargha *et al*[1] initially used sugar as the carrier of alkylating agent to synthesize mannomustine which was effective in the treatment of leukemic lymphadenosis and lymphadenoma . It led to the study of cytostatic sugar derivatives known as the hexitols . In this series , diacetyldianhydrogalactitol (DADAG) exhibited higher antitumor activity and less toxicity and was more amphiphilic than other hexitols and easily penetrate the blood brain barrier into brain[2] . So , DADAG was the most promising member in the hexitols . Despite the fact that the antitumor effects of DADAG have been studied by many authors , the mechanism of its action remains unclear . This study was to investigate the apoptosis induced by DADAG in HL-60 cells and the relationship between apoptosis and Bcl-2 family proteins and caspase-3 .

MATERIALS AND METHODS

Materials Diacetyldianhydrogalactitol (DADAG) was supplied by the Guangxi Institute of Traditional Chinese Medicine . DADAG was dissolved in 0.9 % NaCl solution and was prepared immediately prior to use . MTT , propidium iodine (PI) and RNase A were from Sigma Chemical Co . . Polyclonal rabbit anti-Bcl-X_L (1∶100) and monoclonal mouse anti-Bad (1∶100) antibodies , z-VAD . fmk (0.1 μmol· mL^{-1}) and z-DEVD .fmk (0.1 μmol· mL^{-1}) were obtained from Santa Cruz Biotechnology InC . .

Cell culture Human promyelocytic leukemia HL-60 cells were maintained in RPMI-1640 medium supplemented with 10 % fetal calf serum , 2 μmol· mL^{-1} glutamine , 100 u· mL^{-1} benzylpenicillin , and 100 u· mL^{-1} streptomycin at 37 ℃ in a 5 % CO_2 atmosphere .

MTT assay The logarithmically growing HL-60 cells were plated onto 96-well plates . DADAG was added to the wells for the desired final concentration . After 72 h treatment with DADAG , the absorbance (*A*) was measured on DG-3022 ELISA microplate Reader at 570 nm . IC_{50} value was determined using a Bliss Software .

Electron microscopy HL-60 cells treated with 8 μg· mL^{-1} DADAG for 24 h were collected . Cells were fixed , postfixed , dehydrated and embedded . Sections stained with uranyl acetate and Reynold′ lead were photographed with the use of a JEM-1200 electron microscope .

Internucleosomal DNA damage HL-60 cells treated with 8 μg· mL^{-1} DADAG for various times were centrifuged and washed once with phosphate-buffered

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saline (PBS) , the cell pellet was solubilized in 400 μL lysis buffer. The total DNA in cells was extracted with phenol-chloroform-isopropanol (25∶24∶1) , separated on 1.8 % agarose gel electrophoresis , stained with ethidium bromide and photographed with UV illumination.

Flow cytometry After treated with 8 μg·mL^{-1} DADAG for 12 , 24 , 36 and 48 h , the cells were washed twice with cold phosphate buffer solution and then stained with PI for 30 min at 37 ℃. The cells were measured by flow cytometer (Becton Dickinson) . The resulting DNA histograms were acquired using LYSIS II software.

Western blotting analysis Cells treated with 8 μg·mL^{-1} DADAG for various times were lysed for 30 min at 4 ℃. Cell lysates were centrifuged at 15 000 × *g* for 20 min at 4 ℃. Equivalent amounts of protein (50 μg) were resolved by 12 % SDS-PAGE and transferred onto PVDF for detection with monoclonal antibodies and appropriate secondary antibody.

***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 ℃. Assays were performed in 96-well plates using the ApoAlert CPP32 colorimetric assay. The manufacture′s instructions were followed.

RESULTS

1 Antiproliferative activity of DADAG *in vitro*

Under concentrations of 2.56 , 3.20 , 4.00 , 5.00 and 6.25 μg·mL^{-1} , inhibitory rates of DADAG on HL-60 cells were 16.7 % , 38.5 % , 69.2 % , 74.4 % and 83.3 % . IC_{50} value was 3.7 (3.4 , 4.0) μg·mL^{-1} .

2 Apoptosis of HL-60 cells

After the HL-60 cells were incubated with 8 μg·mL^{-1} DADAG for 24 h , morphological changes typical of apoptosis were observed using electron microscopy assay. The cytoplasm shrinked and the chromatin of the cells became condensed and marginated (Figure 1) . The intergrity of DNA was assessed by agarose gel electrophoresis. DNA ladder was observed clearly after HL-60 cells were treated with 8 μg·mL^{-1} DADAG for 24 h and DNA ladder was observed in a time-dependent manner (Figure 2) .

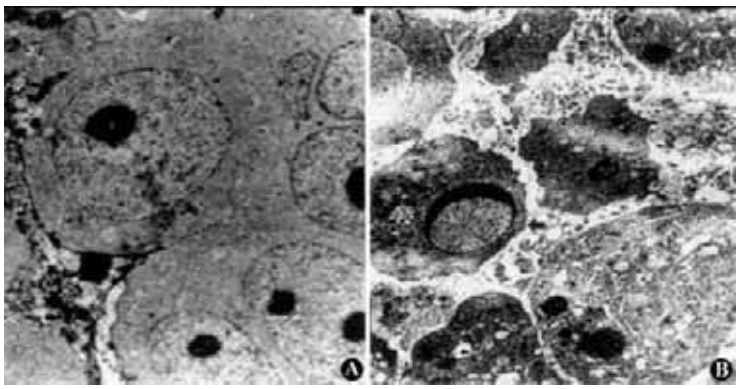

Figure 1 Morphological profiles of DADAG-induced apoptosis in HL-60 cells for 24 h under electron microscope

A. Control cells ; B. Cells treated with 8 μg·mL^{-1} DADAG. The magnification was 3 000 ×

3 Effect of DADAG on the Bcl-2 family members in HL-60 cells

HL-60 cells treated with 8 μg·mL^{-1} DADAG for 6 , 9 , 12 , 24 , 36 and 48 h were collected. Western blotting analysis showed that the Bcl-X_L protein level was progressively decreased in a time-dependent manner. On the contrary , the Bad protein level was up-regulated following DADAG treatment. This change occurred after 6 h and reached peak at 24 h (Figure 3) .

4 Effect of DADAG on the activity of caspase-3 in HL-60 cells

HL-60 cells treated with 8 μg·mL^{-1} DADAG for 3 , 6 , 9 , 12 , 24 , 36 and 48 h were collected. The experiments demonstrated that DADAG induced the activation of caspase-3 in HL-60 cells. The direct estimation of caspase-3 activity showed a peak after exposure to 8 μg·mL^{-1} DADAG for 24 h and rapid decreases after 36 h and 48 h of treatment. The effect was inhibited by z-VAD.fmk (Figure 4) .

5 Effects of the caspases inhibitors on DADAG-induced apoptosis in HL-60 cells

In order to clarify the effect of caspases in DADAG-induced apoptosis in HL-60 cells , the influences of z-DEVD.fmk and z-VAD.fmk , two membrane-permeable

inhibitors of caspase activity, on the effect exerted by DADAG on HL-60 cells were studied. The results obtained by means of flow cytometry showed that z-DEVD.fmk, a selective inhibitor of caspase-3, partially counteracted the apoptotic effects induced by DADAG. When 0.1 μmol·mL^{-1} z-DEVD.fmk was added to 8 μg·mL^{-1} DADAG, the apoptotic percentage in HL-60 cells at 24, 36 and 48 h were 15.5%, 16.6% and 20.6%, respectively. While z-VAD.fmk, a general caspase inhibitor, was capable of entirely suppressing apoptosis induced by DADAG. After addition of 0.1 μmol·mL^{-1} z-VAD.fmk to 8 μg·mL^{-1} DADAG, the apoptotic percentage in HL-60 cells at 24, 36 and 48 h were 5.0%, 4.8% and 6.6%, respectively (Figure 5).

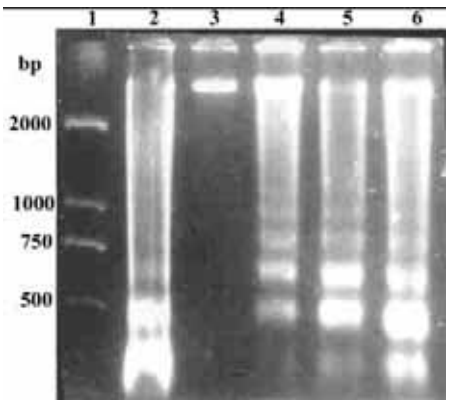

Figure 2 Internucleosomal DNA fragmentation in HL-60 cells treated with 8 μg·mL^{-1} DADAG for indicated times Lane 1. DNA marker; Lane 2. Cells treated with vp-16 for 12 h; Lane 3. Control cells; Lane 4. Cells treated with DADAG for 24 h; Lane 5. Cells treated with DADAG for 36 h; Lane 6. Cells treated with DADAG for 48 h. The DNA samples were ran on agarose gel electrophoresis and detected by staining with ethidium bromide

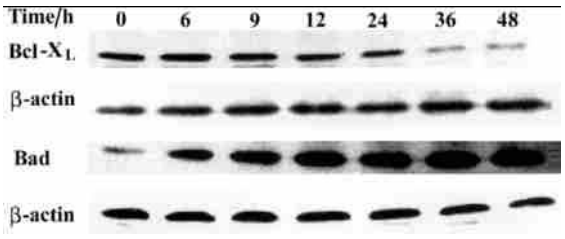

Figure 3 Effect of DADAG on the level of Bcl-2 family members in HL-60 cells. HL-60 cells were treated with 8 μg·mL^{-1} DADAG for indicated times. Western blotting analysis was performed with anti-Bcl-X$_L$ antibody and anti-Bad antibody

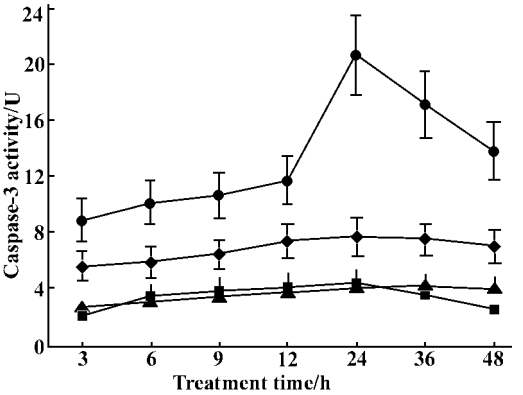

Figure 4 Effect of DADAG on the activity of caspase-3 in HL-60 cells

HL-60 cells were treated with 8 μg·mL^{-1} DADAG for 3 ~ 48 h. Cell lysates were used in the caspase-3 activity assay using the colorimetric substrate DEVD-pNA. Absorbance was read at 405 nm. Caspase-3 activity was calculated by the manufacture′s instructions. ◆—◆ Control; ■—■ DADAG + 0.1 μmol·mL^{-1} z-VAD.fmk; ▲—▲ DADAG-substrate; ●—● DADAG

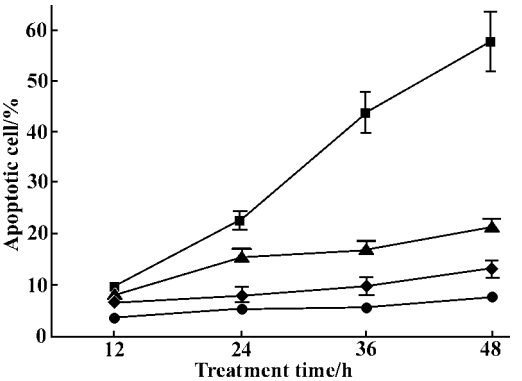

Figure 5 Time course of the effects induced in HL-60 cells by 8 μg·mL^{-1} DADAG on apoptotic cells and the influence of z-DEVD.fmk and z-VAD.fmk on these effects. Assayed by flow cytometry. ◆—◆ Control; ■—■ DADAG; ▲—▲ DADAG + 0.1 μmol·mL^{-1} z-DEVD.fmk; ●—● DADAG + 0.1 μmol·mL^{-1} z-VAD.fmk

DISCUSSION

The members of the Bcl-2 family may play key roles in the regulation of survival and apotosis in p53-null HL-60 cells. Overexpression of proapoptotic Bcl-2 and Bcl-X$_L$ enhances cell survival by suppressing apoptosis in a number of cells subject to a wide range of apoptosis-inducing stimuli[3,4], whereas antiapoptotic Bad, Bax and Bcl-XS promote the response[5]. These proteins have been proposed to regulate apoptosis through both homo- and heterodimerization. This study demonstrated that DADAG

exhibited potent antiproliferative activity in vitro and it was the first report that DADAG induced apoptosis in HL-60 cells. During the process of DADAG-induction of apoptosis, significant up-regulation of Bad protein and apparent decrease of Bcl-X_L protein would favor Bad to successfully compete for Bcl-X_L and Bcl-2, resulting in much more free Bax into homodimers and promoting apoptosis. So, the members of Bcl-2 family played a vital role in apoptosis induced by DADAG in HL-60 cells.

Apoptosis, an evolutionarily conserved form of cell suicide, requires specialized machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell[6]. Understanding caspase regulation is intimately linked to the ability to rationally manipulate apoptosis for therapeutic gain. Among these caspases, caspase-3 has been deemed an attractive candidate as a putative mediator of apoptosis in human HL-60 leukemia cells[7]. The result also showed that the activation of caspase-3 was clearly observed at 24 h by direct estimation of its activity. The event was in accordance with the morphological signs of apoptosis. In order to clarify the central role of caspase-3 activity, HL-60 cells were treated with DADAG plus caspase-3 inhibitor of z-DEVD.fmk or the general caspases inhibitor of z-VAD.fmk before DADAG treatment. The results showed that z-DEVD.fmk reduced but not entirely suppressed apoptosis, whereas z-VAD.fmk was capable of entirely suppressing apoptosis induced by DADAG. So, in the execution of apoptosis, caspase-3 played a central role and other caspases might play secondary roles.

Genetic analysis of apoptosis in the nematode *Caenorhabditis elegans* has revealed the cell death machine to be composed of three core interacting components. CED-4 (equivalent to mammalian Apaf-1) is a nucleotide binding molecule that complexes with the zymogen form of the death protease CED-3, leading to its autoactivation and cell death. CED-9 blocks death by complexing with CED-4 and attenuating its ability to promote CED-3 activation. An equivalent ternary complex was found to be present in mammalian cells involving Apaf-1, the mammalian death protease caspase-9, and Bcl-X_L, an anti-apoptotic member of the Bcl-2 family[8]. In the study, the Bad protein level was up-regulated 6 h after exposure to 8 $\mu g \cdot mL^{-1}$ DADAG in HL-60 cells. While the activity of caspase-3 apparently was increased only at 24 h. The change of Bcl-2 family members led to the increase of the ratio of Bcl-2 family proapoptotic to antiapoptotic proteins and these might activate initiator caspase-9 and then promote caspase-3 activation and apoptosis.

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 both in understanding the diseases and in designing appropriate therapies to counter them. These studies will provide theoretical basis for clinical application of DADAG and for improving the therapeutic index.

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二乙酰二脱水卫矛醇诱导白血病 HL-60 细胞凋亡及其机理

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摘要 : 目的 研究二乙酰二脱水卫矛醇(DADAG) 诱导人白血病 HL-60 细胞凋亡及其机理 。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 ; Western blotting 法和 caspase-3 检测试剂盒分析 DADAG 诱导 HL-60 细胞凋亡与 Bcl-2 家族成员和 caspase-3 的关系 。结果 DADAG 明显抑制 HL-60 细胞增殖和诱导细胞发生凋亡 。8 μg·mL^{-1} DADAG 处理 HL-60 细胞不同时间后 , Bcl-X_L 蛋白水平呈时间依赖性地下降 ,而 Bad 蛋白水平上调 。DADAG 处理 HL-60 细胞 24 h 后 , caspase-3 酶活性达峰值 。Caspase-3 抑制剂 z-DEVD.fmk 可部分逆转 DADAG 诱导 HL-60 细胞凋亡的作用 ,而 caspases 广谱抑制剂 z-VAD.fmk 可完全逆转此作用 。结论 DADAG 诱导 HL-60 细胞凋亡依赖 caspase-3 途径的激活 , 而 caspase-3 的激活可能与 Bcl-2 家族成员密切相关 。

关键词 : 二乙酰二脱水卫矛醇 ; 细胞凋亡 ; HL-60 细胞