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Caspase 3 gene expression and $[Ca^{2+}]_i$ homeostasis underlying desipramine-induced C6 glioma cell apoptosis[1]

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KEY WORDS desipramine; glioma; apoptosis; caspases; calcium

ABSTRACT

AIM: To study desipramine (Des)-induced apoptosis and regulation of caspase 3 gene expression and $[Ca^{2+}]_i$ homeostasis in rat glioma C6 cells. **METHODS**: Apoptotic DNA breaks were quantified by propidium iodide (PI) incorporation using flow cytometry (FCM) and were detected by DNA agarose gel electrophoresis. Expression of apoptotic effector gene caspase 3 was assessed by reverse transcription polymerase chain reaction (RT-PCR). Single cell $[Ca^{2+}]_i$ was measured using fluorescence indicator Fura-3/AM with confocal laser scanning microscopy. **RESULTS**: Des induced apoptotic DNA breaks in a concentration-dependent manner evidenced by hypodiploid peak on FCM histogram and the apoptotic cell percentage induced by Des 10, 20, and 40 μmol/L for 24 h was 5.2 %, 21.9 % , and 41.9 %, respectively. Apoptotic DNA breaks were further confirmed by a typical "DNA ladder" on agarose gel electrophoresis after exposure to Des 40 μmol/L for 24 h. Meanwhile, expression of caspase 3 gene was observed following Des 20 μmol/L treatment. Des 40 μmol/L resulted in an early sustained increase in $[Ca^{2+}]_i$ over 28 min and the elevation magnitude was greatly decreased by removal of extracellular free $[Ca^{2+}]_i$ with calcium-chelator egtazic acid, suggesting that Des elicited $[Ca^{2+}]_i$ influx rather than intracellular calcium mobilization. **CONCLUSION**: Up-regulation of caspase 3 gene expression and disturbance of homeostasis in calcium signaling system might play pivotal roles in Des-induced apoptotic DNA breaks of C6 cells.

INTRODUCTION

Tricyclic antidepressants are the mainstay of management in the treatment of depressive symptoms that usually happen in cancer patients[1]. Recent evidence indicates that tricyclic antidepressants have been shown to be cytotoxic to most malignant cells both *in vitro* and *in vivo* and synergistic with certain chemotherapeutic agents[2,3]. The inhibition of calmodulin and induction of apoptosis are considered being related to this antineoplastic effect of tricyclic antidepressants[4,5]. Since tricyclic antidepressants readily pass the blood-brain barrier and accumulate in the brain, they are very attractive candidates for auxiliary use against tumors of the central nervous system. Rat glioma C6 cells repre-

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sent a suitable model of human glioma for chemotherapeutic studies. Our previous studies have demonstrated that acute exposure of rat glioma C6 cells to desipramine (Des), an active metabolizer of tricyclic antidepressant imipramine, could result in cellular proliferation inhibition in a concentration-and time-dependent manner and apoptotic cell death with *bcl*-2 protein down-regulation[6,7]. Other studies also demonstrated that imipramine could activate the apoptosis process undergoing downregulation of *bcl*-2 gene expression[8,9]. Although it is widely accepted that intracellular calcium signaling and DNA damage might be the common triggers implicated in denomination of apoptosis, gene caspase 3 may play an important role in the executive phase of apoptosis. Some widely used antidepressants such as imipramine and clomipramine have been found to possess antineoplastic effects and induce apoptosis *via* a caspase 3-dependent pathway in human acute myeloid leukemia cell line[10]. In this study, we further characterize C6 cell apoptosis induced by Des and explore whether caspase 3 gene expression and the calcium-messenger system are involved in the regulation of Des-induced apoptosis.

MATERIALS AND METHODS

Chemicals Trizol agent, SuperScript™One-Step™RT-PCR System was purchased from Gibco. Caspase 3 primers (5' CCACTCCCAGTCATTCCTTT-AGTG3', 5' ATGGACAACAACGAAACCTCCGTG3') were obtained from SaiBaiSheng Co. Fura-3/AM was purchased from Gene.

Cell culture C6 cells were cultured in DEME containing 10 % fetal calf serum. Cultures were maintained at 37 ℃ in a humidified incubator under an atmosphere of 90 % air and 10 % CO_2.

FCM analysis of DNA content C6 apoptotic DNA breaks were quantitated as the percent of cells with hypodiploid DNA assessed by PI incorporation by FCM. Cells were fixed with ice-cold 70 % methanol for 30 min, treated with RNase solution at 37 ℃ for 30 min, and stained with PI for 30 min. The minimum of 1×10^6 events was collected and analyzed by software Cell Quest (FACScan, Becton Dickson, USA).

DNA gel electrophoresis Lysed cell samples were centrifuged for 15 min at 13 000×*g*. The supernatants were extracted with equal volumes of phenol/ chloroform/isoamyl alcohol (25:24:1). The DNA samples were loaded onto 1.8 % agarose gels and electrophoresis was performed at 50 mV. Gels were visualized by ultraviolet light after staining with ethidium bromide.

RT-PCR Total RNA was extracted by Trizol agents. The following conditions were used for 35 cycles of PCR amplification: 30 s denaturation at 94 ℃, 30 s annealing at 50 ℃, and 2 min extension at 72 ℃. The amplified products were resolved by gel electrophoresis on 1.8 % agarose.

Measurement of $[Ca^{2+}]_i$ in single cells After an overnight period of attachment, the medium was removed and the cells were loaded for 30 min at 37 ℃ with Fura-3/AM in PBS. The cells were then washed to remove extracellular Fura-3/AM and placed on microscope stage then calcium concentration of individual C6 cell was determined by the fluorescence (LSM510, Zeiss, USA).

Statistics Data were expressed as mean±SD. Statistical analysis of apoptotic cell percentage was performed with two factor analysis of variance and $[Ca^{2+}]_i$ increase percentage was performed by two factor analysis of variance with replication. Duncan test was used to compare two groups.

RESULTS

Quantitation of apoptosis FCM analyses showed classical hypodiploid peak that appeared on the histogram. The average apoptotic cell percentage was about 5.2 %, 21.9 %, and 41.9 % when exposed to Des 10, 20, and 40 μmol/L for 24 h, indicating that Des induced apoptotic DNA breaks in a concentration-dependent manner. Only 0.3 % untreated C6 cells were induced to undergo apoptosis (Fig 1).

Analysis of DNA gel electrophoresis After addition of Des (40 μmol/L) in C6 cells for 24 h, a typical "DNA ladder" appears on agarose gel electrophoresis indicating internucleosomal degradation of DNA into segment of regular 180 bp interval (Fig 2).

Expression of caspase 3 Due to the decomposi-

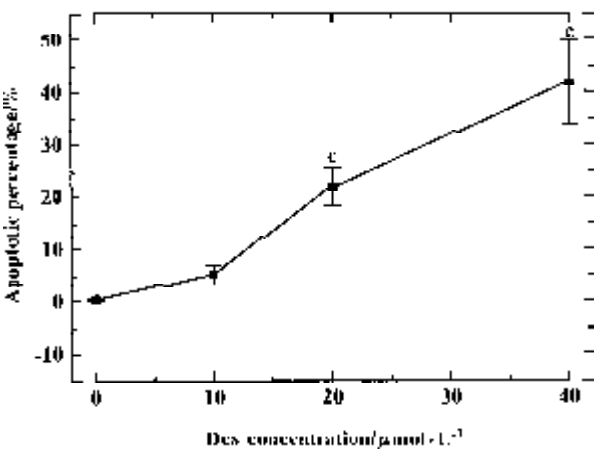

Fig 1. The effect of Des on apoptotic percentage (%) of C6 cells. *n*=3 experiments (each of 1 × 10^6cells). Mean±SD. $^cP<0.01$ *vs* control.

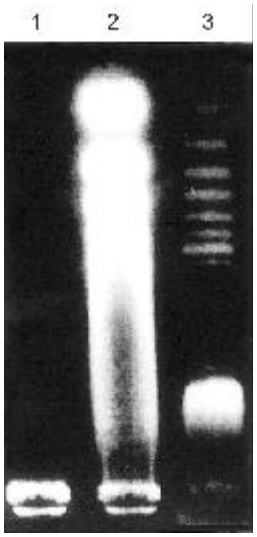

Fig 2. Agarose gel electrophoresis for detecting DNA fragmentation induced by Des 40 μmol/L. Lane 1: control; Lane 2: Des 40 μmol/L; Lane 3: 100 bp Marker.

tion of a large number of C6 cells following exposure to Des 40 μmol/L for 24 h, the expression of gene caspase 3 was detected in low concentration of Des 20 μmol/L. It increased significantly following exposure to Des 20 μmol/L for 24 h, whereas no expression of caspase 3 was detected in the untreated C6 cells (Fig 3).

Effects of Des on $[Ca^{2+}]_i$ in cultured C6 cells Spontaneous oscillations existed in untreated C6 cells. Addition of Des 10 and 20 μmol/L only caused a transient or slight increase in $[Ca^{2+}]_i$. Des 40 μmol/L could elicit an early marked elevation in $[Ca^{2+}]_i$ over 28 min (Tab 1). The elevation of $[Ca^{2+}]_i$ caused by Des 40 μmol/L was largely diminished by adding calcium chelator egtazic acid 0.2 mmol/L and perfusing cells with Ca^{2+}-free buffer PBS, demonstrating that Des elicited $[Ca^{2+}]_i$ influx rather than intracellular calcium mobilization.

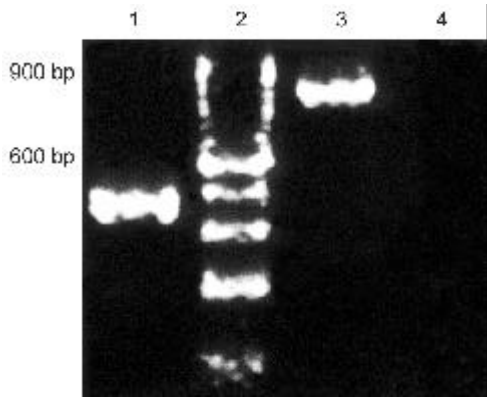

Fig 3. Caspase 3 mRNA level in C6 cells treated with Des 20 μmol/L for 24 h. Lane 1: β-actin; Lane 2: marker; Lane 3: Des 20 μmol/L; Lane 4: control.

Tab 1. Time course of $[Ca^{2+}]_i$ increase (%) at different concentrations of Des in cultured C6 cells. *n*=7 cells. Mean±SD. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

Time/min	$[Ca^{2+}]_i$ increase/%			
	0	10	20	40 μmol/L
4	8±4	18±7^b	21±5^b	133±49^c
8	27±5	37±9^a	50±9^a	291±170^c
12	32±2	44±9^a	60±12^a	436±240^c
16	38±7	50±9^a	68±17^a	746±463^c
20	24±5	49±8^a	59±21^a	895±598^c
24	34±4	33±7^a	59±28^a	993±698^c
28	34±5	29±9^a	62±29^a	1017±757^c

DISCUSSION

In recent years, there have been major insights into the mechanisms by which apoptosis is triggered in cells. The nuclear alterations, which are the pre-emi-

nent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA recognized as a "DNA ladder" on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of apoptosis. Des-induced apoptotic DNA breaks were confirmed by a typical "DNA ladder" on agarose gel electrophoresis as well as evidenced by hypodiploid peak on FCM histogram. However, internucleosomal cleavage of DNA appears to be a relatively late event in the apoptotic process, which in some models may be dissociated from early critical steps. Cell death protease designated as caspase may play an essential role in apoptotic cell death and act upstream of DNA fragmentation[11]. Caspases release proapoptotic factors known to serve as both signal transducers and effective components thus initiate a caspase cascade. Activation of caspase-8 has been shown to recruit downstream "amplifier" proteases, while caspase 3 is regarded as ultimately "effector" in the execution phase of apoptotic cell death[12]. Activation of caspases during apoptosis may result in the cleavage of critical cellular substrates, including poly (ADP-ribose) polymerase, so precipitating the dramatic morphological changes of apoptosis. Our results showed significant increase in the expression of gene caspase 3 following exposure to Des, suggesting Des induced-apoptosis in the C6 cells *via* caspase 3 dependent pathway. This is coherent with our previous results that Des could down-regulate the expression of bcl-2 protein in the C6 cells[7]. Gene *bcl*-2, as the negative regulator of caspase 3, exerts anti-apoptosis action at or before the processing of certain caspases to their catalytically active forms[13].

Changes in $[Ca^{2+}]_i$ provide a chemical signal for early cell death pathway. If $[Ca^{2+}]_i$ can be elevated for a sustained period, cells are induced to undergo apoptosis[14]. Our experiment demonstrated that Des elicited a sustained increase of $[Ca^{2+}]_i$ which was a very early effect compared to morphological changes (*ie* cell rounding and shrinkage) and DNA fragmentation owing to the activation of Ca^{2+}/Mg^{2+}-dependent endonuclease. Thus, we concluded that $[Ca^{2+}]_i$ might be another early initiator in connection with apoptosis. Although the precise mechanism needs further study, our findings provide the evidence for an important role of caspase 3 and homeostasis of calcium signaling system in the regulation of Des-induced apoptosis.

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在地昔帕明诱导胶质瘤 C6 细胞凋亡过程中 caspase 3 基因表达和$[Ca^{2+}]_i$稳态[1]

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关键词 地昔帕明；神经胶质瘤；细胞凋亡；caspases；钙

目的：研究抗抑郁药地昔帕明(Des)对胶质瘤 C6 细胞的凋亡诱导作用以及对凋亡关键效应分子caspase 3 和凋亡早期信号$[Ca^{2+}]_i$的调控作用．**方法**：采用流式细胞术(FCM)和凝胶电泳观察 Des 对 C6 细胞凋亡的 DNA 裂解作用，RT-PCR 分析 caspase 3 基因的表达以及激光扫描共聚焦显微镜测量单个活细胞$[Ca^{2+}]_i$浓度．**结果**：Des (10，20，40 μmol/L) 处理 C6 细胞 24 h 后，FCM 图的 G_1 峰左侧出现凋亡特征性亚二倍体细胞峰，凋亡细胞百分率分别为 5.2 %，21.9 % 和 41.9 %．同时，凝胶电泳显示典型的 DNA“梯带”．Des 20 μmol/L 处理 C6 细胞 24 h 可明显增强 caspase 3 基因的表达，而未经 Des 处理的 C6 细胞则检测不到 caspase 3 基因的表达．此外，Des 40 μmol/L 可使 C6 细胞$[Ca^{2+}]_i$迅速升高并维持超过 28 min，而钙螯合剂依他酸可显著降低 C6 细胞$[Ca^{2+}]_i$增高幅度，提示 Des 致 C6 细胞$[Ca^{2+}]_i$增高主要与细胞外钙内流有关．**结论**：Des 诱导 C6 胶质瘤细胞凋亡可能与 caspase 3 基因表达的上调以及细胞内钙稳态的失衡有关．

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